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NDV entry into dendritic cells through macropinocytosis and suppression of T lymphocyte proliferation

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ABSTRACT

Newcastle disease virus (NDV) causes major economic losses in the poultry industry. Previous studies have shown that NDV utilizes different pathways to infect various cells, including dendritic cells (DCs). Here, we demonstrate that NDV gains entry into DCs mainly via macropinocytosis and clathrin-mediated endocytosis. The detection of cytokines interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), interleukin-4 (IL-4) and interleukin-10 (IL-10) indicates that NDV significantly induces Th1 responses and lowers Th2 responses. Furthermore, NDV entry into DCs resulted in the upregulation of TNF-related apoptosis-inducing ligand (TRAIL) and cleaved caspase-3 proteins, which in turn activated the extrinsic apoptosis pathway and induced DCs apoptosis. Transwell® co-culture demonstrated that direct contact between live NDV-stimulated DCs and T cells, rather than heated-inactivated NDV, inhibited $CD4^+$ T cell proliferation. Taken together, these findings provide new insights into the mechanism underlying NDV infections, particularly in relation to antigen presentation cells and suppression of T cell proliferation.

1. Introduction

Newcastle disease is a contagious bird disease affecting various domestic and wild avian species; its effects are most notable in domestic poultry (Boroomand et al., 2016). The causal agent, Newcastle disease virus (NDV), a variant of avian *paramyxovirus* 1 (APMV-1), is a negative-sense, single-stranded RNA virus. NDV/APMV-1 belongs to the genus *Avulavirus* of the family *Paramyxoviridae*. It has been reported that NDV may penetrate human cervical cancer HeLa cells and avian fibroblasts cells ELL-0 both by direct membrane fusion at the cell surface and through receptor-mediated and dynamin-dependent endocytosis (Sánchez-Felipe et al., 2014). We previously reported that NDV infects DF1 cells via macropinocytosis and clathrin-mediated endocytosis (CME), but not via caveolae-mediated endocytosis (CavME) (Tan et al., 2016), indicating the existence of multiple pathways for host cell infection and immune evasion. To further investigate how NDV evades the host immune system, we hereby examined the NDV infection of dendritic cells (DCs) and its impact on T lymphocytes.

DCs are antigen-presenting cells (APCs) that have the ability to internalize extracellular antigens using endocytic processes, such as receptor-mediated endocytosis, phagocytosis, and macropinocytosis (Liu et al., 2015). These can engulf viruses and bacteria into the cytoplasm and process the antigens into peptides via the cytosolic pathway or ubiquitin-proteasome pathway. The resulting peptides then bind to the major histocompatibility complex (MHC) I or II, which are then presented on the cell surface to facilitate $CD8^+$ or $CD4^+$ T cell recognition and activate downstream immune responses, such as the proliferation of T cells (Mantegazza et al., 2013). *Staphylococcus enterotoxin* B (SEB) is described as a 'superantigen' for its ability to bind to MHC II molecules on antigen presenting cells and β chain of the T cell receptor stimulating a large population of T-cells proliferation (Teresa, 2013). SEB has also been found to bind to another regulator of the T-cell immune response, CD28 (Arad et al., 2011).

Macropinocytosis, also known as cell drinking, is characterized by

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Table 1
Inhibitors used in this experiments and effect on cell.

Inhibitor	Working concentration	Effect on cell
Chlorpromazine (CPZ)	5 μM or 10 μM	Inhibitor of clathrin-mediated endocytosis
Methyl-β-cyclodextrin (MβCD)	2.5 mM or 5 mM	Disrupt lipid rafts in cells by depleting the cholesterol component
Cytochalasin-D (Cyt D)	5 μM or 10 μM	disruption of actin filaments and inhibition of actin polymerization
5-(N-methyl-N-isobutyl) amiloride (EIPA)	20 μM or 40 μM	Inhibitor of the NHEs

the formation of large (> 200 nm in diameter), actin rearrangement-driven, ruffled protrusions of the cell membrane, which are a type of endocytotic mechanism that involves the nonspecific uptake of extracellular material, such as soluble molecules, nutrients, and antigens (Mercer et al., 2009). Macropinocytosis plays a key role in DC-mediated antigen presentation to T cells against pathogens (Liu et al., 2015). Some types of viruses and bacteria, such as human immunodeficiency virus type 1 (HIV-1), vaccinia virus, influenza A virus, and Ebola virus, exploit macropinocytosis for host cell entry and the spread of infection (Edinger et al., 2014; Gobeil et al., 2013; Rizopoulos et al., 2015b; Sakurai et al., 2015). Furthermore, it is essential to determine whether NDV utilizes macropinocytosis for entry into APCs and if so, to determine the consequences of immune responses against NDV infection. Studies on other virus systems have indicated that the failure to proliferate in vitro is linked to deficient DCs function and/or T cell co-stimulation (Marshall et al., 2011).

Elankumaran et al. have demonstrated that NDV imparts its oncolytic effects by both intrinsic and extrinsic caspase-dependent pathways of cell death (Elankumaran et al., 2006). In another study, NDV-induced apoptosis was shown to be dependent on the upregulation of TNF-related apoptosis-inducing ligand (TRAIL) and caspase activation (Liang et al., 2017). These events result in the opening of mitochondrial permeability transition pores and the loss of mitochondrial membrane potential, which is responsible for the activation of apoptosis (Kumar et al., 2012).

TRAIL is a type II transmembrane protein that is produced and secreted by most normal tissue cells (Daniels et al., 2005; Forde et al., 2016). It induces apoptosis by binding to certain death receptors that are involved in cytotoxic lymphocyte-mediated apoptosis as well as plays an important role in T cell-induced DCs apoptosis (Falschlehner et al., 2009). Failure of T cells to proliferate in response to viral infections in vitro has been linked to Fas-FasL-dependent activation-induced cell death (AICD) (Fugier-Vivier et al., 1997; Zarozinski et al., 2000).

In the present study, we investigated the entry pathway of NDV into DCs and its effect on T cell proliferation. NDV entry mainly involves macropinocytosis and CME. Furthermore, NDV infections upregulate TRAIL and activate caspase-3 which in turn causes DCs apoptosis as well as suppresses $CD4^+$ T cell proliferation.

2. Materials and methods

2.1. Cells and the NDV

The NDV velogenic strain Herts/33 was reverse genetics rescued and identified in the Key Laboratory of Animal Infectious Diseases, Yangzhou University. NDV was propagated in 9- to 11-day-old embryonated chicken eggs and titrated onto galline embryonic fibroblast cells (DF-1, ATCC, Manassas, VA, USA) to determine the tissue culture infective dose ($TCID_{50}$) using the method of Reed and Muench (Reed et al., 1938). A $TCID_{50}$ of 1×10^5 $TCID_{50}$/mL will produce 0.7×10^5 Plaque Forming Units (PFU) /mL (Pourianfar et al., 2012). To calculate for the multiplicity of infection (MOI), the number of infectious particles was divided by the number of cells to be infected.

C57BL/6 mice were purchased from Shanghai Sippr-BK Laboratory Animal Co., Ltd. and bred in our own facility at the Shanghai Veterinary Research Institute (CAAS), according to the guidelines of the Institutional Animal Care and Use Committee (IACUC). All animal procedures were performed according to National Institutes of Health guidelines and approved by the IACUC of CAAS. The murine DCs were isolated from the bone marrow and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.1 mg/mL streptomycin, 100 U/mL penicillin, 10 ng/mL GM-CSF, and 20 ng/mL IL-4 at 37 °C in 5% CO_2.

2.2. Inhibitors, antibodies, and reagents

Pharmacological inhibitors, solvent dimethyl sulfoxide (DMSO), and superantigen SEB were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used at the appropriate concentrations that maintained cell vitality (Table 1). RIPA buffer, streptomycin, penicillin, tetramethylrhodamine-phalloidin (TRITC-phalloidin), mouse anti-β-actin monoclonal antibody (mAb), horseradish peroxidase-conjugated (HRP) goat anti-rabbit, and goat anti-mouse IgG antibody (Ab) were purchased from Sigma-Aldrich. The SDS-PAGE sample loading buffer and 4,'6-diamidino-2-phenylindole (DAPI) were obtained from Beyotime (Nantong, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), enhanced chemiluminescent reagent (ECL), TRITC-conjugated transferrin, Alexa Fluor® 594-conjugated cholera toxin subunit B (CTxB), and Texas red-conjugated-dextran, Alexa Fluor® 488 goat anti-mouse secondary antibody, FITC-conjugated anti-mouse CD11c, PE (phycoerythrin)-conjugated anti-mouse CD80 and APC (Allophycocyanin)-conjugated anti-mouse CD86 mAbs were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The mAb against the NDV nucleoprotein (NP; Herts/33 strain) was prepared in our laboratory.

2.3. NDV infection in the presence of inhibitors

DCs were seeded onto 12-well plates at a density of 2×10^5 cells per well 24 h prior to NDV infection. Pharmacological inhibitors were dissolved in DMSO at selected concentrations, after which their effect on the viability of DCs exposed to NDV was tested. After pretreating DCs with the inhibitor at 37 °C for 60 min, the cells were exposed to NDV at an MOI of 5 for 1 h at 4 °C and then incubated for 1 h to 37 °C before complete medium was added. At 5 h post-infection (hpi), the cell supernatant and pellets were harvested, separately. NP expression was analyzed using Western blotting.

2.4. Analysis of NDV-induced activation and apoptosis of DCs by flow cytometry

DCs were cultured for 7 days and then stimulated with NDV as earlier described. At the indicated time, DCs were carefully harvested and pelleted by centrifugation at 300*g* for 5 min. After centrifugation, DCs were resuspended in cold phosphate-buffered saline (PBS) solution with 2% heat-inactivated FBS. The cells were stained with FITC-Annexin V, PI (propidium iodide), or PE-labeled CD80, APC-labeled CD86 and FITC-labeled CD11c mAbs on ice for 20 min in the dark. Isotype-matched mAbs served as the negative controls and were included in all of the experiments. After incubation, the cells were washed thrice with a cold wash solution and resuspended in 200 μL of cold