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Research Article

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Newcastle disease virus suppresses antigen presentation via inhibiting IL-12 expression in dendritic cells

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Abstract: As a potential vectored vaccine, Newcastle disease virus (NDV) has been subject to various studies for vaccine development, while relatively little research has outlined the immunomodulatory effect of the virus in antigen presentation. To elucidate the key inhibitory factor in regulating the interaction of infected dendritic cells (DCs) and T cells, DCs were pretreated with the NDV vaccine strain LaSota as an inhibitor and stimulated with lipopolysaccharide (LPS) for further detection by enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunoblotting, and quantitative real-time polymerase chain reaction (qRT-PCR). The results revealed that NDV infection resulted in the inhibition of interleukin (IL)-12p40 in DCs through a p38 mitogen-activated protein kinase (MAPK)-dependent manner, thus inhibiting the synthesis of IL-12p70, leading to the reduction in T cell proliferation and the secretion of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-6 induced by DCs. Consequently, downregulated cytokines accelerated the infection and viral transmission from DCs to T cells. Furthermore, several other strains of NDV also exhibited inhibitory activity. The current study reveals that NDV can modulate the intensity of the innate–adaptive immune cell crosstalk critically toward viral invasion improvement, highlighting a novel mechanism of virus-induced immunosuppression and providing new perspectives on the improvement of NDV-vectored vaccine.

Key words: Newcastle disease virus; Dendritic cells; Interleukin-12 (IL-12); T cells; Immunosuppression

1 Introduction

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that interconnect innate and adaptive immunity (Pulendran, 2015). DCs can target invasive pathogens and identify hazardous antigens through pattern recognition receptors (PRRs)(Durai and Murphy, 2016) via stimulating downstream pathways such as nuclear factor-κB (NF-κB) and interferon regulatory factor 3/7 (IRF3/7) signaling, leading to the transformation of immature DCs (imDCs) to mature DCs (mDCs) (Yoshimura et al., 2001). The maturation of DCs results in the higher expression of surface markers and proinflammatory cytokines along with efficient antigen presentation to T cells (Rescigno, 2002; Baratin et al., 2015). The generation of an efficient adaptive immune response by T cells through DCs requires three signals, including the antigen-specific T cell receptor (TCR)-peptide-major histocompatibility complex (MHC) complex, co-stimulatory molecules, and cytokines that facilitate the polarization of cluster of differentiation 4-positive ($CD4^+$) helper T (Th) cells (van Panhuys, 2016). The secretion of interferon-γ (IFN-γ) and interleukin-12 (IL-12) by DCs primes Th1-polarized $CD4^+$ T cells, which have been correlated

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with protective immunity against intracellular pathogens like viruses (Hilligan and Ronchese, 2020). Conversely, the Th2-biased response, driven by IL-4 and IL-10, inhibits the production of IFN-γ, IL-12, and IL-2, such that it can regulate the differentiation, proliferation, and function of Th1 $CD4^+$ T cells as well as activated $CD8^+$ T cells (Ronet et al., 2010).

IL-12, expressed by APCs, is a Th1-type cytokine that presents various proinflammatory functions, mainly impacting the secretion of IFN-γ, tumor necrosis factor-α (TNF-α), and IL-2, thus modulating the function of APCs and improving effector T cell responses (Ghilas et al., 2021). IL-12 is a heterodimeric cytokine consisting of disulfide-linked p35 and p40 subunits (Gately et al., 1991) that are coordinately expressed within the cell and then assembled into the biologically active IL-12p70 (Schulz et al., 2000). Mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways, involved in inducing IL-12 production, are activated through Toll-like receptor (TLR)-induced stimulation that leads to the expression of a wide range of cytokines such as IL-12p40 to drive inflammatory responses (Kürten et al., 2020). The p38 MAPK is an important kinase that is activated and phosphorylated by TLR4/myeloid differentiation primary response 88 (MyD88) signaling that mediates the phosphorylation of downstream kinases like MAPK-activated protein kinase 2 (MK2) and induces IL-12p40 expression (Gao et al., 2017; Menon et al., 2017). Protein kinase B (Akt) is another essential factor correlated with TLR4/MyD88 interactions and playing a role in the inflammation mediated by TLR agonists such as lipopolysaccharide (LPS) (Jiang et al., 2018). The two signaling pathways mentioned above target both phosphorylation and the nuclear translocation of NF-κB to control the transcription of multiple proinflammatory cytokines (Zhang et al., 2018; Nie et al., 2019).

Paramyxoviruses are among the most ubiquitous pathogenic viruses in both humans and animals. Unsurprisingly, they have developed diverse immunosuppressive mechanisms to control antigen presentation via modifying the role of DC, such as the maintenance of the immature phenotype (Shrestha et al., 2017), regulation of cytokines (Ayasoufi and Pfaller, 2020), and apoptosis (Gupta et al., 2013). Newcastle disease virus (NDV) of the *Avulavirus* genus in the Paramyxoviridae family is a highly contagious pathogen affecting almost all bird species, causing severe respiratory diseases. NDV presents significant potential as a vaccine carrier for expressing exogenous protein, which has been extensively constructed by reverse genetic technology for the prevention of diseases in humans and animals (Zhang et al., 2019; Park et al., 2021). Importantly, despite the fact that NDV can trigger a synergy of mucosal, cellular, and humoral immunity, it neutralizes host innate immunity through the viral V protein (Nan et al., 2021a) and causes the apoptosis of DCs to inhibit antigen presentation (Tan et al., 2018). Furthermore, recent research from our group has revealed that NDV infection triggers phenotypically mature DCs but these are dysfunctional in stimulating T cell responses (Nan et al., 2021b). Nevertheless, the inhibitory regulation of NDV varies between different virulent types, while the specific mechanism of NDV and DC interaction remains relatively unknown. The present study investigated the key inhibitory factor in manipulating the nature of DCs as well as interaction of infected DCs and T cells, providing an enhanced understanding of NDV immune evasion as well as aiding the improvement of NDV-vectored vaccines.

2 Materials and methods

2.1 Cells and viruses

Bone marrow-derived DCs (BMDCs) were isolated from the bone marrow of six-week-old female C57BL/6 mice and cultured in RPMI 1640 medium with 10% (0.1 g/mL) fetal bovine serum (Gibco, Carlsbad, CA, USA), 20 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF), and 20 ng/mL recombinant mouse IL-4 (rmIL-4) (R&D Systems, Minneapolis, MN, USA) at 37 ℃ for 7 d as previously described (Nan et al., 2021b). The NDV lentogenic strains LaSota, recombinant NDV (rNDV)-enhanced green fluorescent protein (EGFP) and Clone30, the mesogenic strain Mukteswar, and the velogenic strains Herts/33 and F48E9 (chicken) were obtained from Professor Zhigao BU; the NDV velogenic strain Na-1 (goose) was obtained from Professor Zhuang DING; the NDV velogenic strain 167 (pigeon) was obtained from Professor Xusheng QIU. All viruses were propagated in 9-d-old embryonated specific pathogen-free (SPF) chicken eggs.

2.2 Cell treatments

The imDCs were pretreated with NDV at a multiplicity of infection (MOI) of 3 as an inhibitor for 12 h and stimulated with 3 μg/mL TLR4 agonist LPS (Sigma-Aldrich, St. Louis, MO, USA) or TLR7/9 agonist R848 (MedChemExpress, NJ, USA). The cells or supernatant was collected at 48 h for further assays.

As for the co-culture, DCs were pretreated with NDV for 12 h and stimulated with 3 μg/mL LPS for 48 h. Next, 2×10^5 treated DCs were co-cultured with 2×10^6 autologous T cells (1:10) isolated from spleens, and cells as well as supernatant were harvested after 48 h for further assay (Nan et al., 2021b).

For the restoration effect assay, when NDV-LPS-treated DCs were harvested and counted to co-culture with autologous T cells, 20 ng/mL or the indicated dose of IL-12p70, IFN-γ, TNF-α, or IL-6 (R&D Systems) was separately added into the co-cultures. The cells and supernatant were collected after 48 h for further assay.

In order to identify the effect of NDV on T cells, T cells were pretreated with NDV for 12 h and stimulated with 200 ng/mL phorbol 12-myristate 13-acetate (PMA) and 4 μg/mL ionomycin (Dakewe Biotech, Shenzhen, China) for 48 h.

2.3 qRT-PCR

The total RNAs of DCs and T cells were extracted and reverse-transcribed into complementary DNA (cDNA) with random primers 48 h post-treatment. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the Go Taq Master Mix (Promega, Madison, Wisconsin, USA) and specific primers (Table S1) to measure several cytokine-associated genes as well as the nucleoprotein (*NP*) gene of NDV. The relative abundance of messenger RNA (mRNA) was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA and calculated by the $2^{-\Delta\Delta C_T}$ method.

2.4 Proliferation of T cells

Mixed leukocyte reaction (MLR) was performed to test the proliferation of T cells in co-cultures induced by DCs. DCs were pretreated with NDV for 12 h and stimulated with 3 μg/mL LPS for 48 h. Next, 1×10^4 treated DCs were co-cultured with 1×10^5 autologous T cells (1:10) isolated from the spleens in 96-well plates for 48 h. T cell proliferation was measured by the viability assay using cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) at 37 ℃ for 4 h. The stimulation index (SI) of proliferation was evaluated as $SI=(OD_{sample\ well}-OD_{blank\ well})/(OD_{negative\ well}-OD_{blank\ well})$, where OD is optical density, and the $OD_{blank\ well}$ only contained T cells and the $OD_{negative\ well}$ was Mock-treated with DCs co-cultured with T cells.

2.5 Immunoblot analysis

Treated DCs were lysed using radio-immunoprecipitation assay (RIPA) buffer containing a protease and phosphatase inhibitor cocktail for cell lytic extraction. For nuclear extraction, treated DCs were collected and washed by phosphate-buffered saline (PBS) two times and extracted using CelLytic™ NuCLEAR™ (Sigma-Aldrich). The cell pellet was gently resuspended in 1× lysis buffer (including dithiothreitol (DTT) and protease inhibitors) and incubated on ice for 15 min. Then, 10% (volume fraction) IGEPAL CA-630 solution was added followed by vigorous vortexing for 10 s. After centrifugation, the supernatant was harvested as a cytoplasmic fraction. The crude nucleus pellet was resuspended in the extraction buffer and vortexed for 30 min. Nuclear extracts in the supernatant were harvested by centrifugation. The extracted proteins were quantified using the BCA protein assay kit (Beyotime, China). Equal amounts of the extracted protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Following blocking, the PVDF membranes were reacted with anti-GAPDH, anti-p-Akt, anti-Akt, anti-p-p38, anti-p38, anti-p-MK2, anti-MK2, anti-p-p65, anti-p65, and anti-IL-12p70 primary antibodies overnight at 4 ℃ and horseradish peroxidase (HRP)-conjugated secondary antibodies (CST, MA, USA) for 45 min at room temperature. The target proteins were developed using electrochemiluminescence (ECL) reagent and visualized by chemiluminescence.

2.6 ELISA for cytokine production and p-p65

The productions of IFN-α, IFN-β, IFN-γ, TNF-α, IL-6, IL-12p40, and IL-12p70 in the cell-free supernatants of DCs or co-cultures harvested at 12, 24, and 48 h post-treatment were detected using corresponding enzyme-linked immunosorbent assay (ELISA) kits (CST). The expression of p-p65 in the cell lytic extracts and nuclear and cytoplasmatic extracts was measured

through the PathScan® Phospho-NF-κB p65 (Ser536) Sandwich ELISA Kit (CST).

2.7 Flow cytometry analysis

In order to determine the receptors of IL-12 and the expression of CD44, CD25, and CD69 on T cells, the treated cells were stained with anti-CD3-PEcy5 (a specific marker of T cells) and then stained with anti-IL-12Rβ1, 12Rβ2 (receptors of IL-12 that could transduce the IL-12 downstream signals), CD44, CD25, and CD69 (activated markers of T cells) antibodies (eBioscience, CA, USA) at 4 °C for 45 min in the dark. After treatment, cells were washed with PBS and analyzed by flow cytometry. The co-cultured T cells were gated based on CD3 (T cell marker), IL-12Rβ1, 12Rβ2, CD44, CD25, and CD69. For intracellular cytokine staining, cells were pretreated with 2 mmol/L Brefeldin A. After 4 h, cells were labeled with anti-CD11c-APC (DCs) or anti-CD3-PEcy5 (T cells) for surface staining and incubated with fixation/permeabilization solution (BD Cytofix/Cytoperm Kit, BD, NJ, USA). Then, cells were labeled with IL-12p40/70 phycoerythrin (PE) antibodies or anti-IFN-γ PE, anti-IL-6 PE, and anti-TNF-α APC antibodies (eBioscience), and analyzed by flow cytometry.

2.8 Infection efficiency of NDV on treated cells

The infection efficiency of NDV on DCs or T cells was determined using rNDV-EGFP. Overall, cells were pretreated with PBS, 20 ng/mL IL-12p70, IFN-γ, TNF-α, IL-6, or chicken polyclonal serum (1:500, volume ratio) against NDV for 12 h and infected with rNDV-EGFP at an MOI of 3. The efficiency of infection was measured by the proportion of cells expressing EGFP through flow cytometry. qRT-PCR was performed to evaluate the mRNA levels of *NP* gene of NDV in DCs or T cells. Additionally, supernatants were titrated in baby hamster kidney (BHK) cells. To explore the transmission from DCs to T cells, NDV-carrying DCs were co-cultured with T cells by two methods (direct contact and non-contact). In the direct contact method, infected DCs were co-cultured with T cells in six-well plates. However, the Transwell® system (6.5 mm Transwell® with 0.4 μm Pore Polycarbonate Membrane, Corning, NYC, USA) was used to prevent contact between the cells in the top or bottom compartments, which allowed the shuttle of soluble factors in the non-contact method. Infected DCs were seeded in the top chamber, while T cells were placed in the bottom chamber and co-cultured for 48 h to verify whether infectious cell-free NDV was able to transfer from DCs to T cells in the supernatant.

2.9 Statistical analysis

All experiments were carried out in triplicate and all values were presented as mean±standard deviation (SD). All statistics were performed by GraphPad Prism (Version 6). Student's *t*-test and one-way analysis of variance (ANOVA) were applied to compare the significance of differences ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$). Schematic diagrams were created using Office software and BioRender.com.

3 Results

3.1 NDV inhibited IL-12p70 production via blocking IL-12p40

In order to establish whether NDV targeted DCs to inhibit antigen presentation, DCs were pretreated with the NDV LaSota lentogenic strain as an inhibitor, followed by assessing the secretion of various cytokines using LPS (TLR4) or R848 (TLR7) as DC agonists. The results revealed that when uninfected DCs were treated with LPS alone, the production of IL-12p70 reached 325 pg/mL, about 1.5-fold higher compared to the NDV-pretreated group (Figs. 1a and 1b), while IFN-α, IFN-β, IFN-γ, TNF-α, or IL-6 was not decreased (Fig. S1). Moreover, the expression of cell surface molecules such as MHC-II, CD86, CD40, and CD80 was not decreased when infected (Fig. S2). As IL-12p70 was not significantly increased when treated with R848, subsequent experiments focused on the application of LPS. To establish whether decreased IL-12p70 in DCs was caused in the IL-12p35 or the IL-12p40 subunit, the expression of IL-12p35 and IL-12p40 in treated DCs was examined. It was discovered that, in the context of NDV, the *IL-12p40* mRNA level in uninfected DCs was approximately 1.75 times higher than that in NDV-pretreated DCs, while that of *IL-12p35* was similar to the NDV group (Figs. 1c and 1d), suggesting that the downregulated expression of IL-12p40 triggered the suppressed secretion of IL-12p70 in infected DCs. Consequently, IL-12p40 protein levels in NDV-pretreated DCs were significantly lower compared to the uninfected group (Figs. 1e–1g)

in a dose-dependent manner. On the other hand, the ratio of IL-12p70 expressing DCs in the uninfected group was 25.88%, significantly higher compared to the NDV-pretreated group (Fig. 1h). The results presented above indicated that NDV suppressed the secretion of bioactive IL-12p70 by inhibiting the expression of IL-12p40.

3.2 NDV inhibited the activation of p38 MAPK signaling pathway in DCs

In order to further examine the IL-12-dependent regulation of virus control in DCs, we examined the activation of p38 and Akt signaling molecules that have pivotal roles in the activation of IL-12 production (Qian et al., 2018). Western blotting revealed that

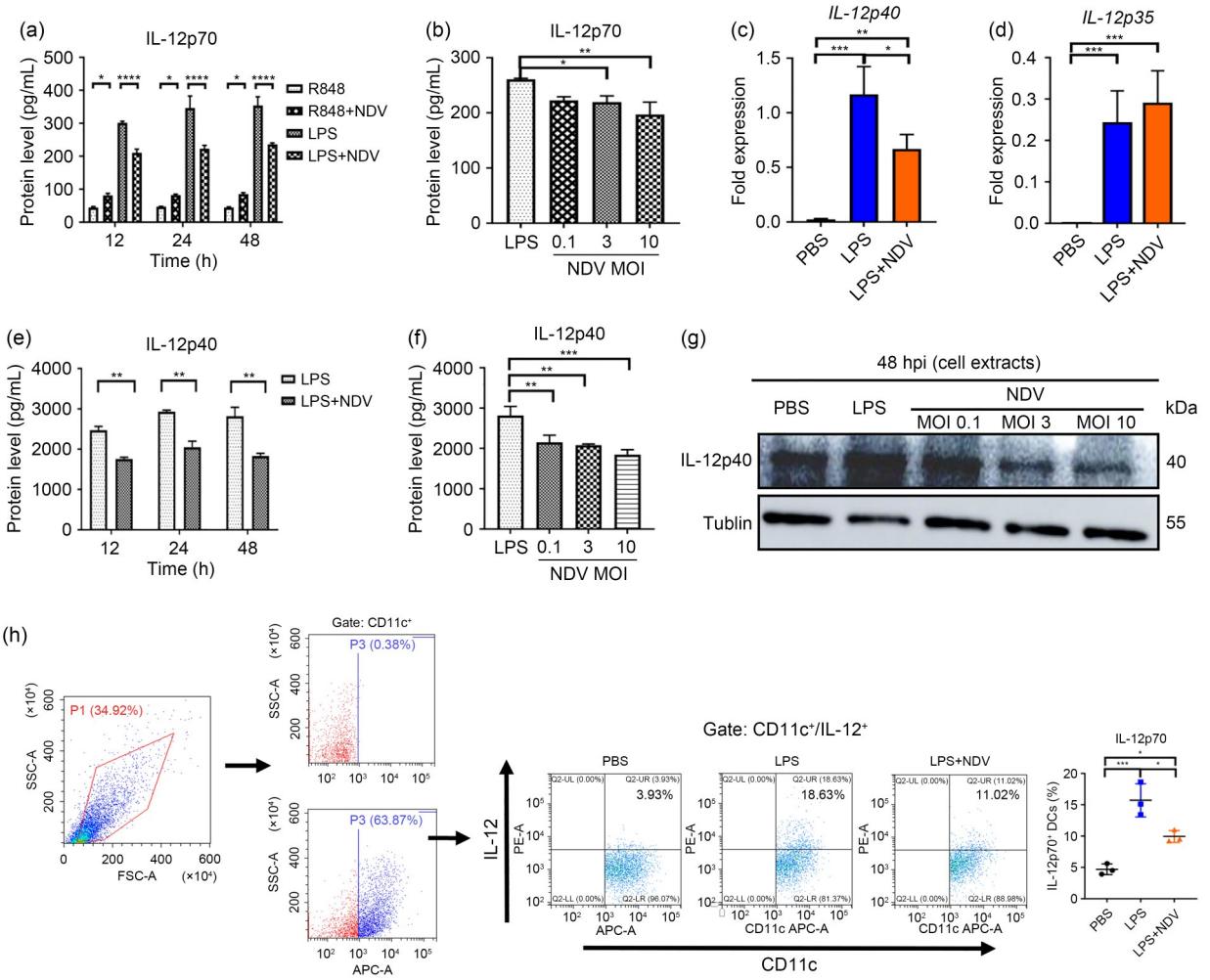

Fig. 1 IL-12 responses in treated DCs. DCs isolated from the bone marrow of mice were pretreated with NDV and stimulated with LPS for 48 h or the indicated time. Then, the supernatant and treated cells were collected for subsequent measurement. (a, b) IL-12p70 secretion in the treated supernatant was detected by ELISA at different time points (12, 24, and 48 h, with NDV at MOI of 3) and MOIs (0.1, 3, and 10, for 48 h). (c, d) The transcriptional levels of *IL-12p40* and *IL-12p35* genes were measured by qRT-PCR. (e, f) IL-12p40 secretion in the treated supernatant was detected by ELISA at different time points (12, 24, and 48 h, with NDV at MOI of 3) and MOIs (0.1, 3, and 10, for 48 h). (g) IL-12 expression in treated cells was detected by immunoblot analysis. (h) To determine the IL-12-expressing DCs, DCs were stained with anti-CD11c and IL-12p antibodies. The absolute number of IL-12 expressing DCs (gated on $CD11c^+/IL\text{-}12^+$) was counted by flow cytometry. Data are expressed as mean±SD of triplicate experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. IL: interleukin; DCs: dendritic cells; NDV: Newcastle disease virus; MOI: multiplicity of infection; LPS: lipopolysaccharide; ELISA: enzyme-linked immunosorbent assay; qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation; PBS: phosphate-buffered saline; hpi: hour post infection; CD: cluster of differentiation; SSC: side scatter; FSC: forward scatter; APC: allophycocyanin; PE: phycoerythrin; UL: upper left; UR: upper right; LL: lower left; LR: lower right.

the levels of key proteins, including Akt, p38, MK2, and p65 involved in the p38 and Akt signaling pathways, were not affected by infection, while NDV preincubation inhibited the phosphorylation of p38, MK2, and p65 considerably, especially p-p38 and p-p65, in a dose-dependent manner (Fig. 2a). Since the change in p-Akt was not significant, we focused on the p-p38 signaling pathway. Inactive NF-κB p65 and p38 subunits shuttle in and out of the cell nucleus, while activated p-p38 and p-p65 (Ser536) translocate within the nucleus and induce the transcription of proinflammatory cytokines such as IL-12p40 (Nie et al., 2019). Importantly, in NDV-treated cells, the nuclear translocation of p-p38 and p-p65 was dampened as expected (Fig. 2b). Similarly, p-p65 from cell lytic, cytoplasmatic, and nuclear extracts assessed by the phospho-NF-κB p65 (Ser536) Sandwich ELISA Kit in Mock was 1.59-, 1.39-, and 1.10-fold higher than that in the infected group, respectively (Figs. 2c–2e). These data revealed that NDV specifically targeted the phosphorylation and nuclear translocation of p38 and p65 to inhibit IL-12p40 expression. The schematic representation of the suppression of IL-12p40 of DCs by NDV can be seen in Fig. 2f.

3.3 NDV inhibits IL-12-induced T cell-stimulating function in co-cultures

In order to explore whether reduced IL-12 production by DCs was directly associated with the limited proliferation of T cells, we detected the receptors of IL-12p70 and the concentrations of downstream cytokines induced by IL-12 in supernatants from DC-T cell co-cultures. The data demonstrated of IL-12p70 receptors in T cells were not significantly affected by NDV (Fig. S3). It was then discovered that proliferation of T cells when pretreated with NDV (Fig. 3a) presented a 45% reduction. In addition, the secretions of IL-12 downstream cytokines, IFN-γ, TNF-α, and IL-6 (Figs. 3b–3d), in Mock-treated co-cultures were 2.17, 1.51, and 1.82 times higher compare to those of the NDV group, respectively, which was consistent with the proportions of IFN-γ-, TNF-α-, and IL-6-producing T cells in co-cultures (Figs. 3f–3h). Interestingly, the secretion of IL-2 in co-cultures was not significantly affected by NDV (Fig. 3e). As IL-12 was critical for guiding T cell immune responses through proinflammatory cytokine release (Wonderlich et al., 2015), supplementing IL-12p70 in co-cultures was performed to determine whether IL-12 addition would reverse the impaired function of T cells (Fig. 3i). Exogenous IL-12p70 indeed restored the proliferation of T cells (Fig. 3j) and their production of cytokines (Figs. 3k–3m) in a dose-dependent manner, particularly IFN-γ, which presented a 9.3-fold increase from 32 to 291 pg/mL. Furthermore, in the presence of TNF-α, IL-6, and especially IFN-γ, the proliferation of T cells triggered by infected DCs was apparently recovered (Fig. 3n). Based on the above, the NDV-specific suppression of IL-12p70 significantly inhibited the proliferation and downstream cytokines produced by T cells.

3.4 NDV inhibited IFN-γ and TNF-α secretion from T cells

Since a major effect of NDV was detected on DC-induced T cell responses in co-cultures, the study focused on identifying whether NDV was directly acting on T cells. To initially examine whether NDV could be transmitted from DCs to T cells in co-cultures like measles virus (MV) (de Witte et al., 2008), rNDV-EGFP was employed as a tracer mark that possessed an identical inhibitory action as the parental strain (Fig. S4). By detecting the percentage of EGFP expressing DCs and T cells, the efficiency of NDV infection was discovered on DCs and T cells in a dose-dependent manner but was negatively associated with the infection period (Figs. 4a–4d). In addition, direct co-cultures as well as a Transwell® system of DCs and T cells were utilized to examine viral transmission from DCs to T cells. The results demonstrated that NDV could be transmitted from DCs to T cells primarily by direct contact in co-cultures, as infection efficiency of the direct contact group was approximately 1.5 times higher than that of the Transwell® system where NDV could only be transmitted to T cells through the supernatant (Figs. 4e and 4f). Based on the fact that NDV could be transmitted to T cells in co-cultures, our group was interested in exploring whether NDV would also directly hinder cytokine expression of T cells. T cells were pretreated with NDV as an inhibitor and induced using agonist PMA+Ionomycin with or without exogenous IL-12p70 for further analysis (Fig. 4g). It was discovered that the secretion of IL-6 was not significantly affected by NDV, while IFN-γ and TNF-α were notably decreased in the NDV-infected group, which could be ameliorated from 147 pg/mL and 360 pg/mL to 460 pg/mL and 486 pg/mL, respectively, by the addition

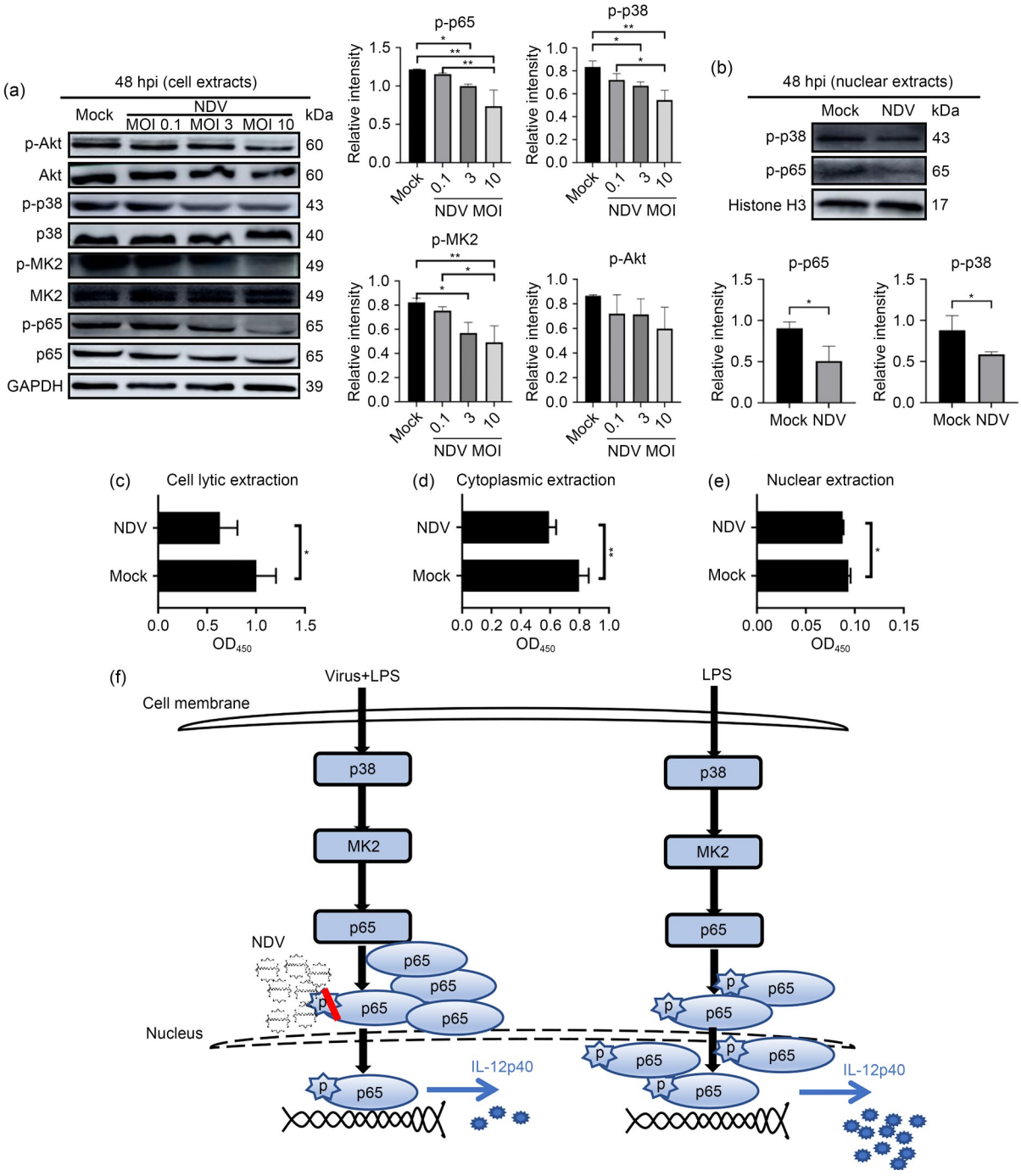

Fig. 2 Activation of p38 MAPK signaling pathway in DCs. DCs were pretreated with NDV at an MOI of 3 or 0.1, 3 and 10 or Mock (PBS), and stimulated with LPS for 48 h. Then, cell lytic and nuclear extracts were detected by immunoblot analysis and ELISA. (a) Expression of key proteins from cell lytic extracts in p38 MAPK and Akt signaling pathways. (b) Expression of p-p65 and p-p38 in nuclear lysates. (c–e) The expression levels of p-p65 in infected cell lytic, cytoplasmatic, and nuclear extracts were evaluated by phospho-NF-κB p65 (Ser536) Sandwich ELISA Kit. (f) Schematic representation of the suppression of IL-12p40 of DCs by NDV. Data are expressed as mean±SD of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$. MAPK: mitogen-activated protein kinase; DCs: dendritic cells; NDV: Newcastle disease virus; MOI: multiplicity of infection; PBS: phosphate-buffered saline; LPS: lipopolysaccharide; ELISA: enzyme-linked immunosorbent assay; Akt: protein kinase B; NF-κB: nuclear factor-κB; hpi: hour post infection; MK2: MAPK-activated protein kinase 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; OD_{450}: optical density at 450 nm; p: phosphorylation; SD: standard deviation.

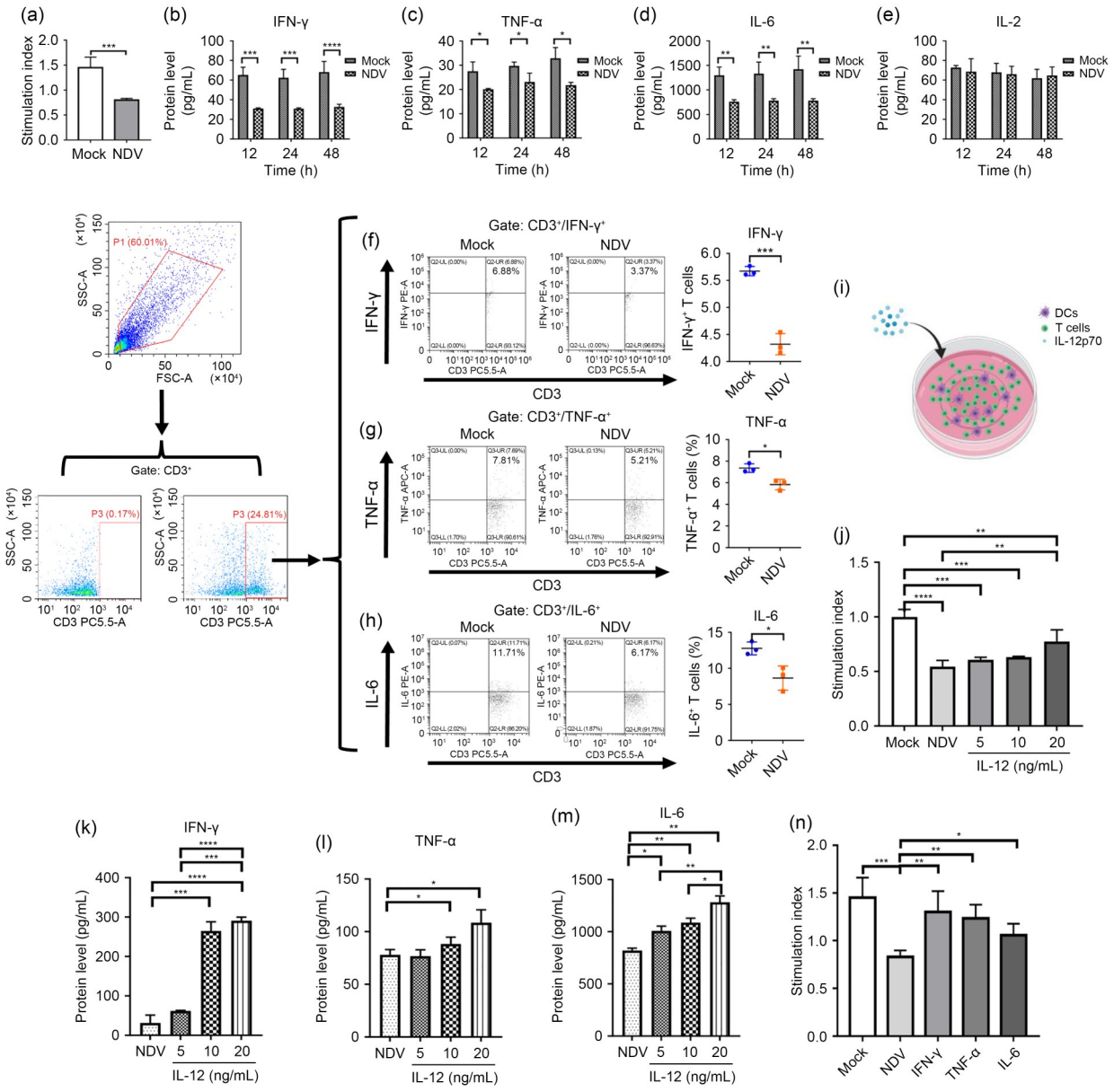

Fig. 3 DC-induced T cell-stimulating function in co-cultures. DCs were pretreated with NDV at an MOI of 3 or Mock (PBS), and stimulated with LPS for 48 h. Then, treated DCs were co-cultured with splenic lymphocytes for 48 h. (a) The proliferation of T cells in co-cultures was measured using MLR assay. (b–e) The secretion levels of IFN-γ, TNF-α, IL-6, and IL-2 in co-cultures were detected by ELISA. (f) To determine the IFN-γ expressing T cells in co-cultures, T cells were stained with anti-CD3 and IFN-γ antibodies, and the absolute number of IFN-γ expressing T cells (gated on $CD3^+$/$IFN\text{-}\gamma^+$) was counted by flow cytometry; (g) Portion of $TNF\text{-}\alpha^+$ T cells; (h) Portion of $IL\text{-}6^+$ T cells. (i) Schematic representation of supplementation with exogenous rmIL-12. (j) Proliferation of T cells in co-cultures with additional rmIL-12 at 0, 5, 10, or 20 ng/mL. (k–m) The secretion levels of IFN-γ, TNF-α, and IL-6 in co-cultures with additional rmIL-12. (n) Proliferation of T cells in co-cultures with additional IFN-γ, TNF-α, or IL-6 at 20 ng/mL. Data are expressed as mean±SD of triplicate experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. DCs: dendritic cells; NDV: Newcastle disease virus; MOI: multiplicity of infection; PBS: phosphate-buffered saline; LPS: lipopolysaccharide; MLR: mixed leukocyte reaction; IFN-γ: interferon-γ; ELISA: enzyme-linked immunosorbent assay; TNF-α: tumor necrosis factor-α; IL: interleukin; rmIL-12: recombinant mouse IL-12; CD: cluster of differentiation; SSC: side scatter; FSC: forward scatter; PC5.5: phycoerythrin-cyanin 5.5; PE: phycoerythrin; UL: upper left; UR: upper right; LL: lower left; LR: lower right; SD: standard deviation.

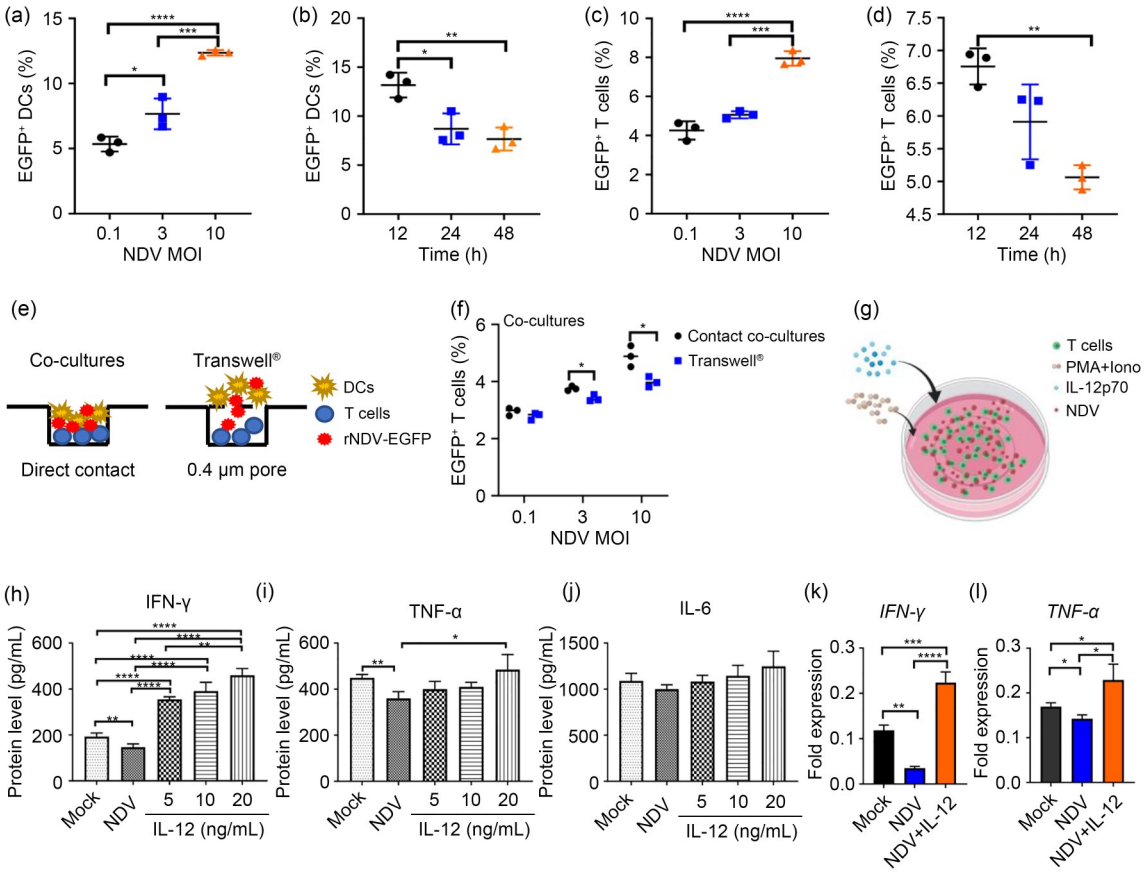

Fig. 4 T cell responses in the context of NDV. DCs and T cells were infected with rNDV-EGFP and then analyzed by flow cytometry. (a) Proportion of EGFP$^+$ DCs (infected DCs) at different infection doses for 48 h. (b) Proportion of EGFP$^+$ DCs (infected DCs with NDV at an MOI of 3) at different hours post-infection (hpi). (c) Proportion of EGFP$^+$ T cells (infected T cells) at different infection doses for 48 h. (d) Proportion of EGFP$^+$ T cells (infected T cells with NDV at an MOI of 3) at different hpi. (e) Schematic representation of co-cultures and the Transwell® system. (f) Infected DCs were co-cultured directly or in a Transwell® system for 48 h. To determine the infection of T cell populations, the cells were stained with labeled antibody against CD3 prior to analysis. The absolute number of T cells (gated on CD3$^+$/EGFP$^+$) of the total counted samples by flow cytometry was used to determine the efficiency of transmission. (g) Schematic representation of the treatment of T cells. T cells were pretreated with NDV at an MOI of 3 or Mock (PBS) and stimulated with 200 ng/mL PMA+4 μg/mL Ionomycin (Iono) and rmIL-12 (0, 5, 10, or 20 ng/mL) for 48 h. (h–j) The secretion levels of IFN-γ, TNF-α, and IL-6 in the supernatant of treated T cells were detected by ELISA. (k, l) The transcriptional levels of *IFN-γ* and *TNF-α* genes in T cells (with 20 ng/mL rmIL-12). Data are expressed as mean±SD of triplicate experiments. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, $^{****}P$<0.0001. NDV: Newcastle disease virus; DCs: dendritic cells; rNDV: recombinant NDV; EGFP: enhanced green fluorescent protein; MOI: multiplicity of infection; CD: cluster of differentiation; PBS: phosphate-buffered saline; PMA: phorbol 12-myristate 13-acetate; IL: interleukin; rmIL-12: recombinant mouse IL-12; IFN-γ: interferon-γ; ELISA: enzyme-linked immunosorbent assay; TNF-α: tumor necrosis factor-α; SD: standard deviation.

of exogenous IL-12p70 (Figs. 4h–4j). The analysis of mRNA levels in T cells further confirmed the ELISA findings of IFN-γ and TNF-α, revealing a 6.43-fold and 1.61-fold increase, respectively (Figs. 4k and 4l). These results suggested that NDV could directly control the role of T cells in co-cultures as a result of viral dissemination from DCs to T cells.

3.5 Infected DCs and T cells inhibited T cell proliferation

As NDV suppressed the function of both DCs and T cells, the roles of infected DCs and T cells on the priming of antigen presentation were subsequently assessed. Ultraviolet (UV)-treated supernatant from NDV-infected DCs was added to the uninfected

co-cultures, resulting in a 1.5-fold higher proliferation of T cells compared to the unirradiated group, suggesting that the virus progeny released from DCs suppressed T cell responses in co-cultures (Fig. 5a). Accordingly, by using chicken polyclonal serum against NDV, it was discovered that the interruption of transmission of NDV from DCs to T cells (Fig. 5b) could significantly alleviate the inhibition of antigen presentation by a 2-fold improvement (Fig. 5c). To better comprehend whether the suppression of IL-12p70 in DCs or

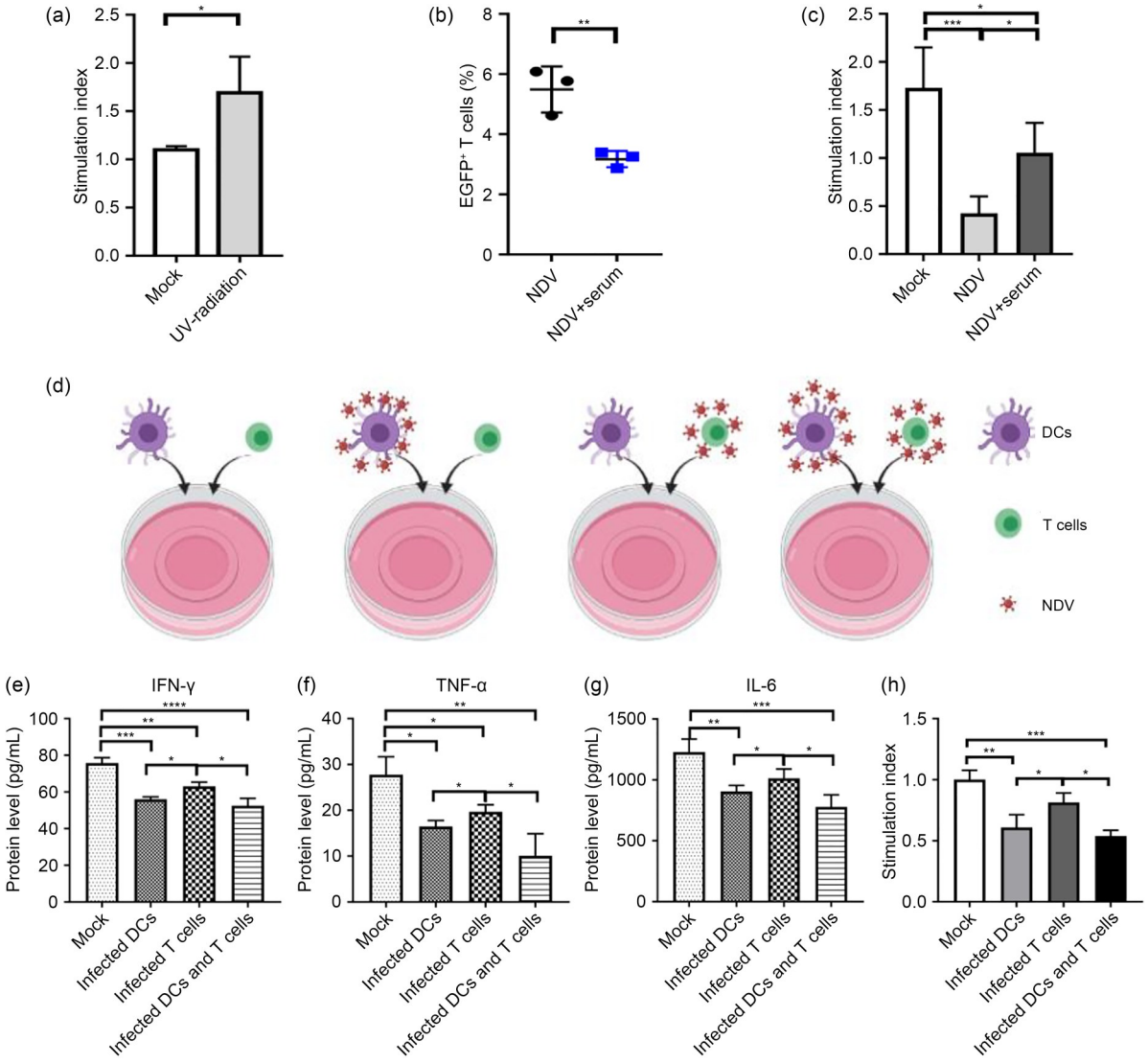

Fig. 5 Roles of infected DCs and T cells in the priming of antigen presentation responses. (a) The supernatant of uninfected DC-T cell co-cultures was replaced by the Mock (PBS)-treated or UV-treated (inactivate NDV) supernatant of infected DCs. The proliferation of T cells in co-cultures was measured by MLR assay. (b) DCs were pretreated with rNDV-EGFP at an MOI of 3 for 12 h and stimulated by LPS for 48 h, and then treated DCs were co-cultured with T cells. PBS (Mock) and chicken polyclonal serum against NDV (1:500, volume ratio) were added to the supernatant of co-cultures and the percentage of EGFP$^+$ T cells was evaluated using flow cytometry. (c) The proliferation of T cells in chicken polyclonal serum-treated co-cultures was measured. (d) Schematic representation of the combination of infected/uninfected DCs and infected/uninfected T cells in co-culture. (e–g) The secretion levels of IFN-γ, TNF-α, and IL-6 in the supernatant of co-cultures were detected by ELISA. (h) Proliferation of T cells in co-cultures. Data are expressed as mean±SD of triplicate experiments. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, $^{****}P$<0.0001. DCs: dendritic cells; PBS: phosphate-buffered saline; UV: ultraviolet; NDV: Newcastle disease virus; MLR: mixed leukocyte reaction; MOI: multiplicity of infection; rNDV: recombinant NDV; EGFP: enhanced green fluorescent protein; LPS: lipopolysaccharide; IFN-γ: interferon-γ; ELISA: enzyme-linked immunosorbent assay; TNF-α: tumor necrosis factor-α; IL: interleukin; SD: standard deviation.

the inhibition of IFN-γ and TNF-α in T cells regulated the suppression of antigen presentation, infected/uninfected DCs and infected/uninfected T cells were combined in co-cultures (Fig. 5d). As expected, the co-cultures of infected DCs and infected T cells demonstrated a nadir of T cell responses, while the combination of infected DCs with uninfected T cells caused the lower priming of T cells than the combination of uninfected DCs with infected T cells (Figs. 5e–5h). This demonstrated that the downregulation of IL-12 in DCs had an essential role in the inhibition of priming on T cells. Moreover, the activation of T cells (CD69, CD25, and CD44) or maintenance of the immunological synapse in co-cultures was not restricted in the context of NDV (Fig. S5). The above data demonstrated that although the infection of both DCs and T cells was responsible for the inhibition of antigen presentation, NDV-induced loss of IL-12p70 production was a key reason.

3.6 NDV promotes its own propagation by inhibiting the expression of cytokines

Besides the induction of antigen presentation, IFN-γ, TNF-α, IL-6, and IL-12p70 also operate in the defense against viral invasion (Dienz et al., 2012; Romanets-Korbut et al., 2016; Chen et al., 2019; Yang et al., 2020). To clarify whether this inhibition of IL-12 could be vital for accelerating NDV replication, DCs were treated with LPS or recombinant IL-12p70 prior to NDV infection, followed by assessing the viral infection efficiency. As demonstrated in Figs. 6a and 6b, the infection efficiences of NDV on LPS- and IL-12p70-treated mature DCs were 2.0% and 5.2%, respectively, which were only 25.5% and 50.0% of untreated DCs. Accordingly, IL-12p70 pretreatment significantly inhibited viral *NP* gene expression and progeny virions that were decreased to approximately 50% and 75% of the PBS group (Figs. 6c and 6d), respectively, while when IL-12p70 was added to T cells or co-cultures, the replication and transmission of NDV were impaired (Figs. 6e–6h). Based on the antiviral effect of IL-12p70, IL-12p70 downstream-mediated cytokines could also lead to resistance against infection. As expected, the antiviral effects of IFN-γ, TNF-α, and IL-6 on T cells were in accordance with IL-12p70 (Figs. 6i–6k) and especially with IFN-γ. The transmission of NDV from DCs to T cells in co-cultures demonstrated that, compared to TNF-α and IL-6, IFN-γ presented an improved antiviral action, while the efficiency of NDV infection in the combination group (IFN-γ+TNF-α+IL-6) was relatively low (4.5% vs. 2.4%, Fig. 6l). These data demonstrated that NDV suppressed the expression of IL-12p70 and downstream cytokines to replicate more effectively.

3.7 Several NDV strains could suppress antigen presentation by inhibiting IL-12 expression

As the NDV lentogenic LaSota strain suppressed antigen presentation via an IL-12-dependent manner, different NDV virulent phenotypes could also inhibit IL-12. The NDV lentogenic strain Clone30, mesogenic strain Mukteswar, and velogenic strains Herts/33, F48E9 (chicken), Na-1 (goose), and 167 (pigeon) isolated from different species and with varying virulence were utilized to examine whether the inhibitory effect was ubiquitous in NDV or was rather based on viral virulence. As presented in Figs. 7a and 7b, all strains operated on suppressing IL-12p40 and IL-12p70. As a result, IL-12 downstream cytokines and the proliferation of T cells were downregulated compared with the LPS-treated groups (Figs. 7c–7f). Importantly, the release of IL-12p40 and IL-12p70 in the lentogenic strain group was approximately half of that in the velogenic strains group, indicating a more robust inhibitory effect of the lentogenic strain. Based on the above findings, various NDV strains were also considered to have the potential of antigen presentation inhibition. The schematic representation of immunosuppression and transmission of NDV from DCs to T cells is presented in Fig. 7g.

4 Discussion

NDV has been considered as a promising candidate for vectored vaccine administered to birds, mammals, as well as humans (Zhang et al., 2019; Sun et al., 2021). The vast majority of previous studies regarding NDV have primarily focused on vaccine development, while limited studies have been performed to delineate the immunomodulatory effect of NDV during antigen presentation. Based on a previous study by our group where vaccine strain LaSota presented an inhibitory effect on the priming of T cell responses (Nan et al., 2021b), the role of NDV in controlling the nature of innate–adaptive crosstalk was further examined. It was discovered that IL-12p40 was reduced upon infection, leading to the incomplete

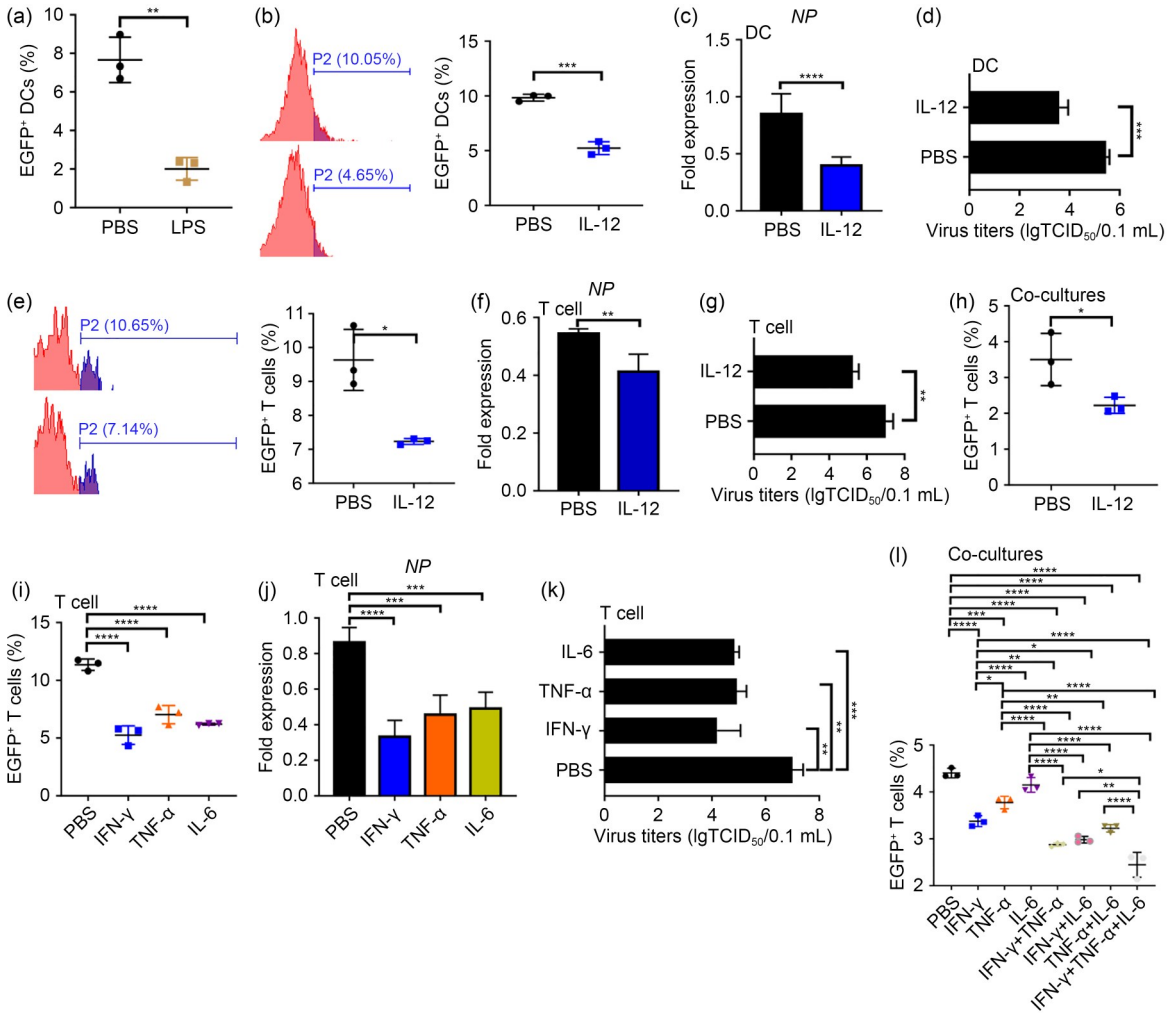

Fig. 6 Infection efficiency of NDV on DCs and T cells. DCs, T cells, or T cells in co-cultures were pretreated with 3 μg/mL LPS, 20 ng/mL IL-12, IFN-γ, TNF-α, or IL-6, or a combination of cytokines. After 12 h, cells were infected with rNDV-EGFP or co-cultured with infected DCs for 48 h. (a) Proportion of LPS-treated EGFP⁺ DCs. (b) Proportion of IL-12-treated EGFP⁺ DCs. (c) Transcriptional level of NDV *NP* gene in IL-12-treated DCs. (d) Virus titers ($TCID_{50}$) in the supernatant of IL-12-treated DCs. (e) Proportion of IL-12-treated EGFP⁺ T cells. (f) Transcriptional level of NDV *NP* gene in IL-12-treated T cells. (g) Virus titers ($TCID_{50}$) in the supernatant of IL-12-treated T cells. (h) Proportion of IL-12-treated EGFP⁺ T cells in co-cultures. (i) Proportion of IFN-γ-, TNF-α-, or IL-6-treated EGFP⁺ T cells. (j) Transcriptional level of NDV *NP* gene in IFN-γ-, TNF-α-, or IL-6-treated T cells. (k) Virus titers ($TCID_{50}$) in the supernatant of IFN-γ-, TNF-α-, or IL-6-treated T cells. (l) Proportion of IFN-γ-, TNF-α-, IL-6-, or their combination-treated EGFP⁺ T cells in co-cultures. Data are expressed as mean±SD of triplicate experiments. * *P*<0.05, ** *P*<0.01, * *P*<0.001, **** *P*<0.0001. NDV: Newcastle disease virus; DCs: dendritic cells; LPS: lipopolysac-charide; IL: inerleukin; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α; rNDV: recombinant NDV; EGFP: enhanced green fluorescent protein; *NP*: nucleoprotein; $TCID_{50}$: 50% tissue culture infectious dose; CD: cluster of differentiation; SD: standard deviation.**

biosynthesis of IL-12p70. Previous studies have revealed that the haemagglutinin-neuraminidase (HN) protein of NDV could downregulate NF-κB expression (Rajmani et al., 2016). Similarly, the results presented in this study revealed that the phosphorylation and nuclear localization of p38 and NF-κB p65 were impaired after NDV infection, which was responsible for the downregulation of IL-12p40. In principle, several viruses like human immunodeficiency virus (HIV) (Cardone et al., 2015) and MV (Romanets-Korbut et al., 2016) also target the expression of IL-12p70, thus blocking the proliferation and activation of T cells. Importantly, the V protein of NDV appears to facilitate the degradation of mitochondrial antiviral signaling protein (MAVS) and phospho-signal transducer and activator of transcription (p-STAT) via ubiquitin-proteasome

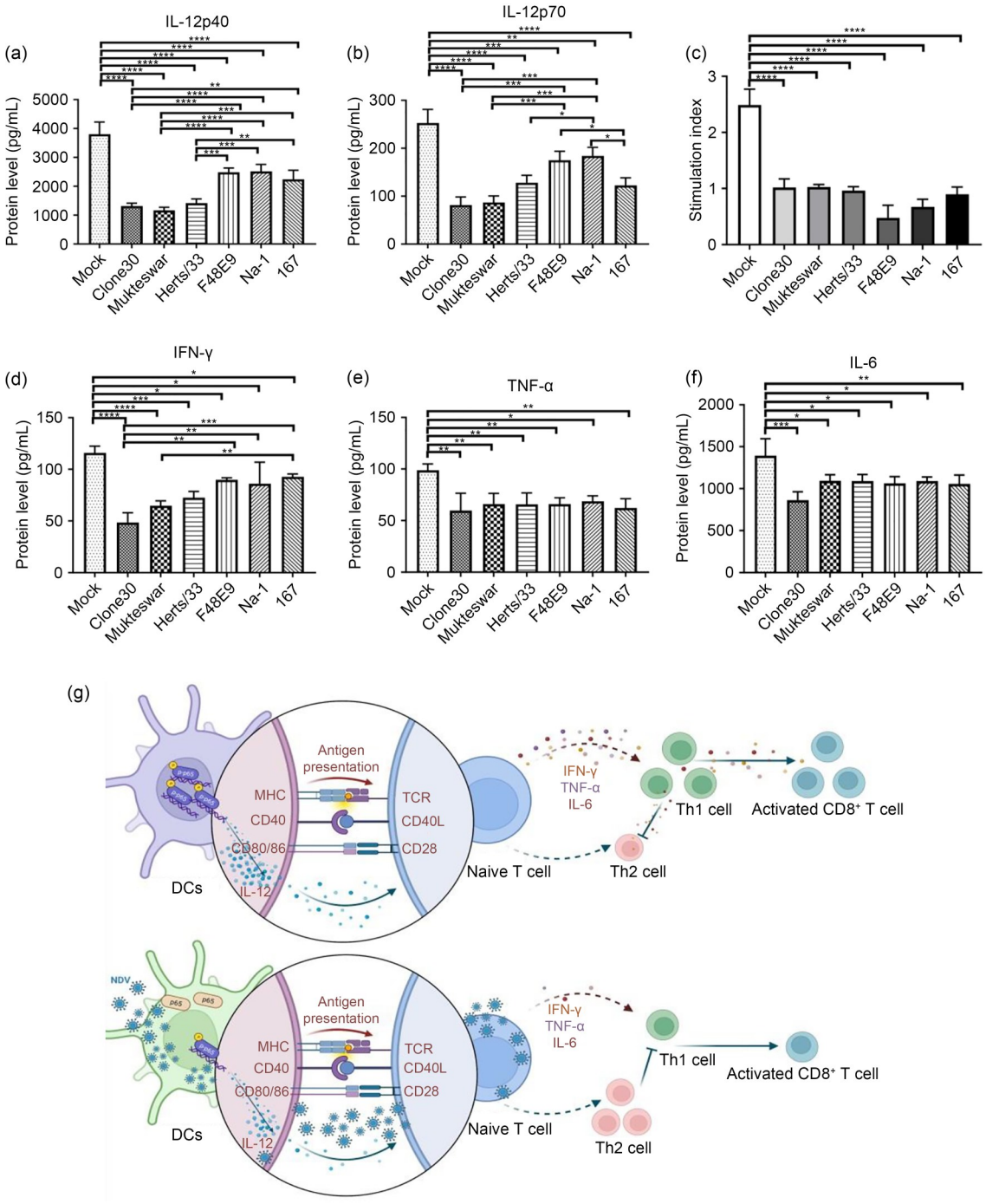

Fig. 7 Inhibitory effects of different NDV strains on antigen presentation. DCs were pretreated with several NDV strains at an MOI of 3 for 12 h and then stimulated with LPS for 48 h. Next, the supernatant and treated cells were collected or co-cultured with T cells for subsequent measurement. (a) IL-12p40 secretion in the supernatant of treated DCs. (b) IL-12p70 secretion in the supernatant of treated DCs. (c) The proliferation of T cells in co-cultures was measured. (d–f) The secretion levels of IFN-γ, TNF-α, and IL-6 in co-cultures were detected. (g) Schematic representation of immunosuppression and transmission of NDV from DCs to T cells. Data are expressed as mean±SD of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. NDV: Newcastle disease virus; DCs: dendritic cells; MOI: multiplicity of infection; LPS: lipopolysaccharide; IL: inerleukin; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α; MHC: major histocompatibility complex; CD: cluster of differentiation; TCR: T cell receptor; Th: helper T; SD: standard deviation.

pathways to restrain IFN-β secretion (Qiu et al., 2016; Sun et al., 2019), which may also be involved in the decreased phosphorylation of p38 and p65. Nevertheless, the more complicated mechanisms of degradation function will be explored in future studies. In addition, the TLR7/9 agonist R848 was not as efficient as LPS on DCs. Of note, LPS could be identified by TLR4 that was distributed on cell surface, while TLR7/9 was endosomal TLR that could only sense engulfed R848 in cells that could contribute to the difference between LPS and R848.

It was subsequently confirmed that suppressed T cell proliferation and production of proinflammatory cytokines were clearly associated with the virus-induced impairment of IL-12 in DCs, which could be restored by the addition of exogenous IL-12. IFN-γ, TNF-α, and IL-6 also had pivotal roles in antigen presentation, since their depletion could significantly impede T cell priming, which could also be rescued following the utilization of exogenous cytokines. Based on the significance of these proinflammatory factors, viruses like HIV (Cardone et al., 2015) and hepatitis (Brady et al., 2003) would specifically target such cytokines for their own benefit. It was then discovered that NDV could be transmitted from DCs to T cells by direct contact, similar to porcine epidemic diarrhea virus that could be transmitted from DCs to T cells to be disseminated to the intestine (Li et al., 2018). Moreover, DC-loaded varicella zoster virus particles have been previously used as "Trojan Horses" that are transferred from the infection site to lymph nodes, enabling viral transmission to T cells (Schönrich and Raftery, 2015). Allowing infected DCs to preserve their robust motility and function as a migratory vector could also be a strategy for the efficient dissemination of NDV to other cells, causing the direct inhibition of T cell response. By using different combinations of infected/uninfected DCs and infected/uninfected T cells, downregulated IL-12p70 in DCs was considered to bring the most significant inhibitory effect that could be attributed to T cell differentiation stimulation as well as the induction of other proinflammatory cytokines (Hilligan and Ronchese, 2020). Furthermore, to regulate the synergistic effect mediated by DCs and T cells, NDV exerts its pleiotropic inhibitory actions directly and indirectly to target various immune cell types.

The reduced levels of IL-12p70 and downstream cytokines affect resistance to viral invasion, but also suppress the function of immune natural killer cells, leading to effective infection by the virus. Therefore, the infection efficiency of NDV was examined, which showed that adding exogenous IL-12p70 suppressed viral propagation on DCs significantly, that is, NDV enhanced viral replication by inhibiting IL-12p70 expression in DCs. Besides IL-12p70, it was further confirmed that reduced IFN-γ, TNF-α, and IL-6 could also provide protection against NDV. As IFN-γ, TNF-α, and IL-6 could suppress T cell infection from NDV more effectively, it was hypothesized that NDV targeted IL-12 in DCs to control downstream cytokines in T cells, in order for NDV to achieve spreading from DCs to T cells more smoothly. Previous studies have proved that the successful establishment of viral infection requires specific strategies to achieve the development of potent antiviral defense mechanisms. NDV appeared to promote viral replication via the inhibition of IL-12p70, IFN-γ, TNF-α, and IL-6 expression.

Although *Paramyxoviruses* could suppress antigen presentation, DCs infected with a vaccine strain or wild-type strain demonstrated a different regulation of cytokines and surface molecular marker (Coughlin et al., 2013). The impact of NDV in different stains, isolation, and virulence on the mediation of DC and T cell responses was then examined. It was discovered that all NDV strains suppressed IL-12 production by DCs, thus compromising the priming of T cells. It is therefore possible that the attenuation of DC and T cell function is a strategy utilized by different strains of NDV to inhibit antigen presentation and thus enhance replication. Intriguingly, there seems to be a disagreement in the result that the lentogenic strain was more efficient in antagonizing cytokines compared to velogenic strains. In principle, velogenic strains with high pathogenicity can accomplish a more rapid replication and destruction of infected cells than lentogenic strains, while the latter with lower pathogenicity and longer duration require a more robust approach in impairing the host's immune responses.

In summary, the present study performed stepwise investigation and identified an important role of suppressed IL-12p70 during NDV infection and its impact on inhibition of T cell responses triggered by DCs. By targeting the phosphorylation of p38 and NF-κB p65, thus regulating the expression of IL-12p70, NDV specifically restricted the immune responses in DCs and T cells, leading to a reduced level of antigen presentation

but supporting viral replication. Our findings provide insights into the novel immunosuppressive mechanisms exploited by NDV to its own benefit, which should be considered for improving the design of NDV-vectored vaccines in the future.

Data availability statement

All data generated during the current study are included within this paper and its supplementary information files.

Acknowledgments

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Author contributions

Ningyi JIN, Bin WANG, Huijun LU, Fulong NAN, and Wenlong NAN: methodology, investigation, formal analysis, and writing – original draft. Xin YAN, Hui WANG, Shasha JIANG, Shuyun ZHANG, Zhongjie YU, Xianjuan ZHANG, He ZHANG, and Fengjun LIU: methodology, investigation, formal analysis, and validation. Jun LI, Xiaoqiong ZHOU, Delei NIU, Yiquan LI, Wei WANG, Ning SHI, Changzhan XIE, and Xiaoni CUI: investigation and formal analysis. Ningyi JIN, He ZHANG, Bin WANG, Huijun LU, and Fulong NAN: funding acquisition, conceptualization, supervision, writing – reviewing & editing, project administration, and data curation. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Fulong NAN, Wenlong NAN, Xin YAN, Hui WANG, Shasha JIANG, Shuyun ZHANG, Zhongjie YU, Xianjuan ZHANG, Fengjun LIU, Jun LI, Xiaoqiong ZHOU, Delei NIU, Yiquan LI, Wei WANG, Ning SHI, Ningyi JIN, Changzhan XIE, Xiaoni CUI, He ZHANG, Bin WANG, and Huijun LU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. All experimental animal procedures were reviewed and approved by the releva nt institutional Ethical Committee of Qingdao University (Approval ID: 20210613C573620220916106).

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Supplementary information

Figs. S1–S5, Table S1