PA-X protein of H1N1 subtype influenza virus disables the nasal mucosal dendritic cells for strengthening virulence

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\textbf{ABSTRACT}

PA-X protein arises from a ribosomal frameshift in the PA of influenza A virus (IAV). However, the immune regulatory effect of the PA-X protein of H1N1 viruses on the nasal mucosal system remains unclear. Here, a PA-X deficient H1N1 rPR8 viral strain (rPR8-\textunderscore PAX) was generated and its pathogenicity was determined. The results showed that PA-X was a pro-virulence factor in mice. Furthermore, it reduced the ability of H1N1 viruses to infect dendritic cells (DCs), the regulator of the mucosal immune system, but not non-immune cells (DF-1 and Calu-3). Following intranasal infection of mice, CCL20, a chemokine that monitors the recruitment of submucosal DCs, was downregulated by PA-X, resulting in an inhibition of the recruitment of CD11b\textsuperscript{+} DCs to submucosa. It also attenuated the migration of CCR7\textsuperscript{+} DCs to cervical lymph nodes and inhibited DC maturation with low MHC II and CD40 expression. Moreover, PA-X suppressed the maturation of phenotypic markers (CD80, CD86, CD40, and MHC II) and the levels of secreted pro-inflammatory cytokines (IL-1\textbeta, IL-6, and TNF-\alpha) while enhancing endocytosis and levels of anti-inflammatory IL-10 \textit{in vitro}, suggesting an impaired maturation of DCs that the key step for the activation of downstream immune responses. These findings suggested that the PA-X protein played a critical role in escaping the immune response of nasal mucosal DCs for increasing the virulence of H1N1 viruses.

\textbf{Introduction}

Influenza A virus (IAV) has eight negative-sense RNA segments, which encode at least ten proteins [1], and then several novel viral proteins were found, including PB1-F2, PB1-N40, PA-X, and PB2-S1 [2,3]. Seasonal influenza epidemics are estimated to cause lots of infection deaths annually, with significant social and economic impact [4]. In addition to seasonal outbreaks, influenza pandemics also occur unpredictably at different intervals [4]. Influenza pandemics have occurred every 10–50 years since the 16th century, the worst of which was the 1918 Spanish flu (H1N1 virus) pandemic [5]. Despite the origin of the virus that caused the 1918 pandemic is still debated, all eight RNA segments of the causative virus were derived from avian influenza viruses (AIVs) [6]. The H1N1 influenza virus that caused the 2009 pandemic (pH1N1) is a triple-reassortant virus containing genes from human, swine, and avian viruses that can transmit to humans and pigs [7,8]. Following the initial pandemic, the emerging influenza viruses continue to circulate as IAVs undergo antigenic drifts and antigenic shifts. Given the consequences of the emergence of pandemic strains, enhanced surveillance of IAVs and studies of the mechanisms underlying their pathogenicity are warranted.

The mucosa of the respiratory tract is an important first barrier that protects the host from pathogenic microorganisms and harmful substances [9]. Importantly, mucosal dendritic cells (DCs) play a significant part in monitoring IAV invasion, linking the innate and adaptive immune systems [10]. After DCs take up IAVs from the nasal cavity, they quickly transform from the immature to the mature state and transport IAVs to nearby draining lymph nodes such as cervical lymph nodes (CLNs). They not only present antigens and activate T cells [11] but also amplify the
immune response cascade through cytokine networks [12]. The ability of DCs to present viruses increases following the infection of viruses, initiating the immune response that defends against viral infection [13,14]. In early stages of influenza infection, inflammatory DCs can be recruited to the trachea by chemokine CCL2 and activated via SIGN-R1 to recruit natural killer (NK) cells to trachea [15]. On the contrary, human respiratory syncytial virus (RSV) can inhibit dendritic cell maturation, which resulted in the decrease of antigen presentation and T cell activation, and incomplete immunity to RSV reinfections [16]. In addition, the 2009 pH1N1 influenza virus was associated with severe depletion of DC subsets [17]. However, it remains unclear that how intranasally infecting H1N1 viruses regulate the immune function of nasal DCs.

IAVs can easily mutate due to the structural characteristics of their segmented RNA, facilitating immune escape. A new fusion protein named PA-X, which contains 191 amino acids derived from the N-terminus of the PA protein and an open reading frame (X-ORF) formed by a ribosomal frameshift in the PA gene was recently identified [18]. Multiple studies showed that PA-X reduced virulence of H5N1 viruses, which are highly pathogenic (HP) [19]. However, PA-X protein played the contrasting part in low pathogenic (LP) IAVs. Analysis of the H1N1 virus, CA09 (A/California/04/2009), showed that PA-X protein increased the viral replication and pathogenicity in murine lungs [20,21]. PA-X protein also enhanced the pathogenicity of H1N1 strain PR8 (A/Puerto Rico/8/1934) in chicken embryos [22]. In addition, PA-X protein promoted virulence by attenuating the innate immune response induced by H1N2 subtype swine influenza viruses (SIVs) [23]. Regarding the underlying mechanism, PA-X has a global host-shutoff activity that is manifested as a sharp decline in host protein synthesis, but not viral genes, following viral infection [19,24–26]. The gene shutoff by PA-X is mainly associated with inflammation, immune response, and apoptosis [18,19]. PA-X protein enhanced IFN-β mRNA expression and anti-haemagglutinin neutralizing antibodies production in mice infected with H1N1 viruses [20]. Therefore, it is possible that PA-X is an important virulence factor in H1N1 viruses as it regulates host immunity. However, the influence of PA-X protein on the viral pathogenicity and host immunity as well as the detailed mechanisms underlying its function in H1N1 viruses remain unclear.

Here, we constructed H1N1 rPR8 and rPR8-∆PAX using reverse genetics technology and used them to evaluate H1N1 pathogenicity in mammals. We also analysed the function of PA-X protein in regulating the nasal mucosal immune system, particularly the crucial component, mucosal DCs. Our findings will help elucidate the infection and immune mechanisms associated with H1N1 viruses.

Materials and methods

Ethics statement

All animal experiments were carried out according to the laboratory animal welfare and ethical guidelines of Jiangsu Administrative Committee for Laboratory Animals, under the authority of Jiangsu Administrative Committee for Laboratory Animals (Permission number: SYXKSU-2017-0044).

Animals

Specific-pathogen-free (SPF) chicken embryos were purchased from Zhejiang Lihua Agricultural Technology Company Limited (Zhejiang, China) and incubated in our laboratory until they were 10 d old. SPF BALB/c and C57BL/6 mice were purchased from Yangzhou University (Center Comparative Medical, Yangzhou, China).

Viruses and cells

H1N1 PR8 strain (A/Puerto Pico/8/1934) [27] was stored in our laboratory. Human embryonic kidney cells (HEK293T), MDCK cells, and Calu-3 cells (alternative models of nasal epithelial cells [28,29]) were cultured in DMEM (Gibco, NY, USA). Douglas Foster-1 (DF-1) cells were cultured in DMEM/F-12 (Gibco, NY, USA). Chicken embryo fibroblast (CEF) cells isolated from embryonated eggs were maintained in Medium 199 (Hyclone, Waltham, USA). All media were supplemented with 10% foetal bovine serum (FBS; Gibco, NY, USA) containing 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, NY, USA). All cells were maintained at 37°C and 5% CO₂.

Virus rescue

The gene encoding the PA protein in strain rPR8, X-ORF 5'-UCCUUUCGU-3', was mutated into 5'-AGUUUCAGA-3' to develop the knockout PA-X protein following a previously described protocol [18,30]. To obtain rescued viruses based on our established reverse genetics approaches [31], plasmids from the
virus were added to dishes plated with HEK293T and 
MDCK cells and the virus was harvested after most of 
the cell lesions had fallen off. Mutant viruses were 
identified using haemagglutinin (HA) titre test, sequen-
cing, and western blotting. Viruses were purified using 
the density gradient centrifugation as previously 
reported [32].

**Western blotting assay**

MDCK cells were infected with rPR8 and rPR8-ΔPAX 
strains and cultured. The cells were collected 12 h post 
infection (h. p. i.), when the multiplicity of infection 
(MOI) was 10. Proteins were extracted, separated on 
12% gels using SDS-PAGE, and transferred onto nitro-
cellulose membranes (Cytiva, Washington, USA). 
The blots were blocked using 5% skim milk (Applygen, 
Beijing, China) and then incubated overnight at 4°C 
with the following primary antibodies: anti-PA-X 
(1:1000) (Biorbyt, Cambridge, England), anti-NP 
(1:3000) (Genetex, Texas, USA), and anti-β-actin 
(1:10000) (Abcam, Cambridge, England). The mem-
brane was then incubated with horseradish peroxidase 
(HRP)-conjugated secondary antibodies (1:8000) 
(Abcam, Cambridge, England). Protein bands were 
detected using an electrochemiluminescence (ECL) 
detection system.

**Virus replication kinetics**

Different cell types, including Calu-3 and DF-1, were 
incubated at a MOI of 0.01. Virus titres were deter-
mined using 50% tissue culture infectious doses 
(TCID<sub>50</sub>). CEF cells were infected with supernatants, 
which were harvested every 12 h for 72 h [31]. HA assay 
was used to identify influenza virus-positive wells [33]. 
TCID<sub>50</sub> values were calculated using the method of 
Reed and Muench [34].

**Pathogenicity study in mice**

Once the rPR8 and rPR8-ΔPAX infection doses were 
10<sup>3</sup>-10<sup>6</sup> EID<sub>50</sub>/50 μL, clinical symptoms and body 
weight changes in six-week-old SPF BALB/c mice 
were recorded, and survival noted to detect 50% 
mouse lethal dose (MLD<sub>50</sub>) (n = 5). The lungs were 
extracted and fixed with paraformaldehyde for histo-
 pathological observation 5 d post-infection (d. p. i.) (n 
= 3). Sections were randomly numbered (after scram-
bling the group numbers) and photographed. Each 
photograph was scored for pathology by two trained, 
double-blinded pathologists, following a previously 
described method [35]. For virus titres, grind each 
sample and take 0.1 mL of supernatant after centrifuga-
tion at 6,800 rpm for 10 min [36]. Chicken embryos 
were inoculated in a 1:10 dilution, with four embryos 
per concentration, and observed for 72 h. Viral titres 
were calculated as described previously [34].

**In vitro DCs culturing**

Murine bone marrow-derived DCs were isolated and 
cultured as previously described [37]. The femurs and 
tibias of C57BL/6 mice were aseptically harvested, 
washed repeatedly until they turned white, and the 
washing fluid collected. The cells were maintained in 
complete RPMI 1640 medium containing 10% FBS, 
100 U/mL penicillin, 100 μg/mL streptomycin, and 10 
ng/mL cytokines (IL-4 and GM-CSF, Peprotech, Rocky 
Hill, USA). After culturing for 60 h, the medium was 
replaced with fresh complete medium. Mature DCs 
with a purity greater than 90% were harvested on the 
fifth day and used in subsequent experiments.

**Analysis of the infectivity to DCs in vitro**

When the MOI was 0.5 (at 6, 48, and 72 h p. i.), the 
expression of NP of infected DCs was measured using 
flow cytometry (FACS) after treating with fixed and per-
meabilized kits (BD biosciences, NY, USA). FITC-NP 
antibodies were purchased from Abcam (Cambridge, 
England).

**Measuring the DCs migration into the nasal 
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When rPR8 and rPR8-ΔPAX infection doses were 10<sup>7</sup> 
EID<sub>50</sub>/50 μL (at 3 h. p. i.) (n = 3), the brain and nasal-
associated lymphoid tissue (NALT) of six-week-old SPF 
C57BL/6 mice were removed, and single nasal cells were 
detached using 0.5% type XIV protease, 0.1% collagenase 
type IV, and 0.1% hyaluronidase, which were from Sigma-
Aldrich (Missouri, USA). A total of 10<sup>7</sup> cells were collected 
using a 100-mesh sieve [27]. Cells were mixed with APC-
Cy7-FVS (used to identify living/dead cells), PerCP-Cy5. 
5-CD11c, PE-CD103, APC-CD11b, and their respective 
isoatypes, and detected using FACS.

**Cryosection and confocal observation**

When the infection doses of rPR8 and rPR8-ΔPAX was 10<sup>7</sup> 
EID<sub>50</sub>/50 μL at 3 h. p. i. (n = 3), the nasal cavity of six-week-
old SPF C57BL/6 mice were separated, placed in a 4% 
paraformaldehyde solution. After 12 h, the paraforma-
dehyde solution was discarded and the nasal tissue put into 
a decalcification solution. After 7 d, the tissue was
embedded in OCT embedding medium (California, USA), and cut into frozen sections. For immunofluorescence staining, the samples were placed in 0.4% Triton X-100 and incubated for 5 min before adding 5% bovine serum albumin for 1 h. Armenian hamster anti-CD11c primary antibody (1:500) was added and mixed with rabbit anti-CCL20 (MIP-3α) pAb (1:500) and mouse anti-CD11b mAb (1:500) overnight and then incubated with goat anti-Armenian hamster IgG (H+L) Alexa Fluor 649 (1:1000), goat anti-rabbit IgG (H+L) Alexa Fluor 594 (1:1000), and rabbit anti-mouse IgG (H+L) Alexa Fluor 488 (1:1000) for 1 h. DAPI was added and the samples incubated for 5 min. Primary antibodies and DAPI were purchased from Abcam (Cambridge, England), and secondary antibodies were purchased from MultiSciences (Hangzhou, China). A Leica TCS SP8 STED confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to observe the cryosections and Image-Pro Plus 6.0 was used to analyse the data [27].

Chemokine expression in Calu-3 cells was analysed using qRT-PCR as previously described [38], when the infection was 0.01 MOI for 6 and 24 h. In brief, after using the RNA extraction kit (Vazyme, Nanjing, China) to extract the RNA of Calu-3 cells according to the instructions, the CCL20 gene in the RNA was amplified with the designed CCL20 gene primers. Primer sequences (5’-3’) were as follows: CCL20 (F: ACTGAGGAGACGCACAATATAT; R: TGTACCAAGAGTTTGCTCCTGG). GAPDH (F: GTCTTCTCTGACTTCAACAGCG; R: ACCACCC TGTTGCTGTAGCCAA).

Migration assay of DCs in vitro

When DCs were infected with the infection dose of 0.5 MOI for 24 h, cells were diluted to 1 × 10^6 cells/mL. A 0.6 mL volume of diluted CCL19 (final concentration 200 ng/mL) was added to a 24-well plate, the chamber (5 μm pore size; Corning, NY, USA) placed into the plate, and diluted DCs added to the chamber. After incubation at 37°C for 4 h, cells on the basal side were counted. A control group was set up consisting of 0.6 mL RPMI 1640 medium without CCL19 added to the lower chamber and 0.1 mL of uninfected DCs added to the upper chamber [37].

Migration ability of the infected DCs to CLNs and their phenotype levels

rPR8 and rPR8-ΔPAX infection doses were 10^7 EID_{50}/50 μL, at 6 h. p. i., the CLNs of six-week-old SPF C57BL/6 mice were extracted and placed on a sterile plate containing PBS and passed through a 100-mesh sieve [39]. A total of 10^7/100 μL cells were collected and mixed with APC-Cy7-FVS (used for identifying living/dead cells), APC-CD11c, PE-Cy7-CCR7, and their respective isotypes. DCs were incubated with FITC-NP to detect their infectivity in the CLNs. To detect DC phenotypes, DCs were mixed with PE-CD40 and FITC-MHC II and detected using FACS.

Analysis of endocytosis, phenotypes, activation, and migration of DCs

When the MOI was 0.5, the morphology of DCs in different groups was observed under an optical microscope at 24 h. p. i. A positive control consisting of 100 ng/mL lipopolysaccharide (LPS)-stimulated DCs was included. LPS was obtained from Sigma-Aldrich (Missouri, USA). DCs were randomly selected and their lengths measured. The morphology index was calculated from 30 DCs randomly selected from five separate experiments, with six cells from each experiment (morphological index = longest axis/shortest axis) [40]. To determine endocytosis, cells were incubated with 1 mg/mL FITC-dextran (Sigma-Aldrich, Missouri, USA) with LPS as a positive control at 37°C for 30 min [40] and detected via FACS. Phenotypes, activation, and migration of DCs were determined by FACS after incubating the cells with various antibodies, including PE-CD80, FITC-CD86, PE-CD40, FITC-MHC II, PerCP-Cy5.5-CD69, and PE-CCR7 (BD Biosciences, NY, USA).

Detection of cytokines

When the MOI was 0.5 (at 24 h p. i.), the levels of IL-1β, IL-6, TNF-α, and IL-10 from the supernatants of DCs were measured using ELISA kits (Multisciences, Hangzhou, China) with the manufacturer’s instructions [38].

Statistical analysis

Data are expressed as mean ± standard deviation (mean ± SD) by GraphPad Prism 9 software (San Diego, CA). Unpaired two-sided student’s t-test was used to measure statistical significance between the two groups, and one-way ANOVA was used to test the significance of differences among groups. Analyses were conducted using SPSS 17.0. *P < 0.05, **P < 0.01.

Results

PA-X protein of H1N1 viruses enhanced pathogenicity in mammals

First, we rescued the recombinant virus, rPR8-ΔPAX, lacking the PA-X protein according to the strategy shown in Figure 1a. To determine the stability of
rescued viruses, chicken embryos were inoculated for five consecutive passages. The sequencing results were correct (no other mutations were generated), and the HA titre was stable and higher than 10 Log2 (Figure S1, Table S1). Western blotting results showed that PA-X protein expression in rPR8-ΔPAX was significantly downregulated compared with PA-X expression in rPR8 (Figure 1b). Viral replication ability was also compared in avian original cells (DF-1 cells), as well as mammalian cells (Calu-3 cells). Virus growth curves showed that the replication levels of rPR8 in DF-1 cells had an upward trend compared with the replication levels of rPR8-ΔPAX, particularly in later stages of infection (Figure 1c). For Calu-3 cells, a replacement model for nasal epithelial cells [28,29], during the whole infection period the replication level of rPR8 was significantly higher than rPR8-ΔPAX (P < 0.01) (Figure 1d). Mice were intranasally inoculated at the dose of 10^5–10^6 EID_50 in 50 μL PBS with each virus to assess the influence on pathogenicity in vivo (Figure 1e). Weight loss and mortality results showed that mice died when inoculated with 10^5 EID_50/50 μL of rPR8 (Figures 1f,h,i), while mice inoculated with rPR8-ΔPAX started to die once they were challenged

**Figure 1.** *In vitro* and *in vivo* analysis of biological characteristics of rescued viruses. (a) Schematic diagram of H1N1 PA-X-deficient viruses. The red letters represent nucleotides at the mutation sites. (b) PA-X, NP, and β-actin expression was measured using western blotting assay (MOI of 10 at 12 h p. i.). (c-d) Growth curves of rPR8 and rPR8-ΔPAX viruses in DF-1 (c) and Calu-3 (d) cells with an infection dose of 0.01 MOI at 72 h. p. i. Analysis was based on unpaired two-sided student’s t-test. (e-i) Six-week-old SPF BALB/c mice were inoculated with rPR8 and rPR8-ΔPAX (10^5–10^6 EID_50/50 μL doses). Body weights and survival were recorded in rPR8-infected (f, h) and rPR8-ΔPAX-infected (g, i) mice over a 14-d period (n = 5). (j) Virus replication in lungs (n = 3). (k) H&E staining of lung sections (n = 3). Scale bar = 100 μm. (l) Histopathological scores of lungs on 5 d. p. i. (n = 3). Data are presented as mean ± SD. One-way ANOVA was used to measure statistical significance. *P ≤ 0.05, **P < 0.01. MOI, multiplicity of infection; SPF, specific-pathogen-free; h. p. i., hours post infection; d. p. i., days post infection; ANOVA, analysis of variance.
with $10^4$ EID$_{50}$/50 μL of the virus (Figures 1g, i). The MLD$_{50}$ of rPR8 was $10^{3.66}$ EID$_{50}$/50 μL, while that of rPR8-ΔPAX was $10^{4.37}$ EID$_{50}$/50 μL, indicating an enhanced virulence of the PR8 strain possessing the PA-X protein. Similarly, the virus replication results showed that the viral EID$_{50}$ titre of rPR8 was higher than that of rPR8-ΔPAX ($P < 0.05$) on 5 d. p. i. (Figure 1j), indicating increased rPR8 viral load in the lungs. Histopathological analysis based on H&E staining showed that rPR8 caused severe interstitial pneumonia with massive haemorrhage and extensive infiltration of inflammatory cells. However, the pathological changes in rPR8-ΔPAX-infected lungs were alleviated (Figures 1k, l). In summary, PA-X protein of H1N1 viruses enhanced the mammalian pathogenicity.

**PA-X protein of H1N1 viruses inhibited the viral infection ability to mucosal DCs**

NP protein expression was detected using FACS, and compared with rPR8-ΔPAX, the ability of rPR8 to infect DCs was reduced, particularly 6 and 48 h. p. i. ($P < 0.05$) (Figures 2a, b). In vivo studies showed that the number of mucosal CCR7$^+$ CD11c$^+$ DCs migrating to CLNs decreased after mice were intranasally infected with rPR8 (Figures 2c, d); meanwhile, the level of NP protein in these migrated DCs was also decreased in comparison with rPR8-ΔPAX group ($P < 0.05$) (Figures 2e, f). In conclusion, the PA-X protein of H1N1 viruses inhibited the ability of viruses to infect the mucosal DCs.

**PA-X protein of H1N1 subtype IAVs inhibited CCL20 expression to reduce the DC mobilization to the nasal submucosa**

Analysis of rPR8 and rPR8-ΔPAX strains at 3 h. p. i. (infection dose of $10^7$ EID$_{50}$/50 μL) using FACS demonstrated that the number of DCs recruited to the nasal mucosa of rPR8-infected mice was lower, in particular, both CD11b$^+$ and CD103$^+$ subtypes of DCs were dramatically reduced compared with rPR8-ΔPAX group ($P < 0.05$) (Figures 3a-e). Notably, more CD11b$^+$ CD11c$^+$ DCs were mobilized compared with CD103$^+$ CD11c$^+$ DCs. Furthermore, observation under a confocal microscope revealed that rPR8-infected nasal epithelial cells secreted less chemokine CCL20 to recruit CD11b$^+$ CD11c$^+$ DCs to the nasal submucosa in comparison to the rPR8-ΔPAX group ($P < 0.01$) (Figures 3f-h), in line with *in vitro* results which showed that the rPR8 virus possessing PA-X protein significantly downregulated the expression of CCL20 compared with rPR8-ΔPAX at both 6 and 24 h. p. i. ($P < 0.01$) (Figures 3i-j). Collectively, PA-X protein of H1N1 viruses inhibited the expression of CCL20 in the nasal mucosa, subsequently suppressing the ability to rapidly mobilize DCs, the key cells involved in initiating mucosal immune responses against IAVs.

**PA-X protein of H1N1 viruses repressed the DC maturation and migration to nearby draining lymph nodes**

CCR7 is a migration marker that guides DCs to nearby draining lymph nodes [41]. CCR7 expression in rPR8-infected DCs with an infection dose of 0.5 MOI at 24 h. p. i. was significantly downregulated compared with expression in rPR8-ΔPAX-infected DCs ($P < 0.05$) (Figures 4a, b). Furthermore, a transwell system was established to simulate the migration capability of DCs towards nearby draining lymph nodes. As shown in Figures 4c, d, the number of rPR8-infected DCs migrating toward CCL19 was reduced markedly ($P < 0.01$). Agreed with the results *in vitro*, FACS results demonstrated the number of CCR7$^+$ CD11c$^+$ DCs migrating to CLNs of the mice infected with rPR8 was decreased compared with rPR8-ΔPAX group (Figures 4e, f). In the rPR8 infected group, MHC II expression was significantly downregulated ($P < 0.01$) in the migrated DCs. Similar trends were observed when the other phenotypic marker, CD40, was analysed (Figures 4g, h). Collectively, PA-X protein of H1N1 viruses decreased the ability of DCs to migrate to CLNs and inhibited the phenotypic maturation of DCs.

**PA-X protein of H1N1 viruses enhanced endocytosis and suppressed phenotypic maturation of DCs**

Mature DCs form long extensions much easier than immature DCs [40]. We found that the cell shape index of DCs infected with the rPR8 strain possessing the PA-X protein was lower than that of the rPR8-ΔPAX strain ($P < 0.05$) where PA-X protein was knocked down (Figures 5a, b). Compared with mature DCs, immature DCs can take up antigens more efficiently through endocytosis capacity [42]. Our results showed that rPR8 increased the endocytosis of FITC-labelled dextran in DCs ($P < 0.01$) (Figures 5c, d), suggesting that DCs hardly developed into their mature state. The phenotypic maturation of DCs is characterized by CD80, CD86, CD40, and MHC II [38];
therefore, we inoculated DCs with PR8 or rPR8-ΔPAX with the infection dose of 0.5 MOI at 24 h. p. i., and detected the expression of phenotypic markers by FACS. Compared with rPR8-ΔPAX, the CD80, CD86, CD40, and MHC II expression of rPR8 were significantly decreased ($P < 0.01$) (Figures 5e, f), indicating that PA-X protein of H1N1 viruses inhibited the phenotypic maturation of DCs.

**Figure 2.** Determining the ability of viruses to infect DCs. (a, b) NP expression on DCs was detected at 6, 48, and 72 h. p. i. (MOI of 0.5). (c-f) When the infection dose was $10^5$ EID$_{50}$ in 50 μL PBS, the infectivity of DCs in murine CLNs was detected by FACS. (c) G1 represents CCR7$^+$ CD11c$^+$ DCs in living cells. (d) FACS statistic of (c), the proportion of CCR7$^+$ CD11c$^+$ DCs. (e) is from G1 for analysing the level of NP protein. (f) FACS statistic of (e) based on the MFI. Data are presented as mean ± SD. One-way ANOVA was used to measure statistical significance. *$P<0.05$, **$P<0.01$. DCs, dendritic cells; MOI, multiplicity of infection; h. p. i, hours post infection; PBS, phosphate buffered saline; CLNs, cervical lymph nodes; FACS, flow cytometry; MFI, mean fluorescence intensity; ANOVA, analysis of variance.
Figure 3. In vivo recruitment of DCs into the nasal mucosa. (a-e) Once infection reached $10^7$ EID$_{50}$ in 50 μL PBS in mice, the number of DCs recruited to the nasal mucosa was measured. (a-b) Recruitment of DCs into the nasal mucosa was measured using FACS at 3 h. p. i. (a) G1 represents CD11c$^+$ DCs from living cells. (b) G2 or G3 from G1 represent CD11b$^+$CD11c$^+$ or CD103$^+$CD11c$^+$ DCs, respectively. (c-e) FACS statistic of (a) and (b), the proportion of CD11c$^+$ DCs (c), CD11b$^+$CD11c$^+$ DCs (d), and CD103$^+$CD11c$^+$ DCs (e).
**PA-X protein of H1N1 viruses inhibited the pro-inflammatory cytokine response of DCs**

When DCs were infected with the dose of 0.5 MOI for 24 h, CD69 expression was enhanced, indicating that DCs were activated ($P < 0.01$) (Figures 6a, b). Furthermore, the levels of secreted IL-1β, IL-6, and TNF-α in rPR8-infected DCs were dramatically lower compared with their levels in rPR8-ΔPAX-infected cells ($P < 0.01$), whereas IL-10 secretion was
significantly higher \( (P < 0.05) \) (Figure 6c). Collectively, PA-X protein of H1N1 viruses inhibited the pro-inflammatory cytokine response of DCs.

**Discussion**

Here, our study analysed the immune escape functions of PA-X protein on the innate immunity of mammalian hosts from the perspective of mucosal DCs, which laid the foundation for understanding the pathogenic mechanism of H1N1 viruses. Our findings suggested that PA-X protein of H1N1 viruses (PR8) increased the infection to the virus-productive cells (DF-1 and Calu-3) but not immune cells (DCs). Moreover, PA-X protein inhibited the CCL20 secretion from mucosal ECs and thereby retarded the recruitment of CD11b+ sub-type DCs to the submucosa. Meanwhile, PA-X protein attenuated the migration of CCR7+ DCs to CLNs and further impaired the maturation of DCs for the suppression of downstream immune responses. Finally, the viral pathogenicity was enhanced in mice based on the immune escape mechanism of PA-X (Figure 7).
Multiple functions of PA-X protein in the pathogenicity and host immunity have been reported [43]. Previous studies found no differences in pathogenicity of H1N1 virus in mice infected with H1N1 PR8 viruses possessing PA-X protein within 48 h [24], although other studies have shown that PA-X protein can increase the virulence of PR8 in chicken embryos [22]. However, the virulence induced by the PA-X protein of PR8 viruses in mice has not been fully elucidated. Therefore, a systematic study of pathogenicity in mice was carried out using the MLD50, lung titres, and histopathological observations. Our murine pathogenicity assay indicated that the PA-X protein of PR8 H1N1 viruses acts as a virulence factor, consistent with results from previous analyses of Ca09 (A/California/04/2009) and H1N2 SIVs [20,21,23]. In contrast, other studies have shown that the PA-X protein is a negative regulator of virulence in some HP strains such as H5N1 viruses [19], suggesting that PA-X may play different roles in virulence. DCs are “sentinels” of pathogens and leaders of the innate immune response [32]. PA-X protein of H1N1 viruses inhibited DC infection both *in vitro* and *in vivo* but increased its replication in DF-1 and Calu-3 cells and viral pathogenicity in mice, indicating that viral replication differed in immune and non-immune cells. One possible reason is that DCs, as professional antigen-presenting cells (APCs), have more complex internal structures than non-immune cells, including a strong lysosomal system that may impact viral replication [44]. Another possible explanation for this discrepancy was the host shutoff activity of the PA-X protein, which impaired the antiviral responses by inhibiting host protein synthesis [19]. Non-immune cells are the targets of infection and perform the productive infection. Therefore, the host shutoff activity could turn off other host genes, but not virus genes in the viral productive cells. However, in our study, it was speculated that the PA-X protein not only shut down the host gene expression but also the viral gene expression in DCs, which might reduce the activation of DCs for suppressing immunity, and obtain a long-term replication in productive non-immune cells. In addition, the number of NP-expressed DCs that migrated to CLNs decreased for inhibiting the activation of downstream immune responses through cascade amplification and then enhancing infection and pathogenicity through delayed immune responses.
DCs constituted the first line of defence of the mucosal immunity [45]. On one hand, antigens stimulate the secretion of a series of cytokines that regulate innate immunity [38]. On the other hand, DCs recognize chemokines such as CCL20 that are secreted by mucosal epithelial cells facilitating them to move to the submucosal regions, extend their dendrites to take up pathogenic microorganisms, and then quickly migrate to nearby lymph nodes to present antigens to T and B lymphocytes, inducing an adaptive immune response [32]. DCs that mediate cytotoxic T cell (CTL) responses against IAV infection are divided into CD103+ and CD11b+ DCs [46]. Lack of CD103+ DCs significantly increases mortality in mice [11,47]. CD11b-expressing DCs are associated with inflammatory stimuli and are present in the airways during immune response activation [48–50]. Our data indicated that PA-X protein of H1N1 viruses decreased CCL20 secretion in the nasal mucosa, reducing DC recruitment to the submucosa, particularly CD11b+ DCs. Subsequently, the virus hijacked DCs by inhibiting CCR7 expression, reducing the maturation and migration of DCs to CLNs. PA-X protein of H1N1 viruses can shut off the host activity, inhibiting the innate immune response in both nasal epithelial cells and mucosal DCs and facilitating viral immune escape. Some pathogens have also evolved mechanisms to hijack DCs to favour their infection. For example, human cytomegalovirus (HCMV) can inhibit DC signalling and T cell proliferation, resulting in its long-term persistence [51]. Hepatitis B virus (HBV) can utilize the C-type lectin receptor (CLR) pathway to hijack DC subsets and evade the immune control [52]. Nonstructural protein 2 (NS2) of IAV inhibits the maturation and antigen-presenting capacity of DCs, leading to influenza virus epidemics [53].
The transition from immature to mature DCs is crucial for the downstream cascade of immune responses [54]. Immature DCs (iDCs) have a strong ability to take up and process antigens, although low levels of CD80, CD86, CD40, and MHC II are expressed on the cell surface [55]. Once pathogen is taken up, iDCs are transformed into mature DCs that are highly capable of stimulating T-cell proliferation and differentiation via upregulation of MHC II and co-stimulatory molecules (CD80 and CD86) [56]. Another study found that if the CD40 function of DCs was inhibited, T cells would be inhibited as well [57]. Our data demonstrated that PA-X downregulated the phenotypic markers of DCs, which was different from HBV vaccine that can upregulate the expression of MHC II and CD86, thereby enhancing the effect of immunotherapy in order to perform anti-virus functions [58]. The reduction of phenotype markers by the PA-X protein of H1N1 PR8 viruses may prevent the activation and maturation of DCs and subsequently inhibit the downstream immune cascade, thereby the immune escape is achieved.

Mature DCs can secrete cytokines under pathogenic stimulation, promoting the systemic immune responses and maintaining the immune homeostasis. CD69 is an early activation marker expressed following DC activation that can activate T-cells [59]. CD69-induced signaling can be adjusted not only to APCs but also to the immune response of cytokines and other metabolites in peripheral cells [60]. IL-1β, a key cytokine involved in inflammatory response, is essential for host protection [61]. IL-6, a significant regulator of the proliferation and survival of T cells, is significantly elevated after influenza infection, and T cells play a significant part in respiratory virus elimination [62]. TNF-α can also regulate immune cells, induce cellular inflammatory responses, and inhibit virus replication [63]. IL-10, as an anti-inflammatory cytokine, can inhibit the antigen uptake function of APCs [64]. Our data indicated that PA-X protein of H1N1 viruses inhibited the DC activation by downregulating CD69 and impaired the cytokine response via the downregulation of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and upregulating anti-inflammatory cytokine (IL-10), implying a functional maturation of DCs was suppressed by PA-X protein. Infection with H1N1 PR8 virus affects the maturation of DCs, preventing the mobilization of diverse immune cells to participate in viral clearance or activation of the downstream specific immunity, giving the virus a great opportunity to replicate. Similarly, the transmissible gastroenteritis virus (TGEV) can reduce the maturation ability of intestinal mucosal DCs, thus weakening their ability to present viral antigens to T cells and allowing the virus to infect epithelial cells in large numbers [65]. In addition, one possible explanation for the discrepancy between these cytokines is that the endonuclease activity of PA-X protein was selective [26] and IL-10 might escape the selective host-shutoff activity.

In summary, our results demonstrated PA-X protein disabled H1N1 viruses in stimulating nasal DCs, which might contribute to enhance virulence in mammals. This work would be helpful to reveal the synergistic regulation ability of PA-X protein on different host cells for suitable virulence.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability

All data are available from the authors upon reasonable request.

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