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West Nile virus-infected human dendritic cells fail to fully activate invariant natural killer T cells

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Summary

West Nile virus (WNV) infection is a mosquito-borne zoonosis with increasing prevalence in the United States. WNV infection begins in the skin, and the virus replicates initially in keratinocytes and dendritic cells (DCs). In the skin and cutaneous lymph nodes, infected DCs are likely to interact with invariant natural killer T cells (iNKTs). Bidirectional interactions between DCs and iNKTs amplify the innate immune response to viral infections, thus controlling viral load and regulating adaptive immunity. iNKTs are stimulated by CD1d-bound lipid antigens or activated indirectly by inflammatory cytokines. We exposed human monocyte-derived DCs to WNV Kunjin and determined their ability to activate isolated blood iNKTs. DCs became infected as judged by synthesis of viral mRNA and Envelope and NS-1 proteins, but did not undergo significant apoptosis. Infected DCs up-regulated the co-stimulatory molecules CD86 and CD40, but showed decreased expression of CD1d. WNV infection induced DC secretion of type I interferon (IFN), but no or minimal interleukin (IL)−12, IL-23, IL-18 or IL-10. Unexpectedly, we found that the WNV-infected DCs stimulated human iNKTs to up-regulate CD69 and produce low amounts of IL-10, but not proinflammatory cytokines such as IFN-γ or tumour necrosis factor (TNF)-α. Both CD1d and IFNAR blockade partially abrogated this iNKT response, suggesting involvement of a T cell receptor (TCR)–CD1d interaction and type I interferon receptor (IFNAR) signalling. Thus, WNV infection interferes with DC–iNKT interactions by preventing the production of proinflammatory cytokines. iNKTs may be a source of IL-10 observed in human flavivirus infections and initiate an anti-inflammatory innate response that limits adaptive immunity and immune pathology upon WNV infection.

Keywords: dendritic cell, flavivirus, human, invariant natural killer T cell

Introduction

West Nile virus (WNV) is an RNA genome flavivirus that is transmitted to humans via mosquito bites. While most infections are asymptomatic, some patients, especially elderly people and immunocompromised individuals, develop neuroinvasive disease, and 10% of these cases are lethal [1,2]. Despite this severe outcome, and the recent increase in the prevalence of WNV in the United States, the pathogenesis of WNV in humans remains ill defined, and there is no approved vaccine or therapy for human WNV infection.

WNV infection begins in the skin, and the virus replicates initially in keratinocytes and skin-resident dendritic cells (DCs) [3]. In the skin, or after migration to cutaneous lymph nodes, infected DCs are likely to interact with natural killer T cells (NKTs), an innate effector cell type important for control of pathogen infections [4–6]. NKTs promote anti-viral responses by activating DCs and contributing to innate immune responses controlling viral load and by regulating adaptive immunity [see [7–10] for recent reviews]. NKTs recognize lipid antigens presented by the non-classical major histocompatibility complex (MHC) molecule CD1d. Two categories of NKTs, types I and II, have been described. Type I or invariant NKTs (iNKTs) express a semi-invariant T cell receptor (TCR) that uses a Vα24-Jα18 rearrangement and Vβ11 TCR genes in

humans. iNKTs respond to α-galactosylceramide (αGalCer) and related lipids [5]. This contrasts with type II NKTs (dNKTs), which do not react with αGalCer and express a much more diverse TCR repertoire [11]. iNKTs are activated in response to pathogens either directly through their TCR or indirectly via cytokines. Direct activation is mediated by CD1d-bound lipids derived from microbes (reviewed in [5]), or can involve recognition of an altered complement of CD1d-bound self-lipids that arises during viral or bacterial infection as a result of changes in lipid metabolism or lipid loading onto CD1d [12–15]. Indeed, infection with hepatitis B virus leads to the synthesis of antigenic host lysophospholipids that are loaded on to CD1d, resulting in iNKT activation [16]. Indirect activation of iNKTs is mediated by proinflammatory cytokines such as interleukin (IL)-12, IL-18 or type I interferons (IFNs) released by DCs in response to pathogen-derived products. iNKTs are activated through this mechanism in response to the Toll-like receptor (TLR)-9 ligand cytosine–phosphate–guanosine (CpG) oligonucleotides [17–19], as well as cytomegalovirus (CMV) [18–20] and Dengue virus [21]. Finally, iNKTs may be activated by a combination of weak responses to CD1d-presented self-antigens and inflammatory cytokines or type I IFN [13,22,23].

The nature of the DC response to virus infection influences immune regulatory pathways mediated by innate lymphocytes. Depending on the virus and the type of DC, infected DCs may undergo apoptosis or survive, modulate surface molecules recognized by lymphocytes and produce combinations of type I IFNs and pro- or anti-inflammatory cytokines [24]. Murine models of skin infection with the related Dengue flavivirus revealed two stages of DC infection – initial infection of resident dermal DCs within 12–24 h and a second wave of infection within 48 h of DCs derived from monocytes recruited to the inflamed dermis [25]. Similarly, in a murine model of dermal WNV infection, bone marrow-derived monocytes infiltrate the skin, cluster near infected fibroblasts and then differentiate into DCs [26]. These reports suggest that human monocyte-derived DCs are a relevant model for the study of DC-intrinsic flavivirus infection. Indeed, prior reports showed that human monocyte-derived DCs are permissive for WNV infection and produce type I IFN upon contact with the WNV dsRNA genome replication intermediate [27–30]. In human epithelial cells, WNV interferes with the host IFN response by inhibiting the type I IFN receptor (IFNAR) mediated activation of signal transducer and activator of transcription-1 (STAT-1) [31]. This blockade of IFNAR signalling did not apparently occur in human DCs, indicating that DCs may have distinct mechanisms to control WNV infection [27].

While these prior studies have contributed to our understanding of human DC responses to WNV, the ability of WNV-infected human DCs to activate innate lymphocytes has not been studied. Herein, we have determined the impact of WNV Kunjin (WNV_{KUN}) infection on interactions between human DCs and iNKT cells. In WNV-infected human monocyte-derived DCs, we studied viral replication and protein expression, co-stimulatory molecule and CD1d surface expression and production of type I IFNs, proinflammatory cytokines and IL-10. We then determined the ability of WNV-infected DCs to activate human blood iNKTs and investigated the role of CD1d and IFNAR in the iNKT response. Our data show that WNV-infected DCs produce significant amounts of type I IFN but fail to activate the proinflammatory function of iNKTs.

Materials and methods

Generation of monocyte-derived DCs

Heparinized peripheral blood was obtained from healthy volunteers with informed consent according to a venipuncture protocol approved by the OMRF Institutional Review Board. Leucocyte buffy coats from anonymous donors were purchased from the Oklahoma Blood Institute. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium gradients (Mediatech Inc., Manassas, VA, USA). $CD14^+$ monocytes were isolated by negative selection using an EasySep human monocyte enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada). Monocytes were cultured at 10^6/ml in RPMI, 10% fetal calf serum (FCS) with 30 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF) and 20 ng/ml IL-4 (recombinant cytokines from Peprotech, Rocky Hill, NJ, USA) for 6 days to promote DC differentiation as described previously [32]. Differentiated DCs were $CD14^-CD11c^+CD209^+$human leucocyte antigen D-related $(HLA\text{-}DR)^+$.

WNV stocks

WNV_{KUN} (a BSL2 isolate) and *Aedes albopictus* C6/36 mosquito cells were a kind gift from Dr M. Diamond (Washington University, Saint Louis, MO, USA). To generate virus stocks, exponentially growing C6/36 cells were infected with WNV_{KUN} and grown at 28°C for 4 days [33]. Supernatants were collected, clarified by centrifugation, aliquoted and frozen at −80° C. One aliquot was used to titre the virus, using either plaque assays [33] or a flow cytometry-based assay adapted from [34].

Assessment of DC activation

On day 6 after differentiation was initiated, DCs were harvested, replated at 5×10^5/ml and either left unstimulated, infected with WNV_{KUN} [multiplicity of infection (MOI) 1, 5 or 50] or activated with polyIC (pIC, 50 μg/ml) or lipopolysaccharide (LPS) (100 ng/ml) + IFN-γ (2000 IU/ml). After 24–48 h, both stimulated and unstimulated DCs were

assessed for changes in cell surface markers using flow cytometry or placed in Trizol for later isolation of mRNA. DC supernatants were collected from duplicate wells, each containing 50 000 DCs in 200 μl, for measurement of secreted cytokines.

Monoclonal antibodies (mAbs) and flow cytometry

Cells were preincubated with human FcR-binding inhibitor (eBioscience, San Diego, CA, USA) and 2% human serum, and labelled with optimally titred mAbs in fluorescence activated cell sorter (FACS) buffer [phosphate-buffered saline (PBS), 5% newborn calf serum, 0.1% sodium azide]. DCs were stained with six to seven parameter combinations of fluorochrome-labelled mAbs specific for CD14 (clone M5E2), CD11c (B-LY6), CD209 (9E9A8), HLA-DR (L243), CD40 (5C3), CD86 (IT2.2) and CD1d (51.1) [obtained from BD Biosciences, San Jose, CA, USA, eBioscience or Biolegend, San Diego, CA, USA]. iNKT cells were stained with mAbs specific for CD69 (FN50), OX-40 (Ber-ACT35) and CD107a/lysosomal-associated membrane protein 1 (LAMP1) (H4A3), CD3 (OKT3) and CD1d-PBS-57 tetramer (obtained from the NIH tetramer facility). The following reagents were obtained through the National Institutes of Health (NIH) Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: monoclonal anti-West Nile virus Envelope (Env) protein, clone E34 (produced *in vitro*), NR-10137 and clone E24 (produced *in vitro*), NR-10136; and monoclonal anti-WNV non-structural protein 1, clone 22-NS-1 (produced *in vitro*), NR-10145. Anti-WNV Env and NS-1 mAbs were labelled with an Alexa Fluor 647 monoclonal antibody labeling kit (Invitrogen, Carlsbad, CA, USA), and intracellular staining was accomplished using a BD cytofix/cytoperm kit. A live/dead fixable Aqua dye (Invitrogen) and a mAb to activated caspase 3 (BD Biosciences) were used to determine DC apoptosis and viability. Samples were run on an LSRII instrument (BD Biosciences) and the data were analysed with FlowJo software (TreeStar Inc., Ashland, OR, USA) software.

Analyses of gene expression

RNA was extracted using an RNeasy/Trizol hybrid protocol and cDNA was synthesized using high-capacity cDNA reverse transcription with RNase inhibitor kit (Applied Biosystems). Relative expression of WNV *ENV* was determined using the $2^{-\Delta Ct}$ method with normalization to actin, beta (*ACTB*) expression using *Taq*Man technology. A Ct of 40 for the WNV *ENV* gene was used in the calculations for the uninfected samples. Specific primer/probe sequences were *ACTB* forward: 5′-ATCCTGGCCTCGCTGTCCAC-3′, reverse: 5′-GGGCCGGACTCGTCATAC-3′ probe: 5′-6FAM TCCAGCAGATGTGGATCAGCAAGCA tetramethylrhodamine (TAMRA)−3′; WNV *ENV* forward: 5′-TTCT CGAAGGCGACAGCTG-3′, reverse: 5′-CCGCCTCCATATT CATCATc-3′ probe: 5′-6FAM ATGTCTAAGGACAAGCCT ACCA TAMRA-3′ [35]. Relative expression of *CD1D* was determined using the ΔΔCt method with normalization to *HPRT* expression using Sybr green technology. Specific primer sequences were: *HPRT* forward 5′-TTGGTCA GGCAGTATAATCC-3′, reverse 5′-GGGCATATCCTACAA CAAAC-3′ [36]; *CD1D* forward 5′-GTGGCCTCCTTGA GTCA-3′, reverse 5′-ACAGGCTTTGGGTAGAATC-3′.

iNKT purification and expansion

PBMCs were purified from buffy coats by centrifugation on a Ficoll gradient, and resuspended in 10% FCS-RPMI. The next day, the PBMCs were incubated with allophycocyanin (APC)-labelled CD1d-PBS-57 tetramer (obtained from the NIH tetramer facility), incubated with anti-APC beads (Miltenyi Biotec, San Diego, CA, USA). After washing again, the tetramer$^+$ cells were selected positively using magnetic columns (Miltenyi Biotec). Enriched cells were stained with CD3 and CD1d-PBS-57 tetramer and sorted (Aria; BD). The sorted cells (>98% iNKT) were then cultured with irradiated heterologous PBMCs in 10% FCS-RPMI containing IL-2 (100 U/ml) and IL-7 (100 U/ml). Cells were stimulated with αGalCer (100 ng/ml) (Funakoshi, Tokyo, Japan) or α-CD3 + α-CD28 (1 μg/ml each), and expanded in 96-well round-bottomed plates. Fresh medium with cytokines was added every 72 h. Before use in experiments, cells were rested in cytokine-free medium for 24–36 h.

iNKT–DC co-culture

Rested iNKTs were incubated with WNV-infected or control DCs (5 : 1) in 10% RPMI in 96-well round-bottomed plates. After 24 h, the supernatant was collected for cytokine detection, and the cells washed and stained with CD3, CD1d-PBS-57 tetramer, CD69, CD107a and OX-40 to assess activation. In some experiments, an α-CD1d blocking mAb (clone 51.1, 10 μg/ml), an anti-IFNAR chain 2 mAb (clone MMHAR-2, 5 μg/ml) or an isotype control mAb, or purified IL-12p70 (100 ng/ml) were added at the beginning of the culture.

Cytokine assays

Cytokines secreted by DCs and iNKTs were measured using Luminex assays in the OMRF Serum Analyte and Biomarker core facility (Oklahoma City, OK, USA). Procarta-Plex kits (eBioscience) for DC cytokines (IFN-α, IFN-β, IL-12p70, IL-10, IL-23, IL-18, IL-1β) and NKT cell cytokines (TNF-α, IL-22, IFN-γ, IL-4, IL-10) were used according to the manufacturer's instructions.

Statistics

Statistical analyses were performed using Prism GraphPad software. The data involving multiple donor DCs and NKTs each exposed to different (three or more) stimuli were analysed using repeated-measure (RM) one-way analyses of variance (ANOVAs), followed by multiple

comparison tests as indicated in the figure legends. Reported significance values compare each stimulated value to the corresponding unstimulated value. In some cases, a comparison between unstimulated and stimulated cells was made with a paired *t*-test. The significance of the changes in iNKT responses (parameter fold induction) in the presence or absence of α-CD1d blocking mAb or the α-IFNAR mAb was determined by ratio paired *t*-tests.

Results

WNV replicates its RNA genome within DCs, leading to expression of viral Env and non-structural (NS-1) proteins without significant cell death

We used WNV_{KUN}, a naturally attenuated (BSL2) strain isolated from an infected individual [37], which is 98% identical at the amino acid level to the highly pathogenic North American WNV_{NY99}. While WNV_{KUN} showed enhanced sensitivity to the host type I IFN response in mice, the virus limited the type I IFN response of human epithelial cells *in vitro*, indicating that at least some of the WNV host interference mechanisms are intact in WNV_{KUN} [31,37]. Thus, WNV_{KUN} is an informative model for understanding the innate response of human DCs to WNV infection.

To determine an optimal time-frame for detection of viral RNA, human monocyte-derived DCs were incubated with WNV_{KUN} at a multiplicity of infection (MOI) of 5 for 4–44 h. These preliminary studies showed that RNA corresponding to the WNV Env protein peaked at ∼44 h (Fig. 1a). In subsequent experiments, DCs from multiple donors were exposed to WNV at MOI = 1 and MOI = 5 for 44 h. WNV-exposed DCs contained significant amounts of RNA corresponding to the WNV Env protein at both MOI, although donor variability was observed (Fig. 1b). However, infection at MOI = 5 led to few DCs expressing the viral Env protein (detected using intracellular staining with a specific mAb) after 24 or 44 h (1-7%), despite the robust production of viral RNA (Fig. 1c).

Because of the low percentage of DCs expressing viral protein, we next infected DCs at MOI = 50 and harvested the DCs 24 and 44 h post-infection. The amount of viral Env RNA was comparable to that found in DCs incubated with WNV MOI = 5 (Fig. 1b). However, the fraction of DCs expressing Env protein (5–28%) was significantly higher upon exposure to MOI = 50 compared to MOI = 5 (Fig. 1c). Infected (MOI = 50 for 24 h) DCs also expressed the viral NS-1 protein (12–47%) (Fig. 1d).

To determine if WNV replicated within DCs, the DCs were incubated with WNV at MOI = 50, and the virus was washed away after 6 h. RNA was collected at 6, 12, 24 and 44 h post-infection. The amount of WNV Env RNA increased steadily between 6 and 24 h, after which it declined by 44 h to a level comparable to when the virus had not been washed out (Fig. 1e). This indicates that the WNV replicated its RNA genome within DCs. Taken together, the data shown in Fig. 1 suggest that virus infection was asynchronous at the population level, leading to detectable amounts of intracellular viral proteins in only 5–50% of DCs, depending on the MOI and time-point post-infection.

To determine if virus infection induced apoptosis, we determined levels of active caspase 3 in infected (MOI = 50) DCs at 24 h post-infection. Fewer than 2% of the DCs contained active caspase 3, and most of those did not contain the Env protein (Fig. 2). Use of a viability stain showed very little cell death in the DC population. Similar results were obtained at 48 and 72 h post-infection with MOI = 5 (not shown). Taken together, these experiments show that human monocyte-derived DCs exposed to WNV were permissive to production of WNV RNA and protein without significant apoptosis or cell death.

WNV-activated DCs produce type I IFN, but not significant amounts of other inflammatory cytokines

To determine DC production of soluble mediators post-infection, we assayed DC culture supernatants for the presence of type I IFNs (IFN-α and IFN-β) and pro- and anti-inflammatory cytokines, including IL-12p70, IL-18, IL-1β, IL-23 and IL-10. We initially measured secreted cytokines at 24 and 48 h post-infection with MOI = 5 and determined that the peak of cytokine production was at 48 h. At 24 h post-infection, low amounts ($\bar{x} < 50$ pg/ml) of IFN-α were produced (Fig. 3a). At 48 h post-infection, DCs infected with MOIs of 1 or 5 showed significant production of IFN-α (300–700 pg/ml) and lesser amounts of IFN-β (10–30 pg/ml) (Fig. 3a,b). This is consistent with a recent report that IFN-α, more than IFN-β, is a crucial mediator of the anti-viral response to WNV in mice [38]. Infection at MOI = 50 did not lead to increased amounts of secreted IFN-α ($\bar{x}$ = 7.5 pg/ml at 24 h and $\bar{x}$ = 198 pg/ml at 48 h) compared to the values at MOI = 5. Although donor variability was found and prior studies reported human sex differences in type I IFN production [39,40], we did not identify a sex difference in the amounts of IFN-α produced by WNV-infected DCs; females, $\bar{x} \pm$ standard deviation (s.d.) = 700 ± 426 pg/ml and males, $\bar{x} \pm$ s.d. = 635 ± 478 pg/ml.

At 48 h post-infection with MOI = 1 or 5, WNV infected DCs produced minimal amounts of IL-12p70 ($\bar{x}$ = 2 pg/ml) (Fig. 3c). Infected DCs also did not produce significant amounts of IL-10 (Fig. 3d), IL-18, IL-1β or IL-23 (not shown). DCs infected at MOI = 50 for 24 or 44 h did not secrete significant amounts of IL-12p70 ($\bar{x}$ = 9 pg/ml) and IL-10 ($\bar{x}$ = 23 pg/ml) relative to uninfected DCs. However, uninfected DCs stimulated via TLRs and other receptors with polyIC (pIC) and LPS/IFN-γ were capable of robust production of IL-12p70 and IL-10 (Fig. 3c,d). In sum, WNV infection at MOI = 5 or MOI = 50 elicited DC

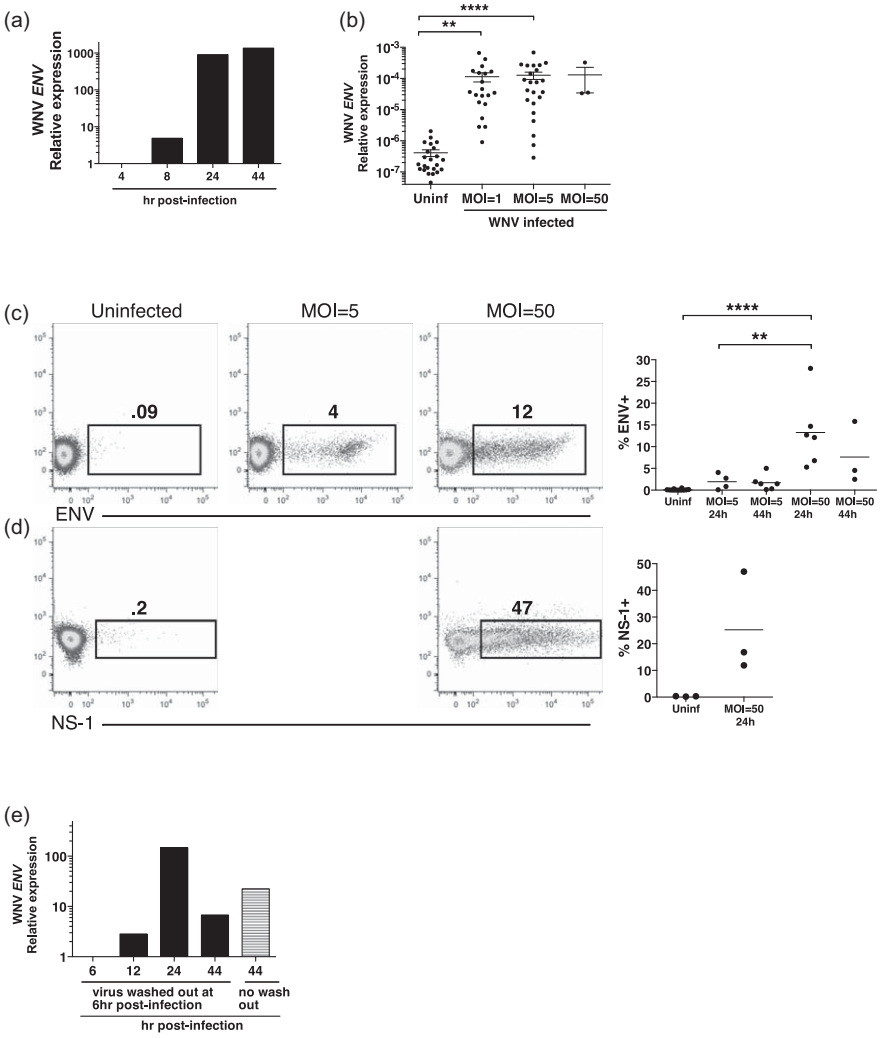

Fig. 1. West Nile virus (WNV) infects human monocyte-derived dendritic cells (DCs). (a) DCs were infected with multiplicity of infection (MOI) = 5 WNV Kunjin isolate (WNV_{KUN}). RNA was collected at the indicated time-points (4–44 h) post-infection, and the cDNA assessed for expression of the *ENV* gene by quantitative polymerase chain reaction (qPCR). Shown is the expression of the WNV *ENV* gene relative to the host *ACTB* gene, with the data normalized to the values at the 4-h time-point. The data are representative of two independent experiments. (b) DCs were left uninfected (Uninf), or infected with MOI = 1 or MOI = 5 WNV_{KUN} for 44 h. Symbols represent individual donors ($n = 22$). Three samples were infected at MOI = 50 for 24 h. The expression of the WNV *ENV* gene relative to the host *ACTB* gene is shown. Calculations were made using the $2^{-\Delta Ct}$ method, as described in the Methods section, and a Ct of 40 for the WNV *ENV* gene was used in the calculations for the uninfected samples, as no signal for viral RNA was detected. The significance of the data was evaluated using a Friedman test followed by Dunn's multiple comparison test (comparing each stimulated value to the corresponding unstimulated value). $^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$. (c,d) The presence of intracellular viral proteins envelope (Env) protein and non-structural (NS-1) protein in DCs (infected at the indicated MOIs) was determined by flow cytometry after 24 or 44 h. These data are representative of three to six similar experiments, and the results of all experiments varying MOIs and time post-infection are compiled. (e) DCs were infected with WNV at MOI = 50, and the virus washed away after 6 h or left in for 44 h. RNA was collected at the indicated time-points post-infection, and the cDNA assessed for expression of the *ENV* gene by qPCR. Shown is the expression of the WNV *ENV* gene relative to the host *ACTB* gene, with the data normalized to the signal at the 6-h time-point. The data are representative of two independent experiments.

production of type I IFN, but not IL-12p70 or IL-10. In subsequent experiments, we infected DCs at MOI = 5.

WNV infection led to increased expression of co-stimulatory molecules by DCs

To determine if WNV infection induced DC up-regulation of molecules important for T cell stimulation, we measured surface expression of the co-stimulatory molecules CD40 and CD86. WNV infection induced the up-regulation of CD40 and CD86 to a level comparable to that induced by pIC, a ligand for TLR-3 and retinoic acid-inducible gene 1 (RIG-I) (Fig. 4). WNV exposure also led to increased expression of HLA-DR, CD83, PDL1 and OX40L, but decreased expression of the IFN-γR (not shown). The up-

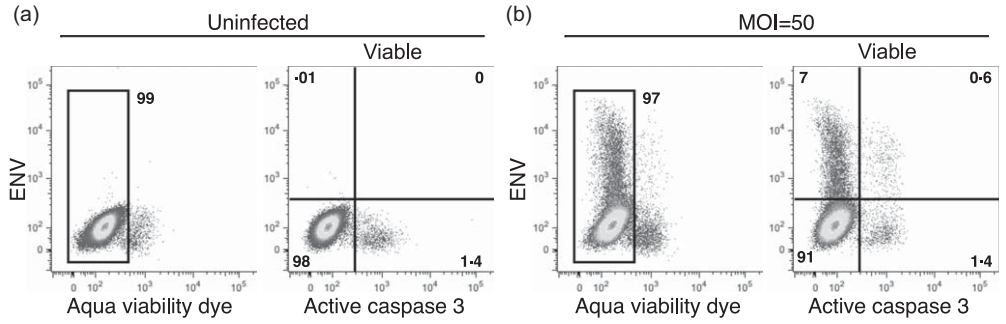

Fig. 2. West Nile virus (WNV) infection did not induce significant dendritic cell (DC) apoptosis. DCs were (a) left uninfected or (b) infected with WNV Kunjin isolate (WNV_{KUN}) at multiplicity of infection (MOI) = 50. After 24 h, cells were stained with monoclonal antibodies (mAbs) specific for envelope (Env) protein and activated caspase 3, and a fixable Aqua viability dye. The data are representative of five similar experiments.

regulation of co-stimulatory molecules was uniform on the population, despite the fact that not all DCs showed expression of viral proteins. This suggests that type I IFNs or other mediators released from infected DCs activated most DCs in the population.

WNV-activated DCs showed decreased levels of cell surface CD1d

We determined the impact of WNV infection on DC expression of CD1d, a lipid ligand-bound molecule recognized by iNKTs. WNV infection of DCs induced increased levels of *CD1D* mRNA (Fig. 5a). However, display of cell surface CD1d on WNV exposed DCs actually decreased relative to uninfected cells (Fig. 5b,c). This effect was not unique to WNV exposure, as incubation of DCs with pIC and LPS/IFN-γ also led to decreased surface CD1d (Fig. 5b,c). Incubation of DCs with the CD1d ligand αGalCer did not induce changes in CD1d surface expression (Fig. 5d) or elicit DC activation (not shown). This suggests that triggering of pattern recognition receptors leads to decreased surface expression of CD1d, and that the decrease in CD1d is unlikely to be due to a viral protein that blocks CD1d trafficking, as proposed for other viruses [41].

WNV-activated DCs activated iNKTs and stimulated their production of low levels of IL-10 but not proinflammatory cytokines

iNKTs are activated optimally by CD1d, CD40 and IL-12p70. WNV-activated DCs produce type I IFN but not IL-12p70, and express elevated CD40 but reduced CD1d. We determined if this DC profile led to activation of iNKTs. iNKTs were purified from blood of healthy donors based on their binding of CD1d-PBS-57 tetramers, expanded *in vitro* and rested prior to incubation with DCs. Because DC production of cytokines was optimal at MOI = 5, DCs were infected with WNV (MOI = 5) overnight and then cultured with iNKTs for 24 h. Unique combinations of DCs

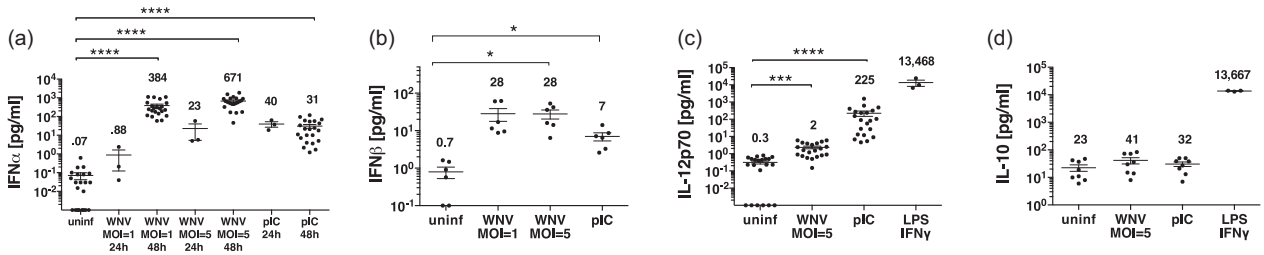

Fig. 3. West Nile virus (WNV) infection induces dendritic cell (DC) production of interferon (IFN)-α but not proinflammatory cytokines. DCs (50 000/well) were left unstimulated (uninf), infected with WNV Kunjin isolate (WNV_{KUN}) at multiplicity of infection (MOI) = 1 or MOI = 5, or stimulated with poly-inosine-cytosine (pIC), and supernatants were harvested after 24 or 48 h. DC supernatants were assessed for amounts of (a) IFN-α, (b) IFN-β, (c) interleukin (IL)-12p70 and (d) IL-10 using Luminex assays. For IL-12p70 and IL-10, DCs were stimulated with lipopolysaccharide (LPS)/IFN-γ as a positive control in some experiments. Symbols represent individual donors. Numbers within the graph are the mean amount of cytokine (pg/ml). Significance was evaluated using a repeated-measure (RM) one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test (comparing each stimulated value to the corresponding unstimulated/uninfected value). $^{*}P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

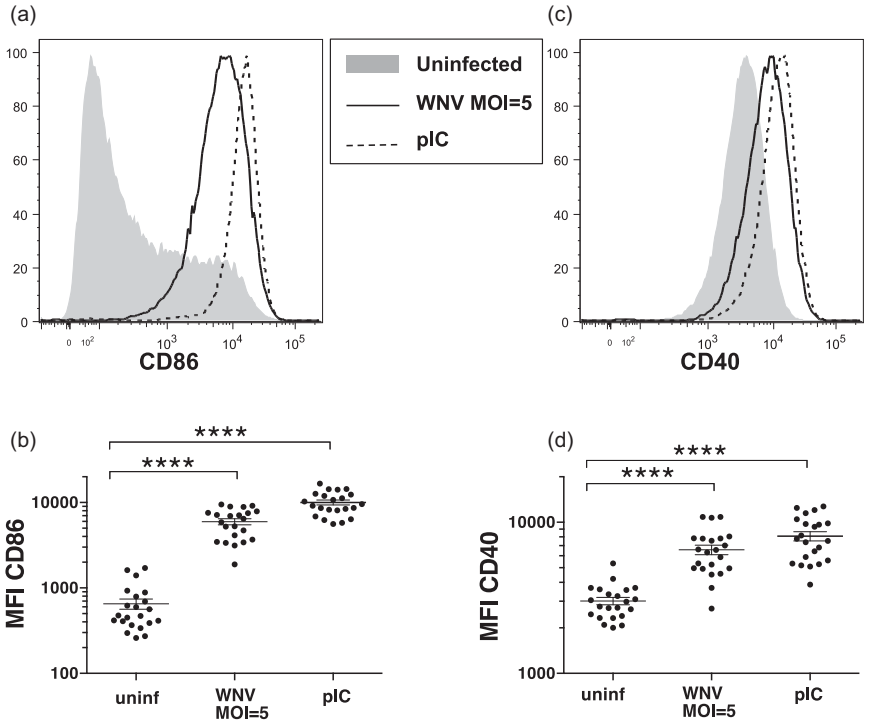

Fig. 4. West Nile virus (WNV)-infected dendritic cells (DCs) increase expression of T cell co-stimulatory molecules. DCs were left unstimulated, infected with WNV Kunjin isolate (WNV_{KUN}) at multiplicity of infection (MOI) = 1 or MOI = 5 or stimulated with poly-inosine-cytosine (pIC), and cells were harvested after 48 h. (a,b) CD86 and (c,d) CD40 surface expression (MFI = mean fluorescence intensity) on DCs was determined using flow cytometry. Symbols represent individual donors. Significance was evaluated using a repeated-measure (RM) one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test (comparing each stimulated value to the corresponding unstimulated value). $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

and iNKTs isolated from multiple donors were tested in this assay. iNKTs exposed to WNV-infected DCs up-regulated CD69 relative to those iNKTs exposed to unstimulated DCs (Fig. 6a,d,e). The CD69 increase was significant, although less pronounced than that induced by αGalCer-pulsed DCs, indicating that WNV-infected DCs were capable of activating iNKTs. In contrast, pIC activation of DCs led to minimal up-regulation of CD69 by

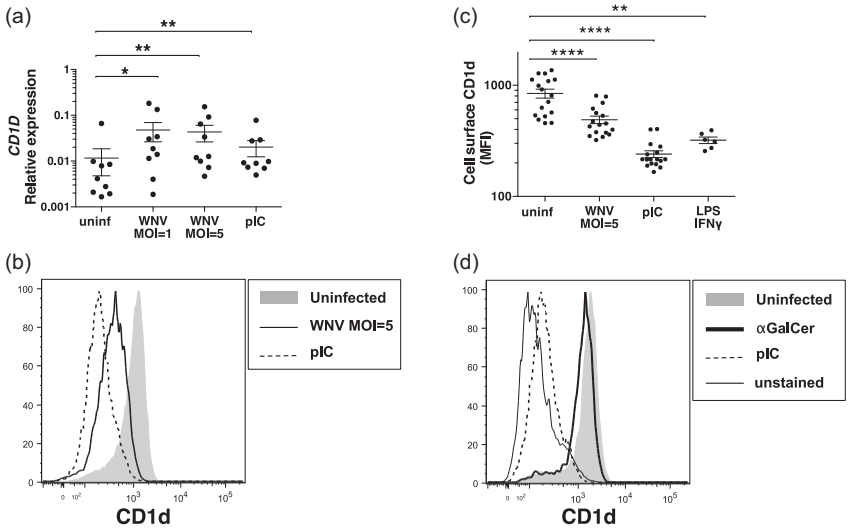

Fig. 5. West Nile virus (WNV) infection decreases dendritic cell (DC) display of surface CD1d. DCs were left unstimulated, infected with WNV Kunjin isolate (WNV_{KUN}) at multiplicity of infection (MOI) = 1 or MOI = 5 or stimulated with poly-inosine-cytosine (pIC), lipopolysaccharide/interferon-gamma (LPS/IFN-γ) or α-galactosylceramide (αGalCer), and cells were harvested after 48 h. (a) DC cDNA was generated from RNA and assessed for expression of the *CD1D* gene by quantitative polymerase chain reaction (qPCR). Shown is the expression of the *CD1D* gene relative to the host *ACTB* gene. (b,d) CD1d surface expression (MFI) on DCs treated as indicated was determined using flow cytometry. (d) The signal of unstained DCs on the fluorescence channel used for anti-CD1d. (a,c) Symbols represent individual donors. Significance was evaluated using a repeated-measure (RM) one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test (comparing each stimulated value to the corresponding unstimulated value) or a paired *t*-test (for unstimulated *versus* LPS/IFN-γ). $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$.

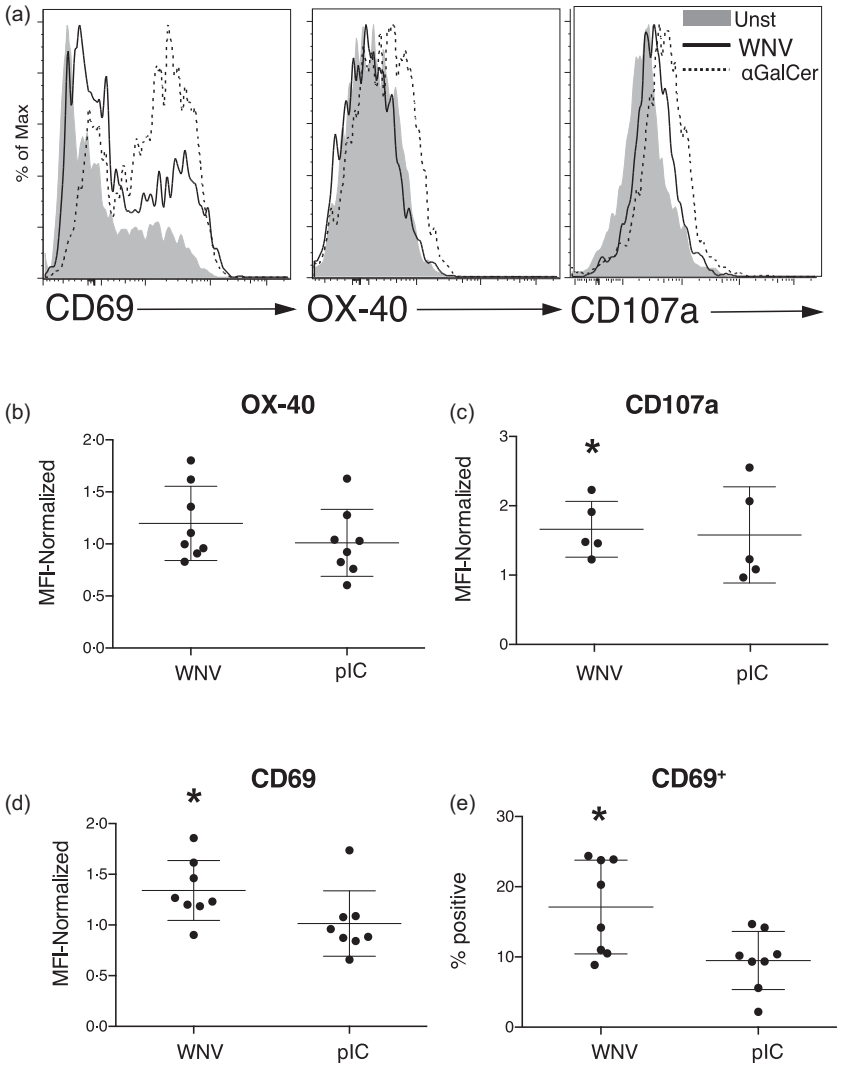

Fig. 6. West Nile virus (WNV)-infected dendritic cells (DCs) activate invariant natural killer T cells (iNKTs). DCs were left unstimulated, infected with WNV_{KUN} at multiplicity of infection (MOI) = 5, incubated with the CD1d ligand α-galactosylceramide (αGalCer) (100 ng/ml) or stimulated with polyinosine-cytosine (pIC) (50 μg/ml) for 24 h. DCs (10 000/well) were then co-cultured with iNKTs (50 000/well) that had been isolated from blood, expanded *in vitro* and rested. Purified iNKTs were $CD3^+$ and bound the CD1d-PBS−57 tetramer. (a) After 48 h, iNKT expression of CD69, OX-40 and CD107a [lysosomal-associated membrane protein 1 (LAMP1)] after incubation with unstimulated (Unst), WNV-infected or αGalCer-exposed DCs was determined by flow cytometry. (b,d) The surface expression of OX-40, CD107a and CD69 on iNKTs in multiple experiments is compiled. In each experiment, mean fluorescence intensities (MFIs) on iNKTs incubated with stimulated (WNV or pIC) DCs were normalized to expression on iNKTs incubated with unstimulated DCs. (e) The percentage of $CD69^+$ iNKTs is shown. The mean percentage of $CD69^+$ iNKTs after exposure to unstimulated DCs was 10·1%. Symbols represent individual experiments with unique donor DC and iNKT combinations. Significance was evaluated using a repeated-measure (RM) one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test (comparing each stimulated value to the corresponding unstimulated value). $^*P < 0{\cdot}05$.

iNKTs (Fig. 6d,e). Other iNKTs activation markers such as OX-40 and CD107a (LAMP1) were not up-regulated significantly by WNV-infected DCs (Fig. 6a–d).

To determine the iNKT production of cytokines elicited by WNV-infected DCs, we measured amounts of secreted cytokines (IFN-γ, IL-10, TNF-α, IL-4, IL-17, IL-22) using Luminex assays. Because iNKTs from different donors varied in their baseline production of cytokines when incubated with unstimulated DCs, the iNKT production of cytokines in response to stimulated/infected DCs is expressed as fold induction over the response to unstimulated DCs. αGalCer-exposed DCs elicited robust production of IFN-γ, IL-10 and TNF-α from iNKTs, indicating that the iNKTs were functionally competent. In contrast, WNV-infected DCs induced iNKTs to produce low amounts of IL-10, but none of the other tested cytokines (Fig. 7a–c, and data not shown). To test whether the absence of IL-12p70 explained the lack of IFN-γ production by iNKTs we added purified IL-12p70 to the cultures of infected DCs and iNKTs, but we did not observe any significant alteration in the production of IFN-γ (Fig. 7d). Taken together, these data show that WNV-exposed DCs fail to completely activate iNKTs.

Blockade of CD1d–TCR interactions or IFNAR inhibits CD69 up-regulation and IL-10 production by iNKTs exposed to WNV-infected DCs

iNKTs are activated in response to pathogens either directly through their TCR, or indirectly via cytokines such as IL-12 or type I IFN [5]. Other studies show that iNKTs may be activated by a combination of weak responses to CD1d-presented self-antigens and inflammatory cytokines or type I IFN [13,22,23].

Because WNV-infected DCs secrete high amounts of IFN-α, we tested if iNKT activation and IL-10 production were mediated by IFNAR-type I IFN interactions. WNV-infected, αGalCer-loaded or unstimulated DCs were incubated with iNKTs in the presence of an IFNAR-blocking mAb or an isotype control mAb, and activation markers

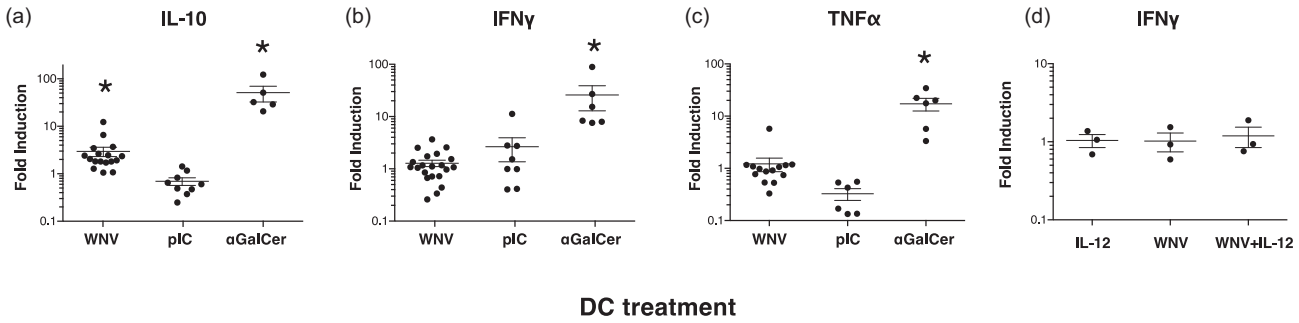

Fig. 7. invariant natural killer T cells (iNKTs) produce interleukin (IL)−10 but not proinflammatory cytokines when exposed to WNV-infected dendritic cells (DCs). DCs were left unstimulated, infected with West Nile virus Kunjin isolate (WNV_{KUN}) at multiplicity of infection (MOI) = 5, incubated with the CD1d ligand α-galactosylceramide (αGalCer) or stimulated with poly-inosine-cytosine (pIC) for 24 h. DCs (1×10^4/well) were then co-cultured for 24 h with iNKTs (5×10^4/well) that had been isolated from blood, expanded *in vitro* and rested. Supernatants were assayed for the presence of (a) IL-10, (b)interferon (IFN)-γ and (c) tumour necrosis factor (TNF)-α using Luminex assays. iNKT production of cytokines in response to stimulated/infected DCs is expressed as fold induction over iNKTs incubated with unstimulated DCs. The mean amount of IL-10 in the cultures of WNV-exposed DCs + iNKTs was 867 pg/ml, although the variability among donors was very high [95% confidence interval (CI) = 390–1345 pg/ml)]. DCs (1×10^4/well) incubated alone in the same assay produced very low amounts of IL-10 ($\bar{x} = 4$ pg/ml). WNV-infected DCs did not elicit iNKT production of other inflammatory cytokines (IL-4, IL-17, IL-22). (d) Addition of IL-12p70 to the cultures did not alter IFN-γ production significantly by iNKTs. Symbols represent individual experiments with unique donor DC and iNKT combinations. The significance of the fold induction for each condition was evaluated using a repeated-measure (RM) one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test (comparing each stimulated (WNV or pIC) value to the corresponding unstimulated value) or a paired *t*-test (for αGalCer *versus* unstimulated). $^*P < 0{\cdot}05$.

and cytokine production determined as described above. Blockade of IFNAR inhibited activation significantly (as judged by CD69 up-regulation) and IL-10 production of iNKTs exposed to WNV-infected DCs (Fig. 8a,b), suggesting that a component of the activation process depends upon IFNAR signalling.

However, as IFNAR blockade did not result in complete inhibition of the iNKT production of IL-10, we also tested the role of the CD1d–TCR interaction using a CD1d-blocking mAb. As shown in Fig. 8c,d, blockade of CD1d also partially inhibited CD69 up-regulation and IL-10 production in response to WNV-infected DCs, suggesting that iNKT activation also depends upon TCR signalling.

Discussion

To understand more clearly early events in the immune response against WNV infections, we studied the ability of WNV-infected human DCs to activate iNKTs, a population of innate lymphocytes that can amplify the adaptive immune response through secretion of different cytokines [7–10]. We studied the effect of WNV infection on DC molecules such as CD1d, CD40 and IL-12p70 that are known to activate iNKTs optimally [42]. Our results show that WNV-exposed DCs synthesized viral RNA and proteins, produced type I IFN but not IL-12p70 or IL-10 and displayed elevated CD40 but reduced CD1d. This infected DC profile induced suboptimal iNKT activation, characterized by CD69 up-regulation and limited production of IL-10 but not proinflammatory cytokines. The iNKT response to infected DCs was dependent upon both CD1d–TCR and IFNAR-type I IFN interactions.

It is well known that iNKTs are able to secrete copious amounts of many different cytokines in a very short time when stimulated *in vitro* with strong agonists, such as the CD1d ligand αGalCer or phorbol esters plus ionomycin. In fact, one of the original defining characteristics of iNKTs was that they could secrete IL-4 and IFN-γ simultaneously. However, iNKT responses *in vivo* are less promiscuous and depend upon their previous history of activation, the cytokine milieu and the activation state of the antigen-presenting cells that interact with them. Our results show a very clear example of this. Interaction with WNV-infected DCs results in iNKT up-regulation of some, but not all, activation markers, and in secretion of IL-10, but no other cytokines, either type 1 (IFN-γ, TNF-α) or types 2 or 3 (IL-4, IL-17, IL-22).

WNV infection of the DCs altered their phenotype in ways that could influence the activation of iNKTs. Despite inducing increased *CD1D* mRNA, WNV infection reduced the surface expression of CD1d, which could influence the strength of TCR signalling in the iNKTs. Indeed, variable concentrations of αGalCer altered the pattern of cytokine production by iNKTs, suggesting that TCR signal strength is an important regulator of iNKT function [43]. The effect of infection on CD1d surface residence is unlikely to be due to viral interference mechanisms that specifically target CD1d, as proposed for viruses such as HSV and HIV-1 (reviewed in [41]), because pattern recognition receptor ligands such as LPS and pIC also induced significant CD1d down-regulation in our assays. As CD1d traffics through

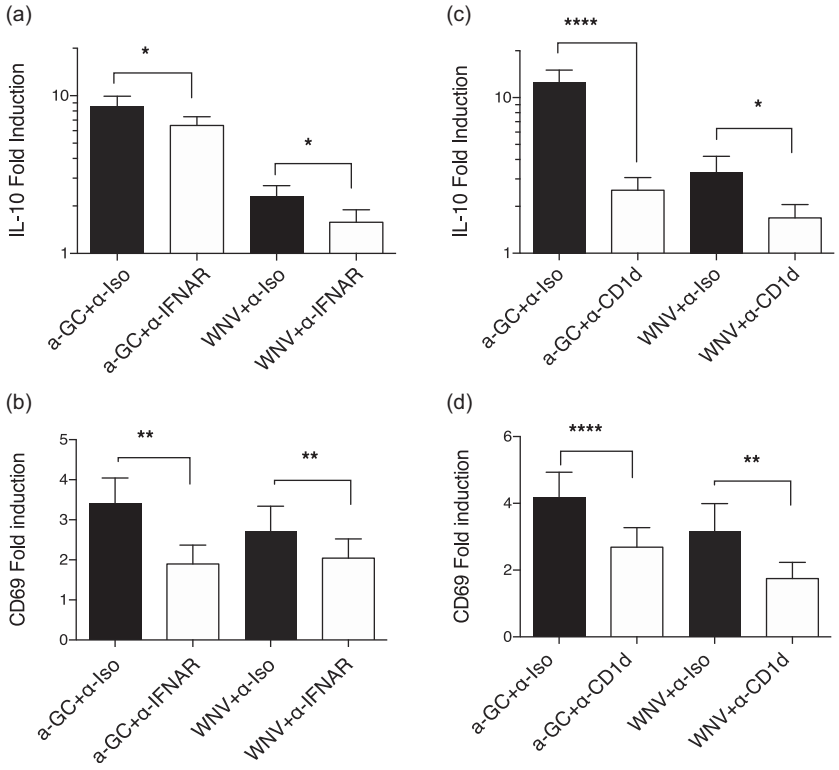

Fig. 8. Invariant natural killer T cell (iNKT) up-regulation of CD69 and interleukin (IL)−10 production after exposure to West Nile virus (WNV)-infected dendritic cells (DCs) depends upon CD1d–T cell receptor (TCR) interactions and type I interferon receptor (IFNAR). (a,b) DCs were infected with WNV Kunjin isolate (WNV_{KUN}) at MOI = 5 for 24 h, incubated with the CD1d ligand α-galactosylceramide (αGalCer) or left unstimulated. DCs were then co-cultured with iNKTs in the presence of a blocking anti-IFNAR monoclonal antibody (mAb) (5 μg/ml) or a corresponding amount of isotype control mAb for 24 h. (a) Supernatants were assayed for the presence of interleukin (IL)−10 using Luminex assays. iNKT production of cytokines in response to stimulated DCs is expressed as fold induction over iNKTs incubated with uninfected DCs. (b) Expression of CD69 was determined by flow cytometry. Changes in CD69 mean fluorescence intensity (MFI) were normalized to the levels in NKT co-cultured with uninfected DCs. (c,d) DCs were infected with WNV_{KUN} at multiplicity of infection (MOI) = 5 for 24 h, incubated with the CD1d ligand αGalCer or left unstimulated. DCs were then co-cultured with iNKTs in the presence of a blocking anti-CD1d mAb (10 μg/ml) or an isotype control mAb for 24 h. (c) Supernatants were assayed for the presence of IL-10 using Luminex assays. (d) Expression of CD69 was determined by flow cytometry. The data [$\bar{x}$ + standard error of the mean (s.e.m.)] summarize individual experiments with unique donor DC and iNKT combinations, $n = 8$ (IFNAR blockade) and $n = 14$ (CD1d blockade). The significance of the response differences in the presence or absence of blocking antibody was determined by ratio paired *t*-tests. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$.

endocytic compartments with Ii chain, the profound changes in MHC-II/Ii trafficking in activated DCs may alter CD1d trafficking and lead to reduced CD1d surface expression [44].

Alternatively, early studies of iNKT responses in the context of viral infections suggested that they were similar to those in the presence of TLR-9 ligand (CpG ODN)-stimulated DCs, which involved mainly IL-12 and type I IFN, and were independent of TCR–CD1d interactions [18,20]. However, later studies have shown that some viruses, as well as innate stimuli, can alter the repertoire of lipids presented by CD1d in infected cells, and induce direct CD1d–TCR-mediated responses of iNKTs [14,16]. These studies suggest that iNKT cell responses to viruses may be influenced both by changes in the lipid repertoire and by cytokine secretion of the infected DCs. Our experiments support this model. We show that the response of iNKTs to WNV-infected human DCs can be blocked partially by α-CD1d and α-IFNAR, suggesting that both direct TCR triggering and IFNAR signalling contribute to the iNKT activation.

WNV-exposed DCs produced IFN-α but not IL-12p70, due probably to IFNAR-induced feedback mechanisms that dampen IL-12 production [45,46]. Although IL-12-independent T cell IFN-γ production promoted by type I IFN is observed in some virus infections [47], the absence of IL-12p70 could explain why the iNKTs did not produce IFN-γ. The presence of type I IFN in the absence of IL-12p70 often leads to production of IL-10 in T cells and myeloid cells [46,48–50]. However, in our experiments, supplementation of IL-12p70 into cultures of infected DCs and iNKTs did not induce production of IFN-γ or

alter the production of IL-10. Taken together, these results suggest that other cytokines and/or co-stimulatory molecules influence the response pattern of iNKTs to WNV-infected DCs.

The ability of freshly isolated iNKTs to produce IL-10 has been controversial. In some experiments PMA and ionomycin stimulation did not induce IL-10 production [51,52], while others showed iNKT IL-10 production after stimulation via anti-CD3 + anti-CD28 [53]. However, many experiments have demonstrated increased IL-10 production of NKTs *in vivo*, especially after restimulation of αGalCer-activated cells [54,55], and a naturally existing subset of NKTs that produce primarily IL-10 was identified recently [54]. Furthermore, IL-10 production by NKTs is important for their protective role in some autoimmune diseases such as type I diabetes [56] and experimental autoimmune encephalomyelitis [57]. Reported populations of forkhead box protein 3 $(FoxP3)^+$ regulatory iNKTs could be the source of this IL-10 [58,59].

Clinical studies show that IL-10 has an important role in regulating immune responses to flaviviruses in both humans and mice. There is a significant positive correlation between plasma IL-10 levels and severity of disease in Dengue virus-infected patients [60], and *in-vitro* blocking of IL-10 improves T cell responses from Dengue patients [61]. In mice, IL-10 modulates the morbidity and mortality of WNV infection and blockade of IL-10 improves the immune response to WNV infection [62]. Interestingly, in these experiments a $CD4^+CD3^+$ T cell population produced primarily the IL-10 *in vivo*, a population that would include iNKTs. Although this work did not evaluate directly whether iNKTs produced IL-10, our results suggest that iNKTs could be partly responsible for this *in-vivo* effect, especially as IL-10 production peaked by 72 h post-infection. Consistent with this, evidence is emerging that iNKTs may be important in flavirus infections. iNKTs become activated in mouse models of Dengue [21], and iNKT activation correlates with disease severity in human Dengue patients [63]. Taken together, our data suggest that upon interaction with WNV-infected DCs, iNKTs could be responsible for IL-10 production in the early stages of WNV infection.

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Disclosure

The authors declare that no competing interests exist.

Author contributions

S. K. and J. A. designed experiments and wrote the manuscript; S. K., S. T., A. S. and J. A. performed experiments and analysed the data; T. P. and E. C. identified blood donors and performed phlebotomy.

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