Activation and cell death in human dendritic cells infected with Nipah virus

Manisha Gupta *, Michael K. Lo, Christina F. Spiropoulou
Viral Special Pathogens Branch, NCEZID, DMCPP, Centers for Disease Control and Prevention, Atlanta, CA 30333, USA

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A B S T R A C T
Nipah virus (NiV) is a highly pathogenic paramyxovirus that causes pulmonary disease and encephalitis in humans with 40–70% fatality. Interactions between NiV and the human immune system remain poorly understood. Here, we demonstrate the effects of NiV infection on DC and T cell function. Using an in vitro system, we found that NiV infects and replicates at low levels in DCs and induces the expression of TNF-α, IL-1α, IL-1β, IL-8, and IP-10. NiV infection activates DCs, and upregulates the expression of CD40, CD80, and CD86. Also have reduced levels of bcl2 and high levels of active caspase 3, suggesting the induction of apoptosis. DCs infected by NiV are unable to efficiently prime CD4 and CD8 T cells, but instead induce apoptosis in T cells. Interestingly, DCs treated with inactivated NiV also show signs of apoptosis. These findings indicate that NiV infected DCs could play an important role in NiV pathogenesis.

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Introduction
Nipah virus (NiV) is a highly pathogenic paramyxovirus belonging to the Henipavirus genus, which it shares with Hendra virus (Mayo, 2002). NiV and Hendra virus are 80–90% homologous at the genetic and antigenic levels (Halpin and Mungall, 2007; Rota et al., 2005, 2006). Subsequent recurring NiV outbreaks have been reported in Bangladesh and India since 2001 (Bellini et al., 2005; Lo et al., 2012; Luby et al., 2009b; Rahman et al., 2012). The fatality rate of NiV found in Bangladesh is ~70%, compared to 40% in Malaysia (Halpin and Mungall, 2007). NiV is transmitted from bats to pigs to humans (as in Malaysia) or directly from bats to humans (as in Bangladesh), and is capable of human-to-human transmission (Chua et al., 2002; Gurley et al., 2007; Luby et al., 2009a) Due to its mode of transmission and high fatality rates, NiV is considered as a high containment biosafety level-4 (BSL-4) virus.

NiV causes severe infection in both humans and animals. Its incubation period ranges from a few days to several weeks (Wong and Tan, 2012). The virus probably enters through aerosol, initially lung endothelial cells and alveolar macrophages. From the lungs, NiV spreads to peripheral organs, like spleen, lungs, heart, and kidneys. NiV infects a variety of cell types, including parenchymal cells, endothelial cells, smooth muscle cells, nerve cells, and monocytes (Wong and Tan, 2012). Ephrin B2 and B3 have been identified as NiV receptors; while ephrin B2 is the higher-affinity receptor used by both Hendra and NiV, ephrin B3 is a functional receptor only for NiV, despite a comparatively lower affinity for the NiV glycoprotein (Bonaparte et al., 2005; Negrete et al., 2005, 2006).

NiV pathology in humans includes vasculitis, endothelial multinucleated syncytia, and thrombosis. Initially, NiV causes non-specific symptoms like fever, headache, and drowsiness, and progresses to pulmonary disease or encephalitis, and death (Lam and Chua, 2002). NiV pulmonary syndrome is an influenza-like illness, with hypoxemia and diffuse alveolar shadowing on chest X-rays. Neurological signs include confusion, motor deficits, and seizures (Lam and Chua, 2002; Wong and Tan, 2012).

Dendritic cells (DCs) play a central role in initiating host innate and adaptive immune responses. Immature DCs capture viruses, process antigens, and becomes mature and activated. Activated DCs produce anti-viral cytokines and chemokines to limit viral replication and spread. Mature DCs upregulate MHC Class II and other co-stimulation molecules on the cell surface, and migrate to draining lymph nodes to activate CD4 and CD8 T cells and initiate humoral and cellular responses to combat infection (Pollara et al., 2005). A number of paramyxoviruses, like measles (Griffin, 2010), respiratory syncytial (Guerrero-Plata et al., 2009), and simian/parainfluenza (Pejawar et al., 2005) viruses, are known to replicate in DCs. Among these, measles virus severely alters DC function, causing T cell anergy and immunosuppression (Griffin, 2010).

In this report, we document the modulation of DC function by NiV infection. Using an in vitro system, we show that NiV productively infects DCs and induces the production of certain chemokines and cytokines. In addition both NiV infection of DCs...
and treatment with inactivated NIV cause the upregulation of co-stimulatory molecules and induce apoptosis; However, DCs infected with NIV are unable to stimulate CD4 and CD8 T cells.

**Results**

**NIV infection of DCs**

Although a prior study had documented NIV infection of DCs, endogenous levels of the functional NIV receptors ephrin B2 and B3 were not evaluated at the protein level (Mathieu et al., 2011). We examined the expression of ephrin B2 and B3 in human monocyte-derived DCs by flow cytometry, and detected low levels of ephrin B3 at the cell surface but not ephrin B2 (Fig. 1A). Our results are contrary to a previous report of low ephrin B2 mRNA expression levels and undetectable ephrin B3 mRNA in DCs (Mathieu et al., 2011). Vero-E6 cells were used as positive controls. Both DCs and Vero-E6 cells show low levels of ephrin B3 expression (Fig. 1A) and did not express significant levels of ephrin B2, whereas ~13% of Vero-E6 cells have high levels of ephrin B2 receptor (Fig. 1A).

We then infected immature DCs with purified NIV or treated with purified inactivated NIV, and then stained the cells for NIV antigen by IFA. Approximately 60–65% cells were positive for NIV antigen in NIV infected cultures (Fig. 1B). A small percentage (2–5%) of DCs was also weakly positive for NIV antigen in cells treated with purified inactivated NIV. Next, we examined viral replication by determining viral RNA levels in DCs (Fig. 1C). Viral genome copy numbers increased two-fold by day 1, but remained relatively constant at days 2 and 3, and gradually decreased at later time points (data not shown). Together these findings suggest that NIV infects DCs, but does not replicate efficiently in these cells.

**NIV induces cytokine and chemokine expression in DCs**

We next determined whether NIV induces cytokine and chemokine expression in DCs. DCs were infected with purified NIV or with inactivated NIV; cell culture supernatants were collected from day 1 to 4 post-infection and tested for the presence of 26 cytokines and chemokines by a multiplex assay. Out of the 26 cytokines tested, only 7 showed significant increases or decreases compared to the mock-infected controls: IL-1β, TNF-α, MIP-1α, IL-10, IP-10, IL-8, and IL-1α (Fig. 2). Expression of IL-1β, TNF-α, IL-10, and MIP-1α peaked at day 1 and decreased by day 4 post-infection. Levels of these cytokines are significantly higher in DCs infected with live virus than in those treated with inactivated virus. Inactivated NIV-treated cells also express TNF-α, IP-10 and MIP-1α. We did not observe any significant differences in IL-8 levels between DCs infected with NIV or treated with inactivated NIV. Also, we saw no significant induction of IFN-α and β in infected DCs (data not shown).

**NIV causes activation-induced cell death in DCs**

We next examined the effects of NIV infection on DC function. Monocyte-derived DCs marked as CD1a+ cells infected with purified NIV or treated with purified inactivated NIV were stained for the presence of activation and apoptotic markers. Fig. 3 shows mean fluorescence intensity of various markers on days 5 and 8 post-infection. We used these time points to observe apoptosis followed by activation signals. Usually, pathogens cause activation and induce death signals in cells that are detectable later after infection. The left panel of Fig. 3A shows histogram plots for activation and co-stimulation molecules on CD1a+ cells, and saw significant upregulation of CD40, CD80, and CD86 levels in infected cells and cells treated with inactivated NIV, or inactivated Ebola virus. We also studied different co-stimulation molecules on the surface of CD1a+ cells, and saw significant upregulation of CD40, CD80, and CD86 levels in infected cells and cells treated with inactivated NIV, or inactivated Ebola virus. The MFI levels of CD40 on CD1a+ cells increased from 96 to 147 in NIV infected, and 162 in those receiving inactivated NIV or with inactivated Ebola virus, but not cells infected with NIV. We also studied different co-stimulation molecules on the surface of CD1a+ cells, and saw significant upregulation of CD40, CD80, and CD86 levels in infected cells and cells treated with inactivated NIV, or inactivated Ebola virus. The MFI levels of CD40 on CD1a+ cells increased from 96 to 147 in NIV infected, and 162 in those receiving inactivated NIV. MFI of CD80 on CD1a+ cells changed

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**Fig. 1.** Nipah virus (NIV) infection of dendritic cells (DCs). Monocyte-derived dendritic cells were tested for NIV receptors ephrin B2 and ephrin B3 by flow cytometry. Vero-E6 cells were used as positive controls. (A) Histograms showing receptor staining. Black lines show isotype controls and red line represent receptor staining. (B) IFA staining for NIV antigen on DCs 24 h post-infection (magnification 20 ×). Viral antigen is stained with FITC and shown in green; the nucleus (blue) is stained with DAPI. (C) NIV replication (determined as viral RNA copy numbers per ng of total RNA) in DCs 0–3 days post-infection. Results are representative of 3 experiments.
Fig. 2. Cytokines and chemokines from DCs infected with NIV. DCs were infected with either purified live or inactivated NIV (MOI=5). Supernatants were collected on 1–4 days post-infection. Expression of 26 cytokines was tested by Luminex method. Only 7 cytokines show a significant difference over uninfected controls. Y-axis shows cytokine concentration in pg/mL; x-axis shows days post-infection. Data is plotted as mean ± SEM. Data are representative of 3 experiments.

Fig. 3. NIV causes activation-induced cell death in DCs. FACS analysis of DC activation and apoptosis on days 5 (A) and 8 (B) post-infection. Left panels in A and B show histograms for MHC class II, CD40, CD80, and CD86 molecules on the x-axis, and cell numbers on the y-axis. Values inside the plots represent mean florescent intensity (MFI) of each molecule. In (A) scatter plots on right-hand panel show bcl2 and active caspase 3 on the x-axis, and FSC on the y-axis. Histogram in lower panel indicates FSC on DCs on day 5 post-infection. In (B) scatter plots show Fas, bcl2, and active caspase 3 levels on the x-axis, and FSC on the y-axis on day 8 post-infection. These results are representative of 7 independent experiments. MFI of isotype controls for CD40, CD80, and CD86 were 30, and for bcl2 and caspase 3 were 20.
from 365 in uninfected controls to 698 in NiV infected, and to 866 in cells treated with inactivated virus. Similarly, MFI of CD86 on CD1a+ cells increased from 414 in uninfected cells to 1854 and 866 in infected and cells treated with inactivated NiV, respectively. These increases suggest that inactivated NiV activates DCs even in the absence of virus replication. These experiments were repeated seven times. The MFI of MHC class II and costimulatory molecules varied only between 5% and 8% in seven independent experiments.

We also examined apoptosis markers (bcl2 and activated caspase 3) on CD1a+ infected smaller cells (FSClow). Usually DCs appear in the 400–800 channels, as shown in the lowest right panel of Fig. 3A. Only 27% of CD1a+ cells were FSClow (< 400 channels) in uninfected controls, compared to 58% and 42% of infected cells or cells treated with inactivated NiV, respectively. Of the CD1a+ cells in uninfected controls, 24% are also FSClow bcl2low, and 13% are FSClow active caspase 3high. However, the number of CD1a+ FSChigh bcl2low cells increased in NiV-infected cells. In NiV-infected CD1a+ cells, 49% of the FSClow bcl2low, and 24% were FSClow active caspase 3high. Similarly, CD1a+ cells treated with inactivated virus, 37% are also FSClow bcl2low, and 16% are FSClow active caspase 3high. Treatment with inactivated Ebola virus does not change the FSClow population or apoptotic marker expression compared to uninfected controls.

However, NiV-infected DCs show high CD40, 80, and 86 levels compared to uninfected controls on day 8 post-infection, although CD80 and CD86 levels are lower on day 8 than they were on day 5 (Fig. 3B). These data indicate that DCs are activated by NiV infection.

We then examined Fas expression in FSClow cells. Approximately 40% and 32% CD1a+ NiV infected cells and inactivated NiV-treated cells, respectively, are Fashigh FSClow, compared to 13% in controls (Fig. 3B) on day 8 post-infection. We did not find any significant change in numbers of Fashigh FSClow on CD1a+ cells between uninfected and NiV infected groups on day 5 post infection (8% versus 9.1%, respectively). The number of FSClow bcl2low cells in infected DCs increased from day 5; 72% and 51% of NiV infected cells and inactivated NiV-treated cells, respectively, are FSClow bcl2low. Similarly, 51% of NiV-infected cells and 43% of inactivated NiV-treated cells are FSClow active caspase 3high compared to 11% of uninfected controls (Fig. 3B). These data suggest that NiV causes activation-induced cell death in DCs independently of viral replication.

NIV-infected DCs induces T cell apoptosis

We found that DCs infected with NiV show an activated phenotype, so we next determined whether these activated DCs can induce T cell priming. We co-cultured infected DCs with T cells, and determined the expression of T cell activation markers such as CD25, CD62Llow, and CD44high on CD4 and CD8 T cell populations on day 8 post-infection. In addition we also determined induction of apoptosis in T cells as we did with the DCs.

We found that DCs infected with NiV partially activate CD4 T cells, as no upregulation of CD25 and CD44 molecules on these cells was observed (Fig. 4A). However, CD4 T cells do down-regulate CD62L: MFI of CD62L was 699 in uninfected controls and 394 in NiV infected cells. Surprisingly, a small percentage (6%) of CD4+ FSChigh cells have the active caspase 3high phenotype (compare to 4% of controls), suggesting that CD4 T cells undergo a partial and insufficient activation. Interestingly, DCs treated with inactivated NiV were able to induce CD4 T cells activation more than DCs infected with virus; 28% CD3+CD4+ cells are CD25+ v. 19% controls; 47% are CD62Llow v. 18% controls; 11% CD44high v. 7% controls, and 15% are FSChigh active caspase 3high v. 4% controls (Fig. 4A). DCs treated with inactivated Ebola virus were able to activate CD4 T cells.

Approximately 12% of CD3+8+ T cells were CD25+ v. 6% controls, and 20% cells were CD62Llow v. 8% controls, again suggesting that DCs infected with NiV partially activate CD8 T cells (Fig. 4B). DCs treated with inactivated NiV were able to activate CD8 T cells better than virus infection: 43% inactivated NiV-infected cells were CD25+ v. 6% controls; 34% were CD62Llow v. 8% controls; 11% FSChigh cells were active caspase 3high v. 4% controls (Fig. 4B). DCs treated with inactivated Ebola virus were able to activate CD8 T cells.

Surprisingly, we found that CD4 and CD8 T cells also expressed low levels of bcl2: 58% and 40% of infected CD4 T cells or inactivated NiV treated cells, respectively, were FSClow bcl2low, compared to 7% of mock control cells and 9% in cells treated with inactivated Ebola virus (Fig. 4C). Similarly, 54% and 20% infected CD8 T cells or inactivated NiV treated cells were FSClow bcl2low (14% uninfected controls and 11% in inactivated Ebola virus samples, Fig. 4C). These data suggest that NiV-infected DCs do not induce sufficient priming of CD4 and CD8 T cells, but induce apoptotic signals in these cells. To verify that the priming is DC-dependent, we examined various activation and apoptotic markers in T cells without DCs. We found no upregulation of CD25 or CD44, and no change in bcl2 levels, suggesting that activation and apoptosis of T cells is DC-dependent (data not shown). We also found that T cells do not replicate Nipah virus. We test this with viral antigen by protein assay and also by RNA levels and did not see any change in the levels compared to controls.

Discussion

Our study is the first to show that Nipah virus alters DC function and thus negatively affects host cell-mediated immunity. Our results confirm a previous report that NiV can infect DCs, but cannot replicate to high titers (Mathieu et al., 2011). In human endothelial cells, NiV can induce various chemokines and cytokines, particularly IP-10 and IL-8 (Lo et al., 2010). IL-8 primarily attracts neutrophils and has less effect on monocytes and T cells (Mahalingam et al., 1999). IP-10 attracts activated T cells and natural killer cells, and is associated with anti-viral activities (Mahalingam et al., 1999). Previous reports have shown that both infectious and non-infectious central nervous system pathology is associated with high levels of these cytokines (Glabinski and Ransohoff, 1999), suggesting that they may play an important role in NiV pathogenesis and chemotaxis. Activated DCs can be a source of these cytokines. In addition, NiV infection upregulates IL-10, an anti-inflammatory immune-suppressor cytokine primarily produced by the monocytes and DCs. In general, IL-10 downregulates Th1 response, inhibits CD8 T cell response (O'Garra et al., 2008), and reduces IFN-γ, IL-2, and TNF-α, thus inhibiting the inflammatory response (Brockman et al., 2009). In our study, IL-10 is up-regulated early in the infection, and then decreases, in a course similar to viral replication. It is possible that IL-10 is involved in downregulating and/or modifying CD4 and CD8 T cell responses in NiV infection. Pathways underlying the induction of these cytokines and their role in the cellular response to NiV infection remain to be determined.

A number of viruses, like human immunodeficiency (Rodriguez-Plata et al., 2012), Ebola (Mahanty et al., 2003), herpes simples (Salio et al., 1999), and measles viruses (Servet-Delprat et al., 2000b), interfere with DC function and affect T cell responses. The measles virus infects DCs and upregulate MHC-class II, CD40, 80, and 86 molecules on DC surface (Schnorr et al., 1997; Servet-Delprat et al., 2000b). DCs do not undergo apoptosis until T cells are co-cultured with them, but adding T cells increases virus
replication and DC death (Fugier-Vivier et al., 1997). DC death is associated with expression of Fas/Fas ligands and TNF-α-related apoptosis-inducing ligand (TRAIL) (Servet-Delprat et al., 2000a; Vidalain et al., 2000). In our study, NiV, even when inactivated, can upregulate co-stimulatory molecules. DCs activated by NiV also express low levels of bcl2 and high levels of active caspase 3 and Fas molecules, and undergo cell death. This DC apoptosis does not require any other cells and is replication-independent. Whether this cell death is Fas-mediated or not is unknown.

In a recent study in measles virus by Hahm et al. (2005) show that measles virus downregulate MHC class II expression in DCs which may be responsible for immunosuppression caused by measles virus. In our study we also found that NiV infection of DCs downregulate MHC class II levels. Whereas there is a significant increase in the levels of co-stimulatory molecules compared to controls on DCs. NiV infected DCs do not activate T cell efficiently. On the other note inactivated NiV and inactivated Ebola virus treated DCs able to unregulate MHC class II levels and also activate T cells. This suggests that NiV may induce insufficient activation of DCs that might be responsible for T cells unresponsiveness. Mechanism by which NiV downregulate MHC class II levels has to be determined. We also observed two distinct populations of DCs one that unregulates CD80 levels and one that does not have high levels of CD80. The reason of this distinct population is possibly due to insufficient signaling of Nipah antigen and causing for T cell unresponsiveness.

DCs co-cultured with T cells do not efficiently activate CD4 and CD8 T cells populations. Both CD4 and CD8 T cells also fail to proliferate in live NiV infected DC cultures (data not shown). In fact, T cells show downregulation of bcl2 and upregulated active caspase 3, suggestive of apoptosis. T cells infected with measles virus undergo anergy and/or death when co-cultured with DCs (Kerdiles et al., 2006; Okada et al., 2000). T cell apoptosis and/or anergy require MHC complex-T cell receptor interaction induced by DCs (Grosjean et al., 1997). T cells also express receptors for NiV, and NiV may infect T cells (Mathieu et al., 2011). To test whether T cell apoptosis is mediated directly by NiV or through DCs, we infected T cells with live or inactivated NiV without DCs. We found no activation or cell death (indicated by bcl2 and active caspase 3) in the absence of DCs, suggesting that T cell apoptosis requires MHC-T cell interaction.
We found that purified gamma-irradiated NiV also induces apoptosis in DCs and T cells in DC/T cells co-cultures. This suggests that cell death in DCs is not replication-dependent. It has been previously shown that measles virus attachment glycoprotein can cause apoptosis in PBMCs (Iwasa et al., 2010). We do not know yet if NiV glycoprotein can also cause apoptosis in DCs. DCs treated with inactivated NiV can activate CD4 T and CD8 cells better than those given live virus and show less cell death. NiV proteins, factors, and mechanism causing DC death remain to be identified.

Under the limitations of in vitro study, this is the first study showing that NiV infection alters DC function. NiV infection of DCs does not induce anti-viral cytokines, such as IFN-α and IFN-β, but induces inflammatory cytokines like TNF-α, IP-10. Also induces cytokines IL-8, IL-10 an indication that it can suppress the host immune response by multiple pathways. NiV infection transiently activates DCs early on and subsequently induces their cell death. However, the transiently activated DCs are unable to activate T cells sufficiently to mount an anti-viral immune response. Our study provides new insights into NiV immune modulation and will possibly help to understand the mechanisms of NiV pathogenesis.

Materials and methods

Virus

NiV (strain 810573, accession AF212302 isolated from a patient of the 1998–1999 Malaysia outbreak) was grown in Vero-E6 cells. All experiments with live virus were done in a BSL-4 laboratory at Centers for Disease Control and Prevention (Atlanta, GA, USA).

Purification of NiV

Vero-E6 cells grown in a monolayer were infected with 5 × 10⁷ pfu/mL of NiV. On day 5, the supernatant was collected, centrifuged at 2000 rpm for 10 min to remove cell debris, and stored at −70 °C. For purification, 12 mL of 25% glycerol in TNE (Tris–NaCl–EDTA) solution was added first, and 22 mL of the supernatant was carefully layered on top, and centrifuged at 25,000 rpm speed for 4 h in an SW28 rotor (Beckman Coulter, Brea, CA, USA). After centrifugation, the supernatant was decanted and the pellet was re-suspended in phosphate buffered saline (PBS) overnight at 4 °C. Viral titer was measured by plaque assay.

Isolation and purification of monocyte-derived DCs and T cells from peripheral blood mononuclear cells (PBMCs)

Aphaeresis from healthy donors was collected randomly and layered on a ficoll gradient medium (ICN Biomedicals, Aurora, OH, USA). Approximately 10 different donors were used to collect blood. Isolated PBMCs were washed, and monocytes were separated using magnetic beads (Miltenyi Biotec, Auburn, CA, USA) following the manufacturer’s protocol. Monocytes were plated on 6-well plates at the concentration of 2 × 10⁶ cells/well, and allowed to adhere for 40 min. Non-adhering cells were removed and adherent cells were re-suspended in RPMI media containing 10% Fetal Calf Serum (FCS), 50 ng/mL interleukin 4 (IL-4), and GM-CSF (granulocyte-macrophage colony stimulating factor). Media was supplemented with 50 ng/mL of IL-4 and GM-CSF on days 0, 3, and 5, as shown before (Rafferty et al., 2002). On day 7, the cells were harvested and tested for DC purity by CD1a-APC and Class II staining. Over 99% of the cells were positive for CD1a and Class II. T cells were isolated using magnetic beads as described above. T cell identity and purity were determined by CD3 staining. Over 99% cells were positive for CD3.

Infection protocol

DCs were infected with 5 multiplicity of infection (MOI) of either purified live or inactivated NiV. Virus was inactivated by gamma radiated using 5 × 10⁶ rads. Inactivation was tested by the absence of virus growth after a double blind passage in Vero E6 cells. DCs were also infected with purified inactivated Ebola virus (1 MOI) grown in Vero-E6 cells as a positive control for DC activation. After infection, DCs were mixed with T cells in a 1:3 ratio, and activation and apoptosis were determined. T cells were incubated with DC infected live or killed Nipah virus until they were tested for activation and apoptosis signals for example day 5 or 8 post infection. In some experiments, purified T cells were directly infected with 5 MOI of purified NiV or treated with purified inactivated NiV. Cells were examined by flow cytometry for activation and apoptosis markers on day 8 post-infection.

Real-time PCR for determining NiV genome copy number

To determine NiV genome copy numbers, we used the NIP3END primer 5′-ACCAAACAGGCAGATATGCCATAC-3′ to reverse transcribe total RNA (8 μL) extracted from DCs infected with purified NiV. We used the Superscript III First Strand Synthesis kit (Invitrogen, Grand Island, NY, USA) according to manufacturer’s protocols. The resulting cDNA was diluted 1:5, and 2.0 μL of cDNA per sample was used in a 25 μL (total volume) RT² SYBR green/ROX real-time PCR reaction using primers adapted from a real-time Taqman RT-PCR previously described (Lo et al., 2012). Synthetic NiV N RNA standards of known quantity (~10³ copies/μL) were reverse-transcribed using primer NIP9N 5′-TACACATACAGCTCTGACGAA-3′ in a 20 μL reaction, and diluted 1:5. 2.0 μL of these cDNA samples were used to make 10-fold dilutions to generate a standard curve for NiV N gene copy number. We normalized the NiV N gene copy numbers to total ng of RNA based on a standard curve plotting ng of RNA versus Ct levels of GAPDH using primers previously described (Lo et al., 2010).

Surface and intracellular staining

To identify surface markers, cells were incubated on ice with 50 μL of mouse anti-human CD1a-APC, Fal-PE, Class I-PE, Class II-PE, CD40-PE, CD80-PE, CD86-PE, or isotype controls for 30 min. All antibodies were purchased from BD Pharmingen (San Diego, CA, USA). After incubation, cells were washed and permeabilized using a cytofix kit (BD Pharmingen) according to the manufacturer’s instructions. After permeabilization, cells were incubated with active caspase 3-PE, Bcl2-PE, and isotype controls for 30 min on ice. Unadsorbed labeled antibodies were washed away by using centrifugation, and cells were fixed with 0.4% paraformaldehyde and stored at 4 °C until processing. Approximately 40,000 cells were counted in each sample. Cells were gated on CD1a markers above Mean Florescent Intensity (MFI) values of the isotype controls (MFI of 20).

T cells were tested for activation markers on CD4 and CD8 T cells using CD25-PE, CD62L-PE, CD44-PE, or isotype control (BD Pharmingen) as described above. For intracellular staining, cells were permeabilized with cytofix/cytoperm buffer and stained for Bcl2 and active caspase 3. Cells were fixed with 0.2% paraformaldehyde and read on FACS Calibur.

We also tested ephrin B2 and B3 levels in monocyte-derived DCs. Vero-E6 cells were used as positive control. Fc receptors were blocked on DCs with rabbit IgG (10 μg/mL). Primary antibodies against ephrin B2 and B3, and isotype controls, were purchased from Abcam Biotech (Cambridge, MA, USA).
All cell staining was performed in a BSL-4 laboratory. Cell signals were acquired on FACS Calibur inside the BSL-4 laboratory, and analyzed with FACS FlowJo (BD Pharmingen) software.

**Immunofluorescent assays (IFA)**

5 x 10^4 monocyte-derived DCs were plated on glass coverslip in 24-well plates. Cells were infected with 5 MOI of purified NiV or treated with purified inactivated NiV for 1 h. After 24 h, cells were fixed in 4% paraformaldehyde for 10 min. All infections were done in the BSL-4 laboratory. Plates were treated with 5% FCS, and 4 post-infection. All infections were performed in a BSL-4 laboratory. Cells were permeabilized with 0.1% Triton-X for 5 min and washed with PBS. Cells were stained with 1:500 dilution of polyclonal antibody against NiV antigens, and anti-mouse FITC antibody made in goats as the secondary antibody (1:1000 dilution). All antibodies were applied for 30 min at room temperature. Cells were washed 3 times with PBS between antibody applications. Cells were mounted in glycerol containing DAPI.

**Cytokine detection using LumineX beads**

Levels of 26 cytokines and chemokines were detected using Luminex technology (Luminex Corp., Austin, TX, USA). One customized cytokine kit was used according to manufacturer’s instructions. The target cytokines were interleukins (IL)-1α, IL-1β, IL-10, IL-6, IL-12p40, IL-12p70, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, tumor necrosis factor (TNF)-α, TNF-β, interferon α2 (IFN-α2), IFN-γ, IFN-inducible protein (IP-10), T-cell growth factor α (TGFA-α), regulated-on-activation, normal T cell expressed and secreted (RANTES), and vascular cell adhesion molecule-1 (VCAM-1).

Samples were collected from DCs infected with 5 MOI of live or inactivated NiV. Culture supernatants were collected on days 1, 2, 3, and 4 post-infection. All infections were performed in a BSL-4 laboratory. Cell supernatants were incubated with antibody-coupled beads for 1 h at room temperature. The beads were then incubated at room temperature with detector antibody for 1 h, and with streptavidin–phycoerythrin for 30 min. Finally, the complexes were washed and re-suspended in running buffer. The beads were washed 3 times between each step according to manufacturer’s instructions. 100 beads per cytokine were acquired using Luminex 400. Mean fluorescent intensity was measured to calculate final cytokine concentrations in pg/mL.

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**References**


Grosjean, I., Caux, C., Bella, C., Berger, I., Wild, F., Banchereau, J., Kaiserlian, D., 1997. The views expressed in this article are solely those of the authors and do not represent the official views or opinions of the Institute.


