PA-X protein of H9N2 subtype avian influenza virus suppresses the innate immunity of chicken bone marrow-derived dendritic cells

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ABSTRACT H9N2 subtype avian influenza (**AI**) is an infectious disease associated with immunosuppression in poultry. Here, the regulation function of PA-X protein was determined on the host innate immune response of H9N2-infected chicken bone marrow-derived DCs (**chBM-DCs**). Based on 2 mutated viruses expressing PA-X protein (**rTX**) or deficient PA-X protein (**rTX-FS**), and the established culture system of chBM-DCs, results showed PA-X protein inhibited viral replication in chBM-DCs but not in non-immune chicken cells (DF-1). Moreover, PA-X protein downregulated the expression of phenotypic markers (CD40, CD86, and MHCII) and proinflammatory cytokine (IL-12 and IL-1β) of chBM-DCs. The mixed lymphocyte reaction between chBM-DCs and chicken T cells showed PA-X protein significantly decreased H9N2-infected chBM-DCs to induce T cell proliferation, implying a suppression of the DC-induced downstream T cell response. Taken together, these findings indicated that PA-X protein is a key viral protein to help H9N2 subtype AIVs escape the innate immunity of chBM-DCs.

Key words: avian influenza virus, chicken bone marrow-derived dendritic cells, H9N2, PA-X protein, innate immunity

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INTRODUCTION

H9N2 subtype avian influenza virus (**AIV**), which can be asymptomatic or often induce mild respiratory symptoms, has been circulating around the world. Notably, it can cause immunosuppression, which leads to the immune failure of chickens and the increased susceptibility of chicken to various pathogenic microorganisms, resulting in serious economic losses (Xing et al., 2008). In addition, H9N2 subtype AIVs can pose a major threat to public health by donating its genes to the emergence of lethal human H5N1, H5N6, H7N9, and H10N8 subtype AIVs (Pu et al., 2015; Wu et al., 2017). Therefore, it is worth exploring the pathogenic mechanism, especially the immune suppression of H9N2 subtype AIVs, which is of great importance for planning the prevention and control strategy of H9N2 avian influenza (**AI**).

Dendritic cells (**DCs**), professional antigen-presenting cells, are the bridge between innate and adaptive immune responses (Chen et al., 2018). During virus infection, DCs produce interferons (**IFNs**) and other regulatory cytokines, then present antigens to T cells to induce adaptive immunity (Worbs et al., 2017). However, current reports on DCs mainly focus on mammals, and studies on chicken DCs started relatively late. In 2006, chicken DCs were first isolated and identified from skin and mucosa of chickens, but the number of DCs isolated was small and the survival time was short, which were not conducive to subsequent experiments (Igyarto et al., 2006). In 2010, scientists successfully established an inducible culture method of chicken bone marrow-derived DCs (**chBM-DCs**) in vitro using self-synthesized recombinant chicken GM-CSF and IL-4 (Wu et al., 2010). We previously successfully established the separation and culture method of chBM-DCs based on the induction of commercial recombinant chicken

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GM-CSF and IL-4 cytokines (Liang et al., 2015). Our previous study found that H9N2 subtype AIVs infection can regulate the immune function of chBM-DCs (Lin et al., 2018). However, the immune regulation mechanism on chBM-DCs is still unclear.

PA-X protein is encoded by influenza virus gene segment 3 whose N-terminal amino acids are encoded by the PA, and the C-terminal amino acids are coded by a X-open reading frame (**X-ORF**) resulting from the +1 ribosomal frameshift (Jagger et al., 2012). PA-X protein has host shut-off activity, which was manifested as a sharp decline in host protein synthesis after virus infection (Hu et al., 2018). Further studies found that PA-X of H1N1 subtype AIVs interacts with splicing regulators including PUF60, RBM39, and PRPF4 protein for the regulation of host shut-off activity (Gaucherand et al., 2019). In addition, PA-X of H5N1 subtype AIVs can inhibit the NF-κB signaling pathway to downregulate the cytokines secretions for immune modulation (Hu et al., 2020), and suppress the IFN mRNA accumulation through a MAVS-dependent pathway (Rigby et al., 2019). PA-X of H5N1 subtype AIVs is a negative virulence regulator that can reduce virulence by inhibiting viral replication and host innate immune responses (Hu et al., 2015). In contrary, PA-X protein of H9N2 subtype AIVs, as a virulence factor, can improve the pathogenicity in mammals by enhancing virus replication, cell apoptosis, and lung injury (Gao et al., 2015), and affect the embryos lethality and lead to more rapid shedding and visceral tropism in birds (Clements et al., 2021).

Here, we used a PA-X protein-deficient virus based on A/chicken/Taixing/10/2010 (H9N2) to investigate the influence of PA-X protein on viral infectivity, phenotypic markers, cytokine expression levels, and the induced T cell proliferation in chBM-DCs, which will supply a better explanation of the pathogenic mechanism of H9N2 virus in chickens.

MATERIALS AND METHODS

Animal Care

Two-wk-old specific-pathogen-free (**SPF**) chickens were hatched from SPF eggs and raised for 2 wk (Sinopharm Yangzhou vac biological engineering company limited, Yangzhou, China). The protocols of all animal studies were approved by Jiangsu Province Administrative Committee for Laboratory Animals (approval number: SYXKSU-2021-0027) and complied with the guidelines of Jiangsu Province Laboratory Animal Welfare and the ethics of Jiangsu Province Administrative Committee of Laboratory Animals.

Cell Culture

Human embryonic kidney (**HEK**293T) cells and Madin-Darby canine kidney (**MDCK**) cells were cultured in Dulbecco's modified Eagle's medium (**DMEM**, Gibco, Grand Island, NE). Douglas Foster-1 (**DF-1**) cells were maintained in DMEM/F-12 medium (Gibco). These media contain 10% fetal bovine serum (**FBS**, Gibco) plus 1% penicillin/streptomycin (Invitrogen, Grand Island, NE). For chBM-DCs culture in vitro, we followed our previously established methods with some modifications (Liang et al., 2015). Briefly, chicken bone marrow precursors were extracted from the femur and tibia and isolated using lymphocyte separation medium (Sigma-Aldrich, St. Louis, MI). The cells were cultured in complete medium that RPMI 1640 (Gibco) containing 12% chicken serum (Gibco) and 1% penicillin/streptomycin, 10 ng/mL recombinant chicken GM-CSF (Abcam, Cambridge, UK, Cat: ab119158) and IL-4 (Abcam, Cat: ab222172). After the cells were cultured at 37°C for 72 h, the half medium was replaced by complete medium. A final concentration of 1 μg/mL lipopolysaccharide (**LPS**, Sigma-Aldrich) was added for 24 h. The stimulatory effects of LPS on chBM-DCs maturation were observed with ordinary light microscope (Olympus, Tokyo, Japan) to detect the colony and dendrite formation. And for the identification of phenotypic maturation of chBM-DCs before and after LPS stimulation, chBM-DCs were incubated with anti-human-CD40-PE (10 μg/1 $\times$ 10^6 cells, Bioss, Beijing, China) and anti-human-CD86-APC (10 μg/1 $\times$ 10^6 cells, Bioss) and their respective isotypes for 30 min under 4°C, and detected by flow cytometry (BD biosciences, Franklin Lakes, NJ) after washing twice with phosphate buffered saline (**PBS**).

Construction of PA-X-Deficient Virus

Eight plasmids of A/chicken/Taixing/10/2010 (TX) (Zhu et al., 2015), including polymerase basic protein 2 (**PB2**), PB1, polymerase acidic protein (**PA**), hemagglutinin (**HA**), nucleoprotein (**NP**), neuraminidase (**NA**), matrix protein (**M**), and nonstructural protein (**NS**), were inserted to pHW2000 vector, and then the 8 plasmids were co-transfected into the co-cultured MDCK and HEK293T cells using transfection reagent (Lipofectamine 3000, Thermo Fisher Scientific, Waltham, MA) to obtain the recombinant virus, named rTX strain (Hoffmann and Webster, 2000). Based on the improvement of the reported methods, the PA gene X-ORF 5′-UCCUUUCGU-3′ sequence of rTX strain was mutated to 5′-AGCUUUAGA-3′ using a point mutation kit (Vazyme, Nanjing, China) to knock down PA-X protein, and the mutated PA was co-transfected with other seven plasmids into the co-cultured MDCK and HEK293T cells to obtain the recombinant virus, named rTX-FS strain (Figure 1A; Jagger et al., 2012). After cytopathic changes were observed, the supernatant was collected and inoculated into SPF chicken embryos to propagate the virus (Li et al., 2014; Chen et al., 2021). Gene sequencing was performed to verify the rescued viruses. To exclude the activation of DCs by other proteins in the allantoic fluid, viruses were purified by centrifugation using discontinuous sucrose density gradients (Qin et al., 2015b).

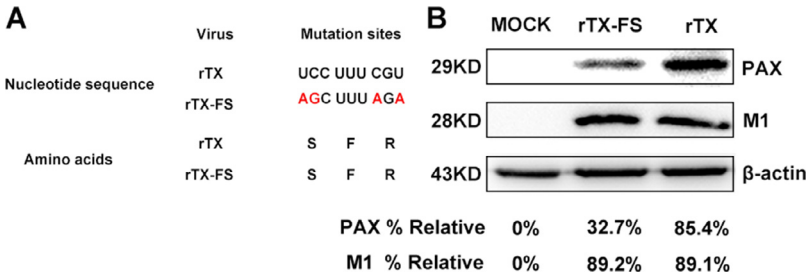

Figure 1. Generation of PA-X-deficient virus. (A) The frameshift motif of UCC UUU CGU was mutated in the PA gene to AGC UUU AGA (red); (B) The knock down of PA-X protein after mutation was verified by western blot (MOI of 10).

Western Blot

MDCK cells were inoculated with recombinant viruses for 12 h. The steps to viral infection were as follow. The cells were inoculated with the infection dose of 10 MOI for 1 h, then the cells were washed with PBS to remove unabsorbed virus particles. In order to make the recombinant virus replicate efficiently, DMEM medium containing trypsin with a final concentration of 2 μg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (**TPCK**) was added for 12 h. TPCK came from Worthington Biochemical (Lakewood, NJ). The total protein was extracted and separated by 12% SDS-PAGE vertical electrophoresis. Primary antibodies including rabbit anti-PA-X (1: 1,000, Biorbyt, Cambridge, UK), rabbit anti-M1 (1: 3,000, SinoBiological, Beijing, China), and mouse anti-β-actin (1: 10,000, Abcam) antibodies. Secondary antibodies including HRP-labeled goat anti-rabbit enzyme-conjugated secondary antibody (1:8,000) and goat anti-mouse enzyme-conjugated secondary antibody (1:8,000). Secondary antibodies were from Abcam. Protein bands were detected by electrochemiluminescence (**ECL**) (Biobest, Shanghai, China). Gray value analyses of PA-X or M1 expression relative to β-actin were detected by Image-Analysis J 1.51j8 software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD).

Viral Infection Level to ChBM-DCs and DF-1 Cells

After infecting chBM-DCs with viruses at 0.5 MOI for 48 h, the fixation/ permeabilization kit (BD biosciences) was used to permeabilized chBM-DCs. DCs were inoculated with mouse anti-NP-FITC antibodies at 1: 20 dilutions according to the manufacturer's instruction (Abcam) for 30 min under 4°C. After 30 min, cells were washed twice with PBS before detecting by flow cytometry. And for DF-1 cells, they were infected with viruses (0.01 MOI) and 200 μL supernatant was collected every 12 h until 72 h. When the 50% tissue culture infectious doses (**$TCID_{50}$**) test was performed, influenza virus-positive wells were identified using the HA assay (Jonges et al., 2010). Calculate $Log_{10}TCID_{50}$ per 0.1 mL using the Reed-Muench method as previously described (Reed and Muench, 1938).

Flow Cytometry

After infection with viruses at 0.5 MOI for 48 h, chBM-DCs were incubated with anti-human-CD40-PE, anti-human-CD86-APC, anti-chicken-MHCII-FITC at 1: 200 dilutions (Abcam) according to the manufacturer's instructions or their isotypes for 30 min under 4° C, and detected by flow cytometry after washing twice with PBS.

Total RNA Extraction and Quantitative RT-PCR

After infection with viruses at 0.5 MOI for 48 h, RNA was extracted from chBM-DCs. Briefly, total RNA was extracted with the HP total RNA kit (Omega Bio-Tek, Norcross, GA), RNA concentration was measured with Nanodrop 2000 (Thermo Fisher Scientific), and cDNA was generated with HiScript qRT SuperMix (Vazyme). The mRNA levels of cytokines were determined by amplification of IL-12, IL-1β, IFN-α, and IL-10 using qRT-PCR with the ChamQ SYBR qPCR Master Mix (Vazyme), and the primers are showed in Table 1. The program settings are as follows: pre-denaturation (95°C, 30 s), cyclic reaction (95°C, 10 s; 60°C, 30 s), melting section (95°C, 15 s; 60°C, 60 s; 95°C, 15 s).

Mixed Lymphocyte Reaction

Responder T cells from splenocytes of chicken were labeled with carboxyfluorescein succinimidylester (**CFSE**). CFSE came from Thermo Fisher Scientific.

Table 1. primers used in the qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
IL-12	TGGAACGATGAGACACCAGC	AGACAGGCAGGTGTAGTTGC
IL-1β	TCGACATCAACCAGAAGTGC	GAGCTTGTAGCCCTTGATGC
IL-10	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC
IFN-α	GACAGCCAACGCCAAAGC	GTCGCTGCTGTCCAAGCATT
β-actin	CCGCTCTATGAAGGCTACGC	CTCTCGGCTGTGGTGGTGAA

ChBM-DCs were infected with viruses at 0.5 MOI for 48 h. CFSE-labeled T cells were co-cultured with infected chBM-DCs with chBM-DC: T cell ratio =1: 5 for 5 d, and detected by flow cytometry.

Statistical Analysis

Results were showed as the means ± SD using GraphPad Prism 8 software (San Diego, CA). Unpaired Student's t tests were employed to compare the variance between the 2 groups. One-way ANOVA analysis of variance followed by LSD was employed to compare the variance between different groups via SPSS 17.0. $^{*}P < 0.05$ was considered significantly different, and $^{**}P < 0.01$ was considered highly significant different.

RESULTS

Generation of PA-X-Deficient Virus

We generated a PA-X-deficient virus (rTX-FS strain) based on the H9N2 TX strain by reverse genetic techniques. The western blot was used to investigate the expression level of PA-X protein in the lysates of cells infected with rTX or rTX-FS strain. As shown in Figures 1A and 1B, the PA-X expression relative to β-actin of rTX-FS strain carrying four nucleotide mutations in the PA gene showed 32.7%, which was lower than the rTX virus that was 85.4%. However, there was no change of M1 protein expression between rTX-FS and rTX with 89.2 vs. 89.1%, respectively, suggesting that the change of PA-X expression was not due to a reduced infection.

ChBM-DCs Were Isolated and Cultured Successfully In Vitro

To explore whether the morphology of the isolated and cultured chBM-DCs met the experimental requirements, we observed the cells with the microscope on d 7. Compared with the cells without GM-CSF and IL-4 addition (Figure 2A), GM-CSF and IL-4-induced chBM-DCs formed conspicuous colonies (Figure 2B) and dendritic structures (Figure 2C), which conformed to the morphological characteristics of chBM-DCs. Furthermore, CD40 and CD86 are essential co-stimulatory molecules of chBM-DCs, which reflect the ability of DCs antigen presentation (Wu et al., 2010). We found that CD40 expression dramatically enhanced ($P < 0.05$) and the expression level of CD86 also showed an upward trend after LPS induction, indicating that immature chBM-DCs can transform to mature chBM-DCs (Figures 2D and 2E). Based on the above results, the isolation and culture of ChBM-DCs had typical morphological characteristics and the function of phenotypic maturation, indicating that the culture method were established and modified successfully, and chBM-DCs can be used for the following experiments.

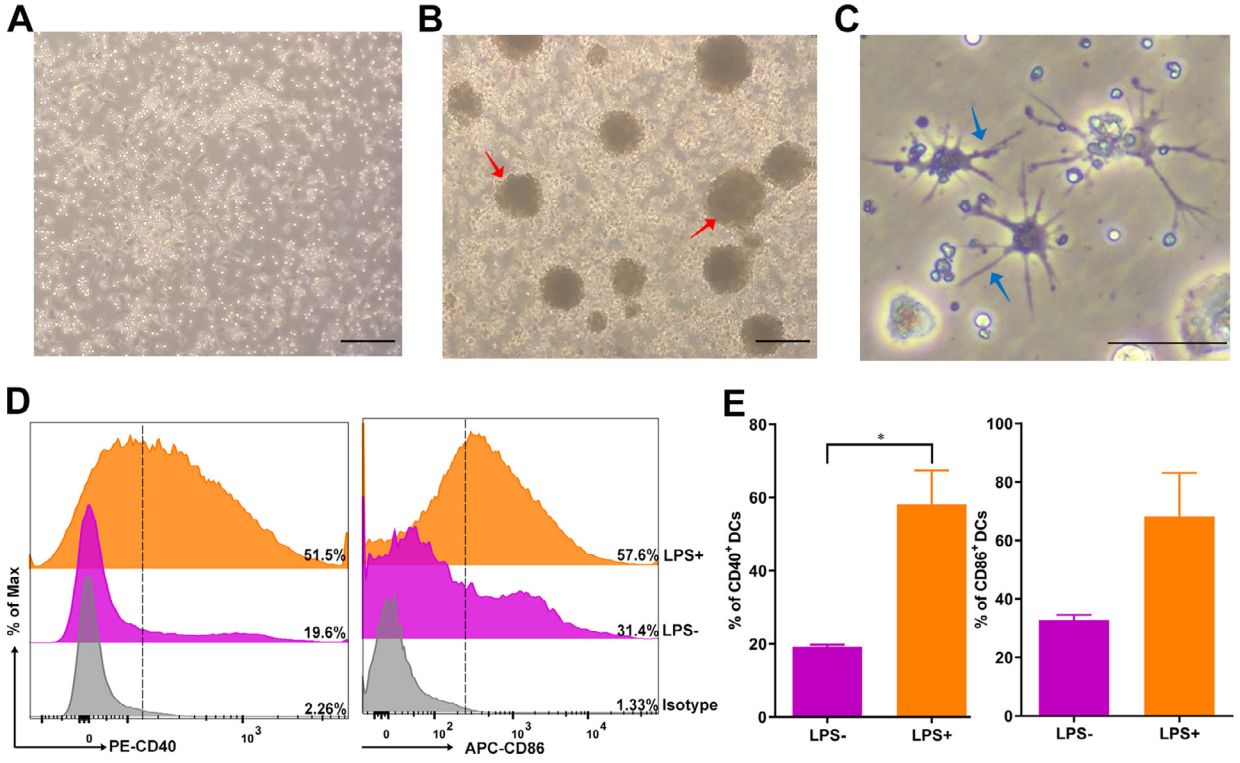

Figure 2. Morphological characteristics and phenotypic identification of chBM-DCs. (A−C) The colonies and dendrites of chBM-DCs were observed by ordinary light microscope on d 7; Scale bars = 50 μm. (A) No chGM-CSF and chIL-4-induced chBM-DCs; (B, C) ChBM-DCs induced by chGM-CSF and chIL-4; The red arrows point to the colonies of DCs (B) and blue arrows points to the dendrite of DCs (C). (D, E) Detection of CD40 and CD86 expression on LPS-induced chBM-DCs. (E) The statistics of flow cytometry results of Figure D. Results were showed as the means ± SD. $^{*}P < 0.05$.

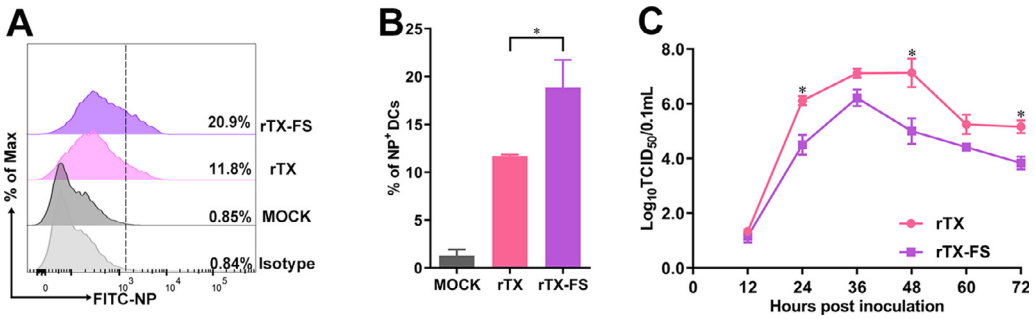

Figure 3. The infection levels of rTX and rTX-FS in chBM-DCs and DF-1 cells. (A, B) ChBM-DCs were infected with rTX or rTX-FS and collected to detect NP protein expression (an index of viral replication) by flow cytometry (MOI of 0.5). (C) Virus growth curve on DF-1 cells (MOI of 0.01). Results were showed as the means ± SD. $^*P < 0.05$.

PA-X Protein Inhibited the H9N2 Infection to ChBM-DCs But Not Non-immune Cells

The infection level of chBM-DCs with rTX or rTX-FS strain was evaluated, meanwhile, a chicken non-immune cell (DF-1) was introduced to compare the differences in infection trend. In chBM-DCs, the NP expression of rTX significantly decreased in comparison to that of rTX-FS ($P < 0.05$; Figures 3A and 3B), suggesting that PA-X protein attenuated the infection level of H9N2 virus in immune cells. However, in DF-1 cells, rTX showed a higher $TCID_{50}$ viral titer than rTX-FS (Figure 3C), indicating that PA-X protein enhanced the viral replication ability of H9N2 virus in non-immune cells. Collectively, the PA-X protein has the different influence on the viral replication of H9N2 subtype AIVs in the non-immune and immune cells.

PA-X Protein Impaired the Phenotypic Maturation of ChBM-DCs

Next, the phenotypic maturation of chBM-DCs after infection with rTX or rTX-FS was evaluated by flow cytometry. Compared with rTX-FS infected group, both CD86 and MHCII expression on the chBM-DCs were dramatically inhibited after rTX infection ($P < 0.05$). Meanwhile, CD40 expression also showed a similar trend (Figure 4). These results indicated that the

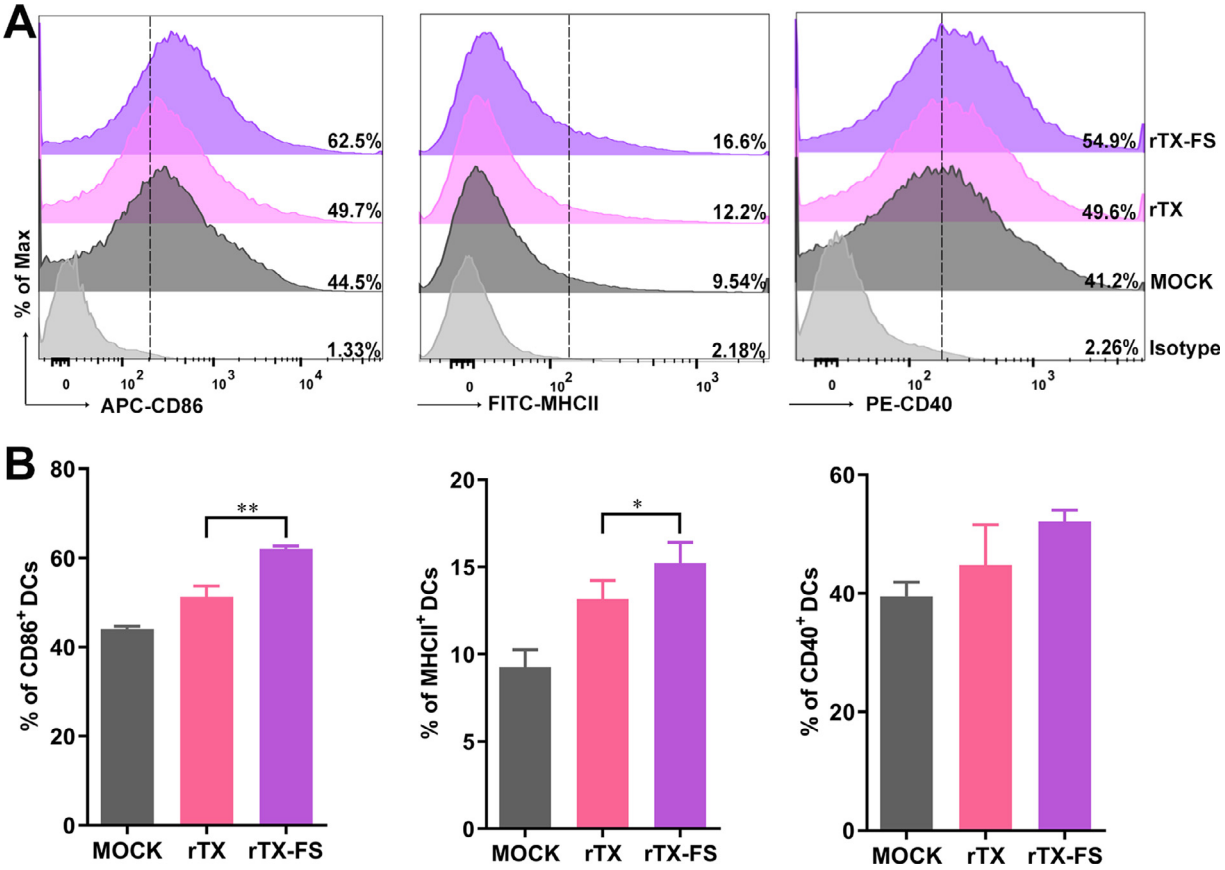

Figure 4. Phenotypic expression of chBM-DCs. (A) ChBM-DCs were infected with rTX or rTX-FS (MOI of 0.5), and CD86, MHCII, and CD40 expression were detected by flow cytometry. (B) Percentage of APC-$CD86^+$, FITC-$MHCII^+$, and PE-$CD40^+$ DCs in flow cytometry (A) was quantitated. Results were showed as the means ± SD.* $P < 0.05$; ** $P < 0.01$.

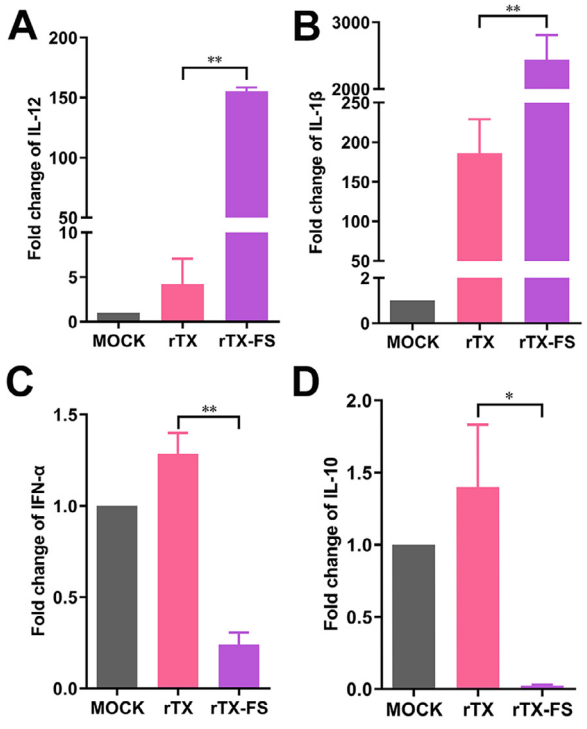

Figure 5. Cytokine mRNA levels of chBM-DCs. (A-D) ChBM-DCs were infected with rTX or rTX-FS (MOI of 0.5). The mRNA expressions of IL-12 (A), IL-1β (B), IFN-α (C), and IL-10 (D) were detected by qRT-PCR. Results were showed as the means ± SD. * $P < 0.05$; ** $P < 0.01$.

PA-X protein of H9N2 subtype AIVs decreased the phenotypic maturation of chBM-DCs.

PA-X Protein Suppressed the Proinflammatory Cytokine Expression of ChBM-DCs

Cytokine expression levels of ChBM-DCs after infection with rTX or rTX-FS were evaluated by qRT-PCR, the transcript expression levels of IL-12 and IL-1β after rTX infection were significantly decreased compared with rTX-FS ($P < 0.01$). Specifically, IL-12 level in the rTX group showed a 4.2-fold change while that in the rTX-FS group had a higher expression with 155.4-fold in comparison to the mock group (Figure 5A). Similarly, IL-1β level in the rTX group with 186.6-fold, while that in the rTX-FS group had a vigorous expression with 2441.0-fold than the mock group (Figure 5B). Although antiviral IFN-α and anti-inflammatory IL-10 expression was maintained at a low level (<2-fold) after viral infection, we found a significant upward of IFN-α and IL-10 after rTX infection compared with the rTX-FS infection group ($P < 0.05$; Figures 5C and 5D). Collectively, the PA-X protein of H9N2 subtype AIVs reduced the proinflammatory cytokine expression in chBM-DCs.

PA-X Protein Inhibited the ChBM-DCs Response to T Cells

Viruses-infected chBM-DCs were co-cultured with CFSE-labeled chicken T cells, and T cell proliferation level was detected using flow cytometry (Figure 6A). Compared with the rTX-FS group, rTX-infected chBM-DCs significantly decreased the T cell proliferation when the ratio of DC: T cell ratio is 1: 5 ($P < 0.01$), indicating the PA-X protein of H9N2 subtype AIVs inhibited the chBM-DCs response to the downstream T cells (Figures 6B and 6C).

DISCUSSION

Here, we explore the regulation role of PA-X protein on the host innate immune response of H9N2-infected chBM-DCs. PA-X protein of H9N2-infected chBM-DCs downregulated the infectivity, phenotypic expression (CD40, CD86, and MHCII) of chBM-DCs. Meanwhile, with the expression of PA-X protein, IL-12 and IL-1β decreased, whereas IFN-α and IL-10 increased. The mixed lymphocyte reaction showed that the chBM-DCs infected with virus contained high-expressed PA-X protein significantly inhibited T cells proliferation. Therefore, these findings indicated PA-X protein contributed to H9N2 subtype AIVs suppressing the innate immunity of chBM-DCs.

The immunosuppression of chickens is the main hazard of H9N2 viruses, leading to the failure of vaccine immunity or secondary infection of chickens. For instance, infection with H9N2 subtype AIVs can aggravate some clinical side effects of live attenuated vaccines that caused severe immunosuppression and immune organ damage in broilers, leading to a decreased effectiveness of Newcastle disease vaccines (Qiang and Youxiang, 2011). However, the mechanism by which H9N2 subtype AIVs lead to immunosuppression remains unclear. Therefore, to achieve an in-depth understanding of the influence on host immune cells, we successfully established an induction culture system of chBM-DCs in vitro, which provided a tool for studying the interconnection between avian pathogens and host immune responses. Some studies showed PA-X protein acts as a virulence factor for H9N2 subtype AIVs (Gao et al., 2015; Clements et al., 2021). Moreover, PA-X protein selectively only to degrade the host's RNA through its endonuclease activity, but not the virus itself (Hu et al., 2015; Nogales et al., 2017; Gaucherand, et al., 2019; Rigby et al., 2019). For non-immune cells, our results in DF-1 cells were consistent with the above studies, PA-X protein did not inhibit virus replication, implying a gene selectivity of host shut-off activity. However, for immune cells, we found that PA-X protein reduced the infection ability of H9N2 virus to the chBM-DCs and inhibited the immune functions of chBM-DCs. Unlike DF-1 cells for viral production, DCs have a powerful antigen presentation system that can process viral antigens to achieve specific immunity (Chen et al., 2018), implying that DCs are not suitable as a cellular factory for virus production. Therefore, the aim that PA-X protein reduced the viral infection could avoid the rapid and excessive activation of DCs for initiating downstream immune response. Moreover, we speculated that

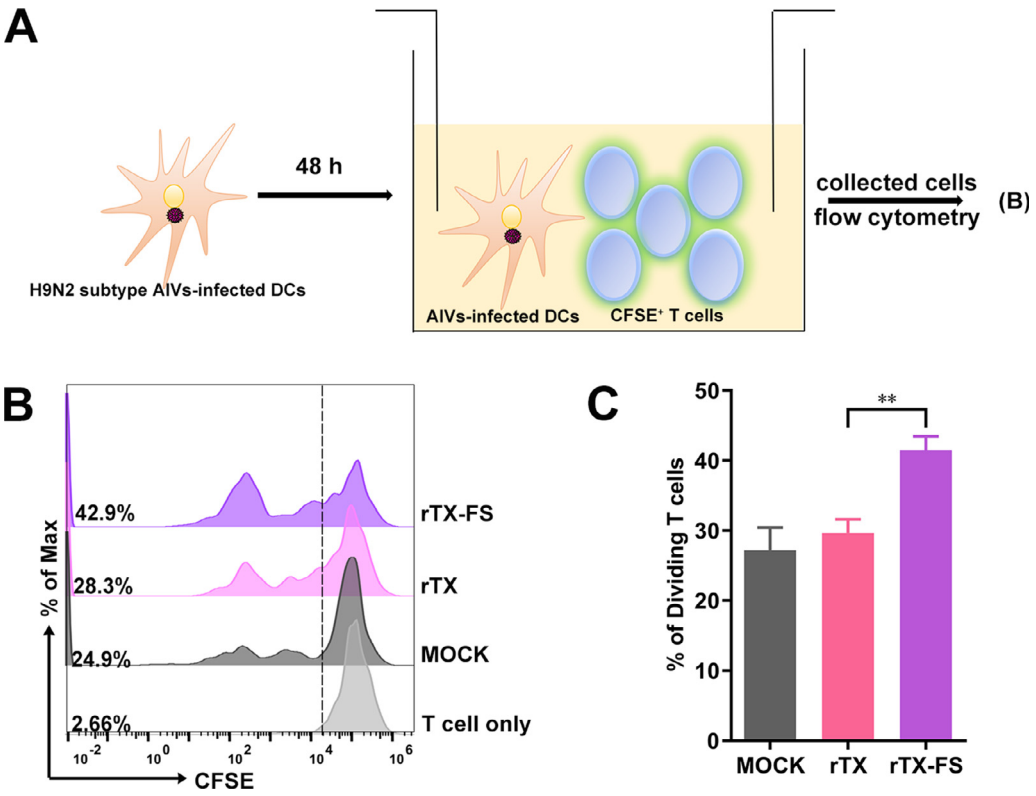

Figure 6. Mixed lymphocyte reaction. (A) rTX or rTX-FS-infected chBM-DCs (MOI of 0.5) were co-cultured with CFSE-labeled chicken T cells with chBM-DC: T cell ratio: 1: 5. (B) T cell proliferation was detected by flow cytometry on d 5. T cells only represented that the T cells is unstimulated by chBM-DCs (C) percentage of CFSE-T cells in flow cytometry (B) was quantitated. Results were showed as the means ± SD. ** $P < 0.01$.

a host cell selectivity of host shut-off activity of PA-X protein based on different cellular structure and function. This unique approach could help the H9N2 subtype AIVs escape from chBM-DCs for retardation of immune activation and thereby strengthen the virus production in non-immune, virus-productive cells through the differentiation regulation of host cells by PA-X protein.

CD40, CD86, and MHCII are the key phenotypes of DC maturation, among which CD40 and CD86 are co-stimulatory molecules (Ma and Clark, 2009; Trzupek et al., 2020), and MHCII as an antigen-presenting molecule, can transmit antigen signals to $CD4^+$ T lymphocytes and elicit specific immune responses (Holling et al., 2004). Our results showed PA-X decreased the phenotypic expression of H9N2 infected-chBM-DCs, which can inhibit the maturation of chBM-DCs. Some studies showed the virus could activate DCs immune function to perform antiviral function, which was usually used in immunotherapy. For instance, hepatitis B virus (**HBV**) vaccine can upregulate the expression of MHCII and CD86, thereby enhancing the effect of immunotherapy (Akbar et al., 1999). However, other studies showed the virus could reduce DCs immune function and achieve immune escape, which was consistence with our study. For instance, our previous study found that the infectious bursal disease virus (**IBDV**), an immunosuppression virus, can impair maturation and functions of chBM-DCs (Liang et al., 2015). After human cytomegalovirus (**HCMV**) infection, the antigen presentation, migration, and T cells activation of DCs were inhibited, resulting in the reduction of antiviral specific immunity and the inability to completely remove the virus, which leaded to viral immune escape and long-term latent infection (Andrews et al., 2005). PA-X protein has the function of host shut-off activity which can shut down the expression of host proteins (Hu et al., 2018). The mechanism of PA-X regulating chBM-DCs phenotype expression may be to shut down the expression of CD40, CD86, and MHCII proteins directly, or to inhibit the upstream transcription factor NF-κB (Giannoukakis et al., 2000; Hu et al., 2020).

Upon pathogen stimulation, mature DCs secrete cytokines, while the host shut-off activity of the PA-X protein affects cytokine expression (Chaimayo et al., 2018; Chen et al., 2021). We found PA-X protein reduced chBM-DCs cytokine mRNA levels, such as IL-12 and IL-1β with a large fold change. IL-12 promotes the differentiation of T helper cell 1 (**Th1**) responses in adaptive immunity (Hama et al., 2009). IL-1β also can induce the differentiation of Th1/17 cells for specific pathogenic microbes (Duhen and Campbell, 2014). Therefore, IL-12 and IL-1β played a significant part in initiating the downstream immune response. PA-X protein of H9N2 subtype AIVs could delay and limit the cascade of immune response via reducing IL-12 and IL-1β expression. The IFNs family regulates the innate immune response to viruses that IFNs signaling pathway can activate antiviral or immune-modulating proteins, thereby inhibiting viral replication (Muramoto et al., 2014). Despite IFN-α expression maintained at a low level after H9N2 virus infection, knocks down of PA-X

protein downregulated the level of IFN-α and increased the infectious ability in chBM-DCs, implying that PA-X protein showed a role of immune escape to avoid excessive activation of immune response via infection control strategy. IL-10 is an anti-inflammatory cytokine that regulates immune responses (Ip et al., 2017). The data showed PA-X protein did not decrease the IL-10 expression, which might also limit the immune activation for viral escape. A previous study reported although H1N1 subtype AIVs inhibited most host genes transcriptional expression, a small subset of transcripts was resistant to the host shut-off activity, suggesting the host shut-off activity of PA-X is selective but not global (Gaucherand et al., 2019). Our data showed the difference in cytokine expression, which may be due to the fact that H9N2 subtype AIVs could selectively inhibit host protein expression by host shut-off activity for viral immune escape.

As DCs mature, they become more capable of migrating to lymphoid tissues for amplifying immune responses through contact with other immune cells such as T cells, implying viral infection requires overcoming these immune functions of DCs (Qin et al., 2015a). Previous study found that DCs were hijacked by HCMV to impair the T cell proliferation, resulting in a long-term viral persistence (Sinclair, 2008). Consistent with the HCMV, PA-X protein hijacked H9N2-infected chBM-DCs to reduce the proliferation levels of T cells, which could possibly limit downstream adaptive immune response (Summerfield and McCullough, 2009).

In conclusion, PA-X protein impaired the immunity of H9N2-infected chBM-DCs, leading to an immune escape, which will supply a better explanation of the pathogenic mechanism of H9N2 virus. Alternatively, the deletion of PA-X protein can not only strongly activate DCs to achieve specific immunity, but also lead to a decrease in viral virulence, which is helpful for the development and application of H9N2 live-attenuated vaccines.

DISCLOSURES

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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