The Combination of Type I IFN, TNF-α, and Cell Surface Receptor Engagement with Dendritic Cells Enables NK Cells To Overcome Immune Evasion by Dengue Virus

Daniel Say Liang Lim, Nobuyo Yawata, Kevin John Selva, Na Li, Chen Yu Tsai, Lai Han Yeong, Ka Hang Liong, Eng Eong Ooi, Mun Keat Chong, Mah Lee Ng, Yee Sin Leo, Makoto Yawata and Soon Boon Justin Wong

_J Immunol_ published online 15 October 2014
http://www.jimmunol.org/content/early/2014/10/15/jimmunol.1302240
The Combination of Type I IFN, TNF-α, and Cell Surface Receptor Engagement with Dendritic Cells Enables NK Cells To Overcome Immune Evasion by Dengue Virus

Daniel Say Liang Lim,* Nobuyo Yawata,† Kevin John Selva,† Na Li,*  
Chen Yu Tsai,* Lai Han Yeong,* Ka Hang Liong,* Eng Eong Ooi,‖ Mun Keat Chong,*  
Mah Lee Ng,* Yee Sin Leo,# Makoto Yawata,*†‡ and Soon Boon Justin Wong*†‡‡

Clinical studies have suggested the importance of the NK cell response against dengue virus (DenV), an arboviral infection that afflicts >50 million individuals each year. However, a comprehensive understanding of the NK cell response against dengue-infected cells is lacking. To characterize cell-contact mechanisms and soluble factors that contribute to the antidendengue response, primary human NK cells were cocultured with autologous DenV-infected monocyte-derived dendritic cells (DC). NK cells responded by cytokine production and the lysis of target cells. Notably, in the absence of significant monokine production by DenV-infected DC, it was the combination of type I IFNs and TNF-α produced by DenV-infected DC that was important for stimulating the IFN-γ and cytotoxic responses of NK cells. Cell-bound factors enhanced NK cell IFN-γ production. In particular, reduced HLA class I expression was observed on DenV-infected DC, and IFN-γ production was enhanced in licensed/educated NK cell subsets. NK–DC cell contact was also identified as a requirement for a cytotoxic response, and there was evidence for both perforin/granzyme as well as Fas/Fas ligand–dependent pathways of killing by NK cells. In summary, our results have uncovered a previously unappreciated role for the combined effect of type I IFNs, TNF-α, and cell surface receptor–ligand interactions in triggering the antidendengue response of primary human NK cells.  

The Journal of Immunology, 2014, 193: 000–000.
NK receptors, such as NKp30, NKp46, NKp44, NKp80, NKGD2, DNAM-1, and 2B4, have potential to trigger NK responses if their ligands are present on the surface of virus-infected cells. Inhibitory NK cell receptors include members of the polymorphic killer cell Ig-like receptor (KIR) family, the KNG2A/CD94 heterodimer and LILRB1. These receptors differ in their recognition specificity. For instance, KIR2DL1 and KIR2DL2/3 interact with group 2 and group 1 HLA-C allotypes, respectively, whereas KIR3DL1 binds to HLA-A/B allotypes that contain the Bw4 motif. NKG2A interacts with HLA-E, and LILRB1 binds to HLA-G with high affinity and to HLA-A/B/C with low affinity. These receptors are expressed in a nonhomogenous manner, leading to the generation of subpopulations of NK cells that form the NK cell repertoires of human individuals. Tissue expression of the cognate ligands for these inhibitory receptors suppresses the activity of NK cells. Conversely, if the ligands for inhibitory NK cell receptors are downregulated on the surface of virus-infected cells, the reduced inhibitory signaling can lead to NK cell activation when other activating NK receptors are engaged (missing-self recognition) (11). Only KIR and NKG2A among the above-listed receptors are known to be involved in this missing-self NK cell response, whereas LILRB1 is known to convey inhibition but does not trigger a missing-self response (12).

Although peripheral NK cells isolated from dengue patients have been shown to display markers of activation when analyzed ex vivo during the acute phase of infection (13, 14), the soluble factors and membrane-bound interactions that activate the response of NK cells to dengue-infected primary cells have not been defined. To elucidate these mechanisms, we cocultured purified NK cells with autologous monocyte-derived DC that had been infected with DenV, and used this system to screen for key regulators that regulate antedengue NK cell responses.

**Materials and Methods**

**Ethics statement**

This research was conducted according to the principles expressed in the Declaration of Helsinki, and was approved by the Institutional Review Board, National University of Singapore. All volunteers who participated in this study provided written informed consent.

**Virus stock**

DenV serotype 1 strain S3638 (DenV1-S3638, a generous gift from Eng Eong Ooi), Duke-National University of Singapore Graduate Medical School) was propagated in the C6/36 mosquito cell line. Six days post-infection, cell-free supernatant was collected after centrifugation of culture supernatant at 10,000 × g for 10 min, aliquoted, and stored at −80°C as virus stock. Limiting dilution plaque assays were then carried out using the baby hamster kidney fibroblast cell line (BHK-21) to determine the titer of the virus stock. Low-passage clinical isolates of DenV serotype 1 (DenV1-07K4383) or DenV serotype 3 (DenV3-05K4176) were purchased from Mah Eong Ooi, Duke–National University of Singapore Graduate Medical School, whereas human rIL-4 and 800 U/ml GM-CSF (both from eBioScience) were added to inhibit protein transport. For assessing NK cell missing-self responses, 1 × 10⁶ PBMC were cocultivated with K562 cells (American Type Culture Collection) at an E:T ratio of 5:1 for 14 h in a round-bottom tube with a final volume of 300 μl complete medium supplemented with 2000 U/ml IL-2 (R&D Systems). Brefeldin A (Golgiplug; BD Biosciences) was added during the last 13 h (12).

**Abs and reagents**

Blocking Ab against IFN-αβ receptor chain 2 (IFNAR2, clone MMHAR-2) was purchased from PBL InterferonSource. Neutralizing Abs against TNF-α (clone T22, (clone MAH1)), and 2B4 were purchased from eBioScience. Neutralizing Abs against IL-15 (clone 34593), IL-18 (clone 12-2H), and the gp130 signal-transducing subunit of the IL-27R (clone 28126) were purchased from R&D Systems. F(ab')₂ fragments of blocking Ab against Fas (clone ZB4; Millipore) and its isotype control (clone P3.6.2.8.1; EBiogene) were generated using the F(ab')₂ Preparation Kit (Pierce Biotechnology). Recombinant human DNAM-1-Fc, NKGD2-Fc, NKG2A-Fc, NKG2D-Fc, NKp44-Fc, NKp46-Fc, and NKp80-Fc fusion proteins, and human IgG1-Fc control protein were purchased (R&D Systems). Cellular receptor-Fc proteins were detected using PE-conjugated F(ab’)_2 fragments of donkey anti-human IgG (Jackson ImmunoResearch Laboratories). The following Abs used in our experiments were purchased from eBioScience: PE-conjugated anti-CD56 (clone MEM188), PerCP-Cy5.5-conjugated anti-CD3 (clone OKT3), allophyocyanin-conjugated anti-IFN-γ (clone 4S.B3), FITC-conjugated anti-CD107a (clone eBioHA3), PE-conjugated anti–HLA-E (clone 3D12HLA-E), and FITC-conjugated anti-Fas (clone DX2). FITC-conjugated anti–HLA-G (clone MEM-G9) was purchased from AbD Serotec. FITC-conjugated anti-CD48 (clone J4-57), PerCP-Cy5.5-conjugated anti-CD158b (GL183), PE-Cy7–conjugated CD158a/h (EB6), and PE-conjugated anti-NKGD2 (Z199) were purchased from Beckman Coulter. V500-conjugated anti-CD3 (SP34-2) was from BD Biosciences. FITC-conjugated anti-CD158a/h (HPMA4), allophyocyanin-conjugated anti-CD56 (MEM-188), PE-Cy7–conjugated anti–IFN-γ (4S.B3), and allophyocyanin-Cy7–conjugated anti–IFN-γ (4S.B3) were from BioLegend. Viobio-conjugated anti-CD158e (DX9) was from Miltenyi Biotec. The supernatant of HB114 hybridoma (provided by Eng Eong Ooi) containing anti-DenV premembrane (prM) Abs (mouse IgG2a isotype) was used to stain for dengue infection of DC, and secondary staining used either Alexa Fluor 488–conjugated goat anti-mouse or Alexa Fluor 647–conjugated goat anti-rabbit secondary Abs against DenV prM (mouse IgG1 isotype). Recombinant soluble Fas protein was purchased from PeproTech, whereas human rTNF-α and universal human type 1 IFN were purchased from eBioScience and PBL InterferonSource, respectively.

**Flow cytometry**

NK cell staining was performed in FACS wash (PBS containing 1% BSA and 0.02% sodium azide) by incubating with Abs against cell surface markers at 4°C for 30 min. Samples were washed twice with FACS wash to remove unbound Abs, fixed, and permeabilized using the Cytofix/ Cytoperm solution kit (BD Biosciences). Intracellular staining was performed by resuspending the samples in Perm/Wash buffer (BD Biosciences) containing Abs and incubating at 4°C for 30 min. Samples were then washed twice with Perm/Wash buffer and stored in FACS wash prior to flow cytometry data acquisition.

DC expression of membrane-bound ligands for DNAM-1, NKG2D, or natural cytotoxicity receptors (NCR) was assessed by incubating cells with the appropriate recombinant receptor-Fc proteins in FACS wash at 4°C for 30 min. Prior to incubation with the receptor-Fc proteins, 500 U/ml human rIL-15 and 1000 U/ml human rIL-18 (both from eBioScience) were added to inhibit protein transport. For determining the percentage of subpopulations of NK cells that form the NK cell repertoires of human individuals, Tissue expression of the cognate ligands for these inhibitory receptors suppresses the activity of NK cells. Conversely, if the ligands for inhibitory NK cell receptors are downregulated on the surface of virus-infected cells, the reduced inhibitory signaling can lead to NK cell activation when other activating NK receptors are engaged (missing-self recognition) (11). Only KIR and NKG2A among the above-listed receptors are known to be involved in this missing-self NK cell response, whereas LILRB1 is known to convey inhibitor but does not trigger a missing-self response (12).
1 h. Samples were washed twice, stained with PE-conjugated F(ab′)2 fragments of donkey anti-human IgG for 30 min, and then washed twice before fixation and permeabilization. To assess for the extent of DenV infection, fixed and permeabilized DC were stained with anti-PRM Ab (culture supernatant from hybridoma clone HB1114), washed with Perm/Wash buffer, then stained with fluorochrome-conjugated goat anti-mouse IgG2a Ab for 30 min, followed by additional washes with Perm/Wash buffer before being resuspended in FACS wash prior to flow cytometry data acquisition. Flow cytometry was performed on either a FACSCalibur (BD Biosciences) or a LSR II instrument (BD Biosciences), and the results were analyzed with Flowjo software (Tree Star). All experiments were performed with at least three replicates using blood from different donors. Details of the replicate experiments are described in the respective figure legends.

Cytotoxicity assay

The DELFIA bis(acetoxymethyl) 2,2′-6′,2′-terpyridine-6,6′-dicarboxylate (BATDA) assay (Perkin Elmer) was used to quantify cytolsis of Dengue-infected DC by NK cells. BATDA is a fluorescence-enhancing ligand that permeates the membrane of target cells and upon entering the cell is rapidly hydrolyzed into a form (2,2′-6′,2′-terpyridine-6,6′-dicarboxylic acid [TDA]) that is impermeable. Upon cytosis, TDA that is released into the culture medium chelates with the europium solution that is added and that is used to quantify target cell cytotoxicity. Culture supernatant conditioned by mock- or DenV-infected DC was carefully aspirated away from pelleted cells and set aside. A total of 1 × 105 DC was loaded with 1 μM BATDA reagent that had been diluted in 1 ml infection medium supplemented with 50 μM 2-ME. After loading for 20 min at 37°C, the DC were washed three times with infection medium and resuspended in the original culture medium that had been set aside earlier. A total of 1 × 105 target DC was incubated with NK cells for 6 h in a 96-well round-bottom plate in a final volume of 200 μl. In the assay, 20 μl cell-free supernatant from each experimental well was added to 200 μl europium-containing solution. The resulting fluorescence signal was measured using a VICTOR3 multilabel counter (Perkin Elmer). Maximum TDA release was determined by sampling culture supernatant from loaded DC that had been treated with lysis buffer (Perkin Elmer). Spontaneous TDA release was determined by sampling supernatant from loaded DC that had been left in culture medium. The percentage specific release was calculated as follows: (experimental release – spontaneous release)/maximum release – spontaneous release) × 100. All experiments were performed with at least three replicates using blood from different donors. Details of the replicate experiments are described in the respective figure legends.

ELISA

Cytokine secretion by infected DC was quantified using the following ELISA kits: human IL-12p70, IL-15, IL-27, and TNF-α (Ready-SET-Go! ELISA, eBioscience; detection thresholds: 4 pg/ml for IL-12 and TNF-α; 8 pg/ml for IL-15; and 16 pg/ml for IL-27). IFN-α and IFN-β (PBL InterferonSource; detection thresholds: 12.5 pg/ml for IFN-α; 25 pg/ml for IFN-β); IL-18 (MBL; detection threshold: 12.5 pg/ml). Five replicate experiments using blood from different donors were performed.

KIR genotyping and KIR ligand typing

Genomic DNA was extracted from the buffy coat of blood samples using a commercial genomic DNA extraction kit (DNeasy Blood and Tissue kit; Qiagen). Sequence-specific primed PCR (PCR-SSP) KIR genotyping was performed using the system described by Vilches et al. (15). The PCRs used Platinum Taq (Invitrogen), and amplification was performed on a PEP9700 thermal cycler (Perkin Elmer). KIR ligand typing was performed using the KIR HLA ligand sequence-specific polymorphism typing kit (Olerup) and the same thermal cycler.

Statistical analyses

The Student t test was used for statistical analyses of the observed differences between two experimental groups. One-way ANOVA with Bonferroni’s post hoc test was used for statistical comparisons involving three or more experimental groups. Data analysis was performed using GraphPad Prism software version 5 (GraphPad Software).

Results

Soluble and membrane-bound factors regulate the IFN-γ and cytotoxic responses of primary human NK cells against autologous dengue-infected DC

To characterize the antidualge response of NK cells, purified primary resting human NK cells were cocultured with autologous monocyte-derived DC that had been infected with DenV serotype 1. Fig. 1A depicts the gating strategy used in our FACS experiments. Primary NK cells responded to DenV-infected DC by producing IFN-γ, but did not do so when cultured with either autologous un-infected DC or mock-infected DC (Fig. 1B). The degranulation marker CD107a was upregulated on NK cells cultured with DenV-infected DC, but not with uninfected or mock-infected DC (Fig. 1C). The strength of NK cell responses correlated well with the extent of DenV infection in a dose-dependent manner (Supplemental Fig. 1).

In line with previous observations (16, 17), the CD56 bright NK cell subset preferentially upregulated CD107a expression compared with the CD56 dim subset, suggesting that the former subset exhibits greater cytotoxic activity against DenV-infected DC. Degranulation required cell contact, as demonstrated using Transwell inserts to segregate NK cells from DenV-infected DC during culture. NK cells also produced less IFN-γ when segregated from infected DC (Fig. 1B, 1C).

To exclude the possibility that NK cells might only be responsive to DC infected with laboratory-adapted strains of DenV or the particular strain of DenV used in these experiments (DenV1 strain S3638), DC were infected with low-passage clinical isolates of DenV serotype 1 (DenV1-07K4383), or DenV serotype 3 (DenV3-05K4176). These infected DC elicited IFN-γ responses from cocultured NK cells that were similar to those observed with strain S3638 (Supplemental Fig. 2, “no antibody” condition).

To demonstrate the importance of soluble factors in regulating these responses, NK cells were cocultured with DenV-infected DC, but the conditioned culture medium in the well was replaced with fresh culture medium. In this study, the IFN-γ response of NK cells decreased significantly (Fig. 1D). Conversely, the IFN-γ response was partially restored when purified NK cells were incubated with conditioned medium from DenV-infected DC cultures but without the DC themselves (Fig. 1D).

The functional significance of the increase in degranulation against DenV-infected DC depicted in Fig. 1C was assessed. In cocultures with autologous DenV-infected DC, NK cells effectively lysed the DC and showed increasing specific lysis at higher E:T ratios (Fig. 1E). To ascertain the contribution to cytosis by membrane-bound factors on DenV-infected DC, we cocultured NK cells with mock-infected autologous DC in culture medium that had been previously conditioned by DenV-infected DC. The degree of cytosis was slightly higher than in the cocultures containing mock-infected DC without conditioned medium, but appreciably lower than standard cocultures with DenV-infected DC and conditioned medium (Fig. 1E). Similarly, soluble factors were also important for the killing of DenV-infected DC by NK cells because replacing conditioned medium with fresh culture medium virtually abrogated target cell lysis (Fig. 1E). In summary, soluble and membrane-bound factors are both required for NK cell–mediated recognition and lysis of DenV-infected DC. In contrast, soluble factors released by DenV-infected DC are sufficient to trigger a basal IFN-γ response in NK cells that is further enhanced by cell-bound factors.

Type 1 IFNs and TNF-α are released by DenV-infected DC

To identify the soluble factors secreted by DenV-infected DC that regulate the NK cell IFN-γ and cytolytic responses, culture supernatant from DenV-infected DC was assayed for production of IL-12, IL-15, IL-18, IL-27, TNF-α, and type 1 IFN (Fig. 2). In line with previous reports, DenV-infected DC readily produced detectable amounts of IFN-α, IFN-β, and TNF-α, but not IL-12 (18, 19). Using RT-PCR to assay for gene expression of the IL-12 subunits, we identified that the failure of DenV-infected DC to produce IL-12 was due to a lack of IL-12p40 transcription. This is...
FIGURE 1. Soluble and cell-bound factors regulate the response of primary human NK cells against autologous DenV-infected DC. NK cells purified by negative selection were cultured for 2 h with autologous uninfected DC, or DC incubated with heat-inactivated DenV (Mock-infected), or DC that had been infected with DenV1-S3638 at multiplicity of infection = 1 for 28 h (DenV-infected). The IFN-γ and CD107a responses of NK cells were subsequently analyzed by flow cytometry. (A) The gating strategy. NK cells were defined as CD3−CD56+ cells within the lymphocyte gate. (B) Quantification of the IFN-γ response of NK cells. (C) Evaluation of NK cell degranulation, using the CD107a mobilization assay. (B and C) Transwell experiments demonstrate that the responses required cell contact. Results representative of experiments involving five different donors are depicted. (D) Soluble factors produced by DenV-infected DC enhance the IFN-γ response of NK cells. Experimental conditions were those used in (B), but with the additional step of exchanging culture medium conditioned by DenV-infected DC with fresh culture medium. In separate wells, NK cells were cultured in the absence of DC but using culture medium conditioned by DenV-infected DC. Values are mean ± SEM for five different donors. One-way ANOVA performed with Bonferroni’s multiple comparison test. *p < 0.05 for the difference between the indicated groups. (E) Soluble and cell-bound factors regulate the lysis of DenV-infected DC by NK cells. Mock-infected or DenV-infected DC were cocultured with purified autologous NK cells at increasing E:T ratios for 3 h. Lysis of target DC was assessed using the BATDA assay. Cytolysis assays were also performed with NK cells and mock-infected DC cultured in conditioned supernatant from infected DC (Mock-infected DC + conditioned sp.), or with NK cells and infected DC cultured in fresh culture medium (DenV-infected DC + fresh media). Representative data from experiments using three different donors are shown. sp, supernatant.
likely the mechanism by which DenV interferes with IL-12 production in DC, removing an important stimulus of NK cell activity. Similarly, the amounts of IL-15 and IL-27 in culture supernatant from DenV-infected DC were below the limit of detection, and IL-18 production was limited to minute amounts (Fig. 2). The DC used in these experiments were functionally intact, as shown by their ability to secrete IL-12, IL-15, IL-27, and TNF-α when treated with LPS and IFN-γ.

**DenV-infected DC activate NK cells through the combination of TNF-α and type I IFNs**

To determine whether type I IFN or TNF-α regulates the IFN-γ response of NK cells to DenV-infected DC, Abs were used to block IFN-αβ receptor chain 2 (IFNAR2) or to neutralize TNF-α in cocultures of NK cells with DenV-infected DC. Either IFNAR2 blockade or TNF-α neutralization resulted in significant reduction of IFN-γ production by NK cells (Fig. 3A). These experiments were repeated using low-passage clinical isolates of DenV (DenV1-07K4383 and DenV3-05K4176) with similar results (Supplemental Fig. 2). The addition of recombinant B18R protein, a blocking receptor produced by vaccinia virus that neutralizes type 1 and type III IFN, also blocked the IFN-γ response of NK cells to DenV-infected DC (data not shown).

To dissect the requirements for type I IFN or TNF-α in triggering a NK cell IFN-γ response, purified NK cells alone (i.e., not in coculture with DC) were stimulated with these cytokines singly or in combination. Addition of either type I IFN or TNF-α alone did not induce significant IFN-γ production in NK cells (Fig. 3B). Incubating NK cells with both cytokines resulted in a significant IFN-γ response, showing that type I IFN and TNF-α act together to activate NK cells. However, the IFN-γ response was lower than when NK cells were cocultured with DenV-infected DC, which also provided membrane-bound signals (compare Fig. 3A and 3B).

Because soluble IL-15 was undetectable by ELISA in DenV-infected DC culture supernatant (Fig. 2), we asked whether there could be a role for membrane-bound forms of IL-15 in the NK cell response. The addition of neutralizing Abs to block IL-15 activity in NK-DC cocultures did not significantly reduce the IFN-γ response of NK cells (Supplemental Fig. 3). Similarly, neutralizing Abs against IL-18 did not diminish the IFN-γ response of NK cells to DenV-infected DC (Supplemental Fig. 3), suggesting that the small amount of IL-18 produced by DenV-infected DC (Fig. 2) was not functionally significant. Furthermore, the addition of neutralizing Abs to IL-12 and IL-27 also did not reduce IFN-γ production by NK cells (Supplemental Fig. 3).

Having identified TNF-α and type I IFN as crucial cytokines for stimulating IFN-γ production by NK cells in response to DenV infection, we assessed whether these cytokines also regulated the ability of NK cells to lyse DenV-infected DC by performing cytotoxicity assays in the presence of neutralizing Ab against TNF-α or blocking Ab against IFNAR2. Although neutralization of either cytokine alone resulted in some reduction in specific lysis of DenV-infected DC, the simultaneous neutralization of both cytokines resulted in a larger reduction in specific cytotoxic activity (Fig. 3C).

**Ligands for most NK cell–activating receptors are not upregulated on DenV-infected DC**

To identify the membrane-bound factors that regulate NK cell responses to DenV-infected DC, the DC were screened for expression of ligands for activating and inhibitory NK cell receptors. NK cells are known to use NCR to recognize virus-infected cells and interact with myeloid cells (20–24). NCR ligand expression was assessed by staining DenV-infected DC with recombinant proteins consisting of the extracellular domain of NCR fused to the Fc portion of human IgG1 (Fig. 4). The A549 cell line expresses NCR ligands and was thus used as a positive control (25). Although there was interdonor variation in the expression of ligands for NKp30, NKp44, and NKp46 on mock-infected DC and on DenV-infected DC, the majority of replicates showed very little baseline expression of these ligands on mock-infected DC, and no increased expression on DenV-infected DC. Among the NCR, we found that NKp80 ligands were expressed constitutively on a minority of mock-infected DC, but DenV infection did not necessarily increase expression beyond baseline levels.

Ligation of either DNAM-1 or NKG2D has been shown to induce IFN-γ production and cytotoxicity in NK cells (26). Mock- or DenV-infected DC did not bind DNAM-1 or NKG2D receptor-Fc proteins (Fig. 4). There was also no appreciable binding of Abs directed against CD155, CD112, MICA/B, or ULBP-1, 2, 3. We also investigated the expression of CD48. The interaction between CD48 (expressed on target cells) and 2B4 (a member of the signaling lymphocyte activation molecule family that is ubiquitously expressed on NK cells) has been described to activate NK cells.
FIGURE 3. Type I IFNs and TNF-α produced by DenV-infected DC trigger IFN-γ production and a cytotoxic response in cocultured NK cells. (A) Purified NK cells were cultured for 2 h with autologous DC that had previously been infected for 28 h with DenV1-S3638 at multiplicity of infection of 1. Where indicated, DC were incubated with neutralizing Ab for TNF-α (final concentration 5 μg/ml, IgG1 isotype), and NK cells were incubated with blocking Ab for IFNAR2 (final concentration 8 μg/ml, IgG2a isotype) or their respective isotype controls, for 30 min in a 37°C incubator prior to coculture. The IFN-γ response of NK cells was subsequently assessed by flow cytometry. No antibody: NK cells cocultured with DenV-infected DC in the absence of neutralizing Ab. Both: NK cells cocultured with DenV-infected DC in the presence of both neutralizing Ab against TNF-α and IFNAR2-blocking Ab. (B) Purified NK cells alone were stimulated for 2 h with 100 U/ml human rTNF-α, or 1000 U/ml universal type 1 IFN, or both. The IFN-γ response of NK cells was subsequently assessed by flow cytometry. No cytokine: NK cells cultured in the absence of exogenous cytokines. (C) DC that had been previously infected with DenV1-S3638 at multiplicity of infection of 1 for 28 h were cocultured for 3 h with purified autologous NK cells at an E:T ratio of 10:1. Lysis of target DC by NK cells was then assessed using the BATDA assay. Cyto kinase blockade conditions were as for (A). n = 5 different donors for (A) and (B), and n = 4 different donors for (C). All values shown are mean percentages ± SEM. One-way ANOVA performed with Bonferroni’s multiple comparison test. *p < 0.05 for the difference between the indicated groups.

The Fas–Fas ligand pathway is involved in the killing of DenV-infected DC by NK cells

Among the cell surface molecules that were screened, expression of Fas displayed the most prominent changes as a result of DenV infection. Compared with mock-infected DC, the overall percentage of Fas-positive cells increased in DC exposed to live DenV (Fig. 5A). DC exposed to live DenV either stained positive for intracellular dengue prM protein (i.e., were productively infected with dengue), or were prM-negative bystander cells in the culture. Fas expression as indicated by mean fluorescence intensity (MFI) of Ab staining was consistently higher on both infected and bystander DC compared with mock-infected DC (MFI ± SD for prM-positive DenV-infected DC, 23.4 ± 8.1; prM-negative bystander DC, 28.3 ± 6.4; mock-infected DC, 15.1 ± 3.0). In NK–DC cocultures, expression of Fas ligand (FasL) was also rapidly upregulated on the surface of NK cells that had been cocultured with DenV-infected DC (Fig. 5B). FasL can trigger apoptosis in Fas-expressing target cells as well as mediate retrograde signaling to provide costimulation for the secretion of IFN-γ by effector cells (28–30). We thus conducted blocking experiments to determine whether the changes in Fas expression observed in Fig. 5A triggered Fas–FasL interactions that subsequently activated the IFN-γ and cytotoxic responses of NK cells toward DenV-infected DC. Masking Fas on DenV-infected DC using F(ab)² fragments of Fas-blocking Ab did not diminish the secretion of IFN-γ by NK cells (Fig. 5C), suggesting that retrograde signaling by FasL did not play a significant role in this response. In contrast, lysis of DenV-infected DC by NK cells was significantly impaired in the presence of recombinant soluble Fas protein that blocked the ability of FasL on NK cells to engage Fas on DC (Fig. 5D).

In summary, the NK cell responses depicted in Figs. 1 and 5 are consistent with an important role for both the Fas/FasL-dependent pathway and the perforin/granzyme pathway in mediating NK killing of DenV-infected DC.

Licensed/Educated NK cells respond against DenV-infected DC

Next, the contribution of inhibitory NK cell receptors to the response against DenV-infected DC was investigated. These NK cell receptors, which include members of the KIR family and NKG2A, interact with subsets of HLA-A/B/C and the nonclassical HLA class I molecule HLA-E, respectively, that are present on the surface of target cells, and negatively regulate NK cell responses. When cell surface expression of these HLA molecules is reduced, the down-regulation of inhibitory signals can contribute to the activation of NK cell responses (the missing-self response). Upon quantifying HLA class I expression on the cell surface of DenV-infected DC using flow cytometry, we found reduced levels of HLA-A/B/C expression compared with mock-infected DC (MFI ± SD for prM-positive DenV-infected DC, 64.0 ± 13.8; prM-negative bystander DC, 66.1 ± 13.9; mock-infected DC, 125.1 ± 41.9) (Fig. 6A). HLA-E expression was low or absent in both mock- and DenV-infected DC (Fig. 6B). HLA-G was not detected on mock-infected DC, and was upregulated by DenV infection (Fig. 6C).

We then asked whether the reduced expression of HLA-A/B/C on DenV-infected DC has a role as a cell-contact factor in triggering the antiviral NK cell response. As previously described, missing-self responses can vary substantially among the array of NK cell subsets present in each individual due to the process of licensing/education (12). Licensing/Education is conferred upon those NK cell subsets that express one or more of the KIR2DL1, KIR2DL3, KIR3DL1, or NKG2A receptors capable of recognizing the particular HLA class I ligands expressed by the individual cells (27). CD48 was undetectable on mock-infected DC and DenV-infected DC (Fig. 4E).
We hypothesized that a role for missing self in the antidengue response would manifest as enhanced responses among the licensed/educated NK subsets. To test this hypothesis, NK cells were freshly isolated from three donors who were genotyped at the KIR and HLA loci (Fig. 7A) and coincubated with autologous DenV-infected DC as in the previous experiments. For each donor, we now quantified the IFN-γ responses of the individual NK cell subsets that can be defined by combinatorial expression of KIR2DL1/S1, KIR2DL3, KIR3DL1, and NKG2A (Fig. 7B). This information enabled us to group the NK cell subsets.

(12, 31, 32). We hypothesized that a role for missing self in the antidengue response would manifest as enhanced responses among the licensed/educated NK subsets. To test this hypothesis, NK cells were freshly isolated from three donors who were genotyped at the KIR and HLA loci (Fig. 7A) and coincubated with autologous DenV-infected DC as in the previous experiments. For each donor, we now quantified the IFN-γ responses of the individual NK cell subsets that can be defined by combinatorial expression of KIR2DL1/S1, KIR2DL3, KIR3DL1, and NKG2A (Fig. 7B). This information enabled us to group the NK cell subsets.
were cocultured for 3 h at an E:T ratio of 10:1 with autologous DC pre-
ANOV A with Bonferroni’s multiple comparison test. (*p < 0.05. For (B)–(D), the experiments
involved four different donors, and values are shown as mean ± SEM.

**Discussion**

In this study, we have performed a mechanistic screening of soluble
and membrane-bound factors that regulate the NK cell response
against DenV-infected DC. We find that combined stimulation by
type I IFN and TNF-α, rather than monokines such as IL-12 and
IL-15, is critical for triggering the IFN-γ response of NK cells in
dengue infection (Fig. 3A, 3B). The missing-self response induced
by downregulation of HLA class I also contributes to the anti-
dengue IFN-γ response (Figs. 6, 7). Cytotoxicity against DenV-
infected DC required cell-contact factors as well as the presence
of type I IFN and TNF-α (Figs. 1C, 1E, 3C). Both the perforin/
granzyme pathway and the Fas–FasL pathway seem to be involved
in NK cell killing of DenV-infected DC (Figs. 1C, 5A, 5B, 5D).

The rapid kinetics of FasL expression on NK cells during coculture
with DenV-infected DC (Fig. 5B) is consistent with the release of
preformed FasL from intracellular stores, which constitutes the
initial phase in the bimodal pattern of FasL expression following
activation of NK cells (33, 34).

DenV is resistant to the direct antiviral effects of type I IFN and
TNF-α (35, 36) and interferes with NK cell activation by inhib-
iting the release of IL-12 by DC (18, 19). Despite this immune
evasion by dengue, our results demonstrate that purified NK cells
still mount a cytokine response against monocyte-derived DC
infected with DenV through the combination of type I IFN and
TNF-α activation of NK cells (33, 34).
TNF-α, a mode of action described previously in vitro experiments employing only cytokine stimulation (37, 38). Myeloid DC isolated from peripheral blood are known to exhibit the same cytokine secretion profile when infected with DenV ex vivo (i.e., secretion of IFN-α and TNF-α, lack of IL-12 secretion) (39), suggesting that this pathway of NK cell stimulation might be physiologically relevant during dengue infection in vivo.

There are several reasons why monocyte-derived DC were used in the experiments presented in this report. Dermal DC and myeloid DC in peripheral blood have been described as likely targets for DenV infection (39–42). Similar to these DC types that are found in vivo, monocyte-derived DC have high expression of DC-SIGN, which facilitates DenV infection (43). Whereas dermal DC and myeloid DC are difficult to isolate in large numbers for experimental purposes, monocyte-derived DC represent an experimentally tractable source of primary human cells that can be readily infected by DenV. Human foreskin fibroblasts and HUVECs are also primary cells that have been used to study dengue infection in vitro. However, unlike these other cells, monocyte-derived DC have been widely used to study the activation requirements of resting NK cells due to their ability to express the requisite cytokines and membrane-bound ligands for NK receptors (44, 45).

Previous studies have uncovered some of the pathways that enable type I IFN to promote NK cell cytotoxicity, proliferation, and IFN-γ production. In murine models, type I IFN triggers the release and trans-presentation of IL-15 by the high-affinity IL-15R expressed on DC, which subsequently primes NK cells for efficient killing and IFN-γ production (46). There is also evidence that IFN-α receptor signaling can directly activate murine NK cells in the absence of IL-15 (47). In contrast, it has been reported that type I IFN by itself does not effectively activate human NK cells, but can indirectly do so in NK–DC cocultures by upregulating cell surface expression of MICA/B on DC (48). In NK–DC cocultures, DC can also respond to the presence of TLR ligands by activating NK cells through the release of IL-12.

None of these previously described mechanisms are likely to be responsible for the activation of NK cells by DenV-infected DC. In agreement with Hayashi and colleagues (48), we observed that human NK cells are poorly activated by the direct effect of type I IFN alone (Fig. 3B). NKG2D ligands, including MICA/B, were not upregulated on DenV-infected DC, or on bystander DC within the same culture well, despite the presence of secreted type I IFN in the culture medium (Fig. 4C). There was no significant secretion of monokines such as IL-12, IL-15, IL-18, and IL-27 by DenV-infected DC (Fig. 2), and neutralizing Ab against IL-15 failed to diminish the response of NK cells (Supplemental Fig. 3), suggesting that trans-presented IL-15 was not a critical factor in this setting. The inability of DenV-infected DC to secrete significant amounts of IL-12 has several implications for the NK response to DenV. Although the combination of type I IFN and TNF-α can activate NK cells, IFN-γ production stimulated by these cytokines may be more transient than with IL-12 stimulation (49). It is less clear whether the failure of infected DC to secrete IL-12 during infections with DenV (18, 19), or other viruses (50, 51), has any effect on NK cell immunosurveillance for repeat infections. Although IL-12 and STAT4 signaling was shown to be important for generating long-lived memory NK cells in a murine model of murine CMV infection (52), it is not known whether this is applicable to human NK cells. We speculate that the priming of primary antidengue Th1 and CTL responses may also be reduced by the lack of IL-12 production by DenV-infected DC. From this perspective, the NK cell response to DenV described in this work might be a physiologically important means of reversing this defect in IL-12 secretion by

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** HLA class I is downregulated on DenV-infected DC. DC infected for 28 h with DenV1-S3638 were stained for expression of (A) HLA-A/B/C, (B) HLA-E, and (C) HLA-G, or with the respective isotype control Abs (iso). For (C), the staining isotype control is depicted in Fig. 4D. The infection rate was assessed by staining for intracellular DenV prM protein. Mock-infected DC served as controls. Gating for DC during flow cytometry analysis was performed as in Fig. 4A. For each marker, representative data from four different donors are shown.
infected DC by providing an early source of IFN-γ (19), or CD40–CD40L signals during NK–DC interactions (53). NK cells might also enhance the release of DenV Ag by cytolysis of DenV-infected DC, and promote subsequent DC-mediated Ag cross-presentation.

We show that downregulation of HLA class I molecules on DenV-infected DC contributes to the NK cell response as a cell-contact stimulus for missing-self response in the presence of soluble factors secreted by the infected cells. The missing-self response triggered in the NKG2A-expressing subsets due to lack of the nonpolymorphic HLA-E molecule on DenV-infected DC is important in that this conserved receptor–ligand interaction ensures a basic level of NK cell response in all human individuals. As demonstrated by the variation of NK subset responses against DenV-infected DC associated with licensed/educated status (Fig. 7), involvement of the polymorphic HLA and KIR systems also suggests that the magnitude of NK cell responses to DenV-infected DC will differ among human individuals. The molecular determinants of KIR and HLA class I are known to directly alter both cytokine and cytotoxic NK cell responses (12). Furthermore, a DenV-derived peptide loaded onto particular HLA class I allo-types has been shown to modulate KIR binding and affect the baseline NK cell response (54). Collectively, these results dovetail with other studies that have described the impact of HLA variation on T cell responses against DenV (4). An immune-monitoring platform that comprehensively assesses the role of interindividual differences in both innate and adaptive cellular immune responses against DenV may provide useful insights into the pathogenesis of mild versus severe dengue disease.

**Disclosures**

The authors have no financial conflicts of interest.

---

**FIGURE 7.** Evidence for a role of licensing/education in the NK cell IFN-γ response against DenV-infected DC. (A) The KIR genotypes and KIR ligands expressed by three different blood donors. (B) The bar graphs depict IFN-γ responses of NK cell subsets in these three donors against autologous mock-infected DC as negative control (top row), or against autologous DenV-infected DC (middle row). Mock- or DenV-infected DC were prepared as in the previous experiments. As a positive control for NK cell missing-self responses, 1 × 10⁶ PBMC were coincubated with the HLA class I-deficient cell line, K562, at an E:T ratio of 5:1 for 14 h in the presence of 2000 U/ml IL-2 (bottom row). NK–DC cocultures did not contain supplemental IL-2. Flow cytometry was used to assess the expression of KIR2DL1/2DS1, KIR2DL3, KIR3DL1, or NKG2A inhibitory receptors on NK cells, and NK cell subsets were classified according to their licensing/education status based on the KIR genotypes and KIR ligands expressed by each donor. The IFN-γ responses of NK cell subsets that expressed neither NKG2A nor KIR that recognized self-HLA class I ligands are depicted as white bars. The IFN-γ responses of NK cell subsets that expressed either NKG2A or KIR capable of interacting with cognate ligand expressed on autologous cells to confer licensing/education are depicted as black bars. The ≥ 2 ss receptors: NK cell subsets that expressed two or more self-specific receptors. Measurements are from duplicate experiments for each donor. Values are shown as mean ± SEM. Unpaired t test. *p < 0.05.
References


Supplementary Figure S1. NK cell IFN-γ responses correlate with the frequency of DenV-infected DC. (A) The frequency of DenV-infected DC was determined by...
staining for intracellular DenV prM protein 28h after infection and analyzing the cells by flow cytometry (right panel). Mouse IgG2a isotype control was used to determine the background level of non-specific staining (left panel). (B) DC were infected for 28h with DenV1-S3638 at different MOIs in order to achieve different infection rates, and were then co-cultured with purified autologous NK cells for a further 2h. Intracellular staining for IFN-γ was performed, and flow cytometry was used to analyze the response of CD3−CD56+ NK cells. Symbols represent the results from three different donors.
Supplementary figure S2. DC infected with clinical isolates of DenV serotypes 1 and 3 also induce IFN-γ production in NK cells through TNF-α and type I IFN.

Purified NK cells were cultured for 2h with autologous DC that had previously been infected for 28h with (A) DenV serotype 1, clinical isolate 07K4383, or (B) DenV serotype 3, clinical isolate 05K4176 (both at MOI of 1). In other wells, NK cells were cultured with DC exposed to heat-inactivated DenV (Mock-infected DC), or were cultured in the presence of cell-free conditioned supernatant from DenV-infected DC. Where indicated, the DC were incubated with neutralizing antibody against TNF-α (final concentration 5μg/ml, IgG1), or NK cells were incubated with blocking antibody
against IFNAR2 (final concentration 8μg/ml, IgG2a), or their respective isotype controls, for 30 minutes in a 37°C incubator prior to co-culture. The IFN-γ response of NK cells was subsequently assessed by flow cytometry. No antibody: NK cells co-cultured with DenV-infected DC in the absence of neutralizing antibody; Both: NK cells co-cultured with DenV-infected DC in the presence of both neutralizing antibody against TNF-α and IFNAR2-blocking antibody.
Supplementary figure S3. The IFN-γ response of purified NK cells to autologous DenV-infected DC is not dependent on IL-12, IL-15, IL-18, or IL-27. DC infected with DenV1-S3638 at MOI=1 for 28h were then incubated with blocking antibodies for IL-12, IL-15, IL-18, or the matching mouse IgG1 isotype control, for 30 minutes in a 37°C incubator prior to co-culture with purified autologous NK cells for an additional 2h. The IL-27 receptor subunit on NK cells was masked by incubation with anti-gp130 blocking antibody under similar conditions. Intracellular staining for IFN-γ was subsequently performed, and CD3−CD56+ NK cell responses were analysed by flow cytometry. Blocking antibodies and isotype control antibody were used at a final
concentration of 2.5µg/ml. The data is depicted as mean percentages ± SEM, and summarizes results from three experiments using blood from different donors.