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Flavivirus infection induces indoleamine 2,3-dioxygenase in human monocyte-derived macrophages via tumor necrosis factor and NF-κB

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ABSTRACT

Infection with West Nile virus (WNV) via a mosquito bite results in local viral replication in the skin, followed by viremia. Thus, tissue macrophages are ideally located to prevent the dissemination of WNV throughout the host. The current study shows that WNV infection of human monocyte-derived macrophages (MDM) results in increased WNV mRNA, protein, and infectious virions at 24 h p.i. with a decline in titer after 48 h. Concomitant with viral control was the robust induction of indoleamine 2,3-dioxygenase (IDO) and resultant metabolism of L-tryptophan (L-Trp) to kynurenine. In WNV-exposed cultures, IDO protein was induced primarily in noninfected versus viral-infected MDM. Whereas WNV infection increased the production of IFN-α, IFN-β, and TNF, only antibody neutralization of TNF attenuated IDO expression and activity. WNV infection also activated NF-κB, and inhibition of this pathway with BMS-345541 abrogated IDO induction. Similar results were also obtained with MDM infected with the related flavivirus, Japanese encephalitis virus. Whereas IDO-mediated L-Trp metabolism can exhibit antiviral properties, inhibition of IDO activity in MDM with L-1-MT or the addition of excess L-Trp did not affect viral control. However, culturing MDM in L-Trp-deficient medium or overexpression of IDO in cells prior to infection significantly attenuated WNV replication, which was reversed by adding excess L-Trp. Together, these data support that although IDO is not required by MDM for the clearance of established viral infection, the spread of flavivirus infection is limited by IDO expressed in uninfected, neighboring cells. *J. Leukoc. Biol.* 91: 657–666; 2012.

Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus that has recently become the most widely distributed member of the *Flaviviridae* family following the emergence and spread of a highly virulent strain in North America [1]. Infections with neurotropic flaviviruses of the Japanese encephalitis serotype, which include inter alia, Japanese encephalitis, Murray Valley encephalitis, St. Louis encephalitis, and West Nile, result in debilitating or fatal neurological involvement in a significant proportion of clinical cases and in WNV infection, particularly affect the elderly and the immunocompromised [2, 3]. Thus, there is a continuing need to understand disease pathogenesis to develop effective antiviral strategies.

Among the multifarious mechanisms used by cells to combat infection, indoleamine 2,3-dioxygenase (IDO) (EC 1.13.11.52) has been shown to be antiviral in vitro for a variety of viruses [4]. IDO is most potently induced by IFN-γ and, to a lesser extent, by IFN-α/β and LPS, with IL-1 and TNF providing synergistic activity [5, 6]. IDO is the initial enzyme in the kynurenine (Kyn) pathway of L-tryptophan (L-Trp) catabolism. Once expressed, IDO catalyzes the oxidative conversion of L-Trp, the least-abundant, essential amino acid, into *N*-formyl-Kyn [6], which is converted further into Kyn. This depletes L-Trp from the local microenvironment, with the concomitant formation of biologically active Kyn pathway metabolites.

Although infection with dengue virus, HIV, influenza, poliovirus, hepatitis C, or EBV is associated with IDO induction in various tissues and cell types in vivo and in vitro [7–12], the antiviral role of IDO-mediated L-Trp depletion has primarily

Abbreviations: BHK=baby hamster kidney, D-1-MT=1-methyl-D-tryptophan, IDO=indoleamine 2,3-dioxygenase, JEV=Japanese encephalitis virus, Kyn=kynurenine, L-1-MT=1-methyl-L-tryptophan, L-Trp=L-tryptophan, MDM=monocyte-derived macrophage, NEMO=NF-κB essential modulator, NHMRC=National Health and Medical Research Council, NS1=nonstructural protein 1, p.i.=postinfection, siRNA=small interfering RNA, WNV= West Nile virus

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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been investigated in the context of an in vitro IFN-induced effector mechanism against CMV, herpes simplex virus, HIV, and vaccinia virus [7, 13–18]. However, the signaling mechanisms underlying virus-stimulated IDO expression are currently unknown. Whether IDO that is induced in response to virus infection alone controls viral replication is also unknown.

Moreover, emerging evidence suggests that the induction of IDO by certain pathogens, including HIV, enables the pathogen to escape the host immune response [19, 20]. Indeed, numerous reports show that IDO-expressing cells, such as macrophages and DCs, strongly inhibit T cell responses, thus also linking IDO to diverse physiological and pathophysiological immunosuppressive scenarios, including pregnancy, autoimmune diseases, and immune evasion by tumor cells [5].

To date, a role for IDO in WNV infection has not been investigated. Given that flavivirus infections are characterized by significant immunopathology, studies show that monocytes/macrophages can be pathogenic or beneficial for the host [21–26]. This is complicated further by the finding that macrophages themselves are susceptible to WNV infection [27–30]. Thus, the interaction of WNV with host macrophages and the implications for macrophage function require investigation. In this study, we investigated the signaling mechanisms by which WNV and Japanese encephalitis virus (JEV) infection induces IDO expression and activity in human monocyte-derived macrophages (MDM) and determined the extent to which this enzyme represents an antiflaviviral effector mechanism.

MATERIALS AND METHODS

Cell culture

Monocytes were isolated from human blood buffy coats (Australian Red Cross Blood Bank) and matured into MDM by 8–12 days in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated human serum and 25 ng/mL M-CSF (PeproTech, Rocky Hill, NJ, USA) [31]. In certain experiments, MDM were cultured in L-Trp-deficient RPMI-1640 (Invitrogen) without human serum during infection. BHK and HeLa cells were cultured in endotoxin-free DMEM or MEM (Invitrogen), respectively, with 10% FCS (Sigma-Aldrich, St. Louis, MO, USA).

Virus propagation

WNV (lineage II, Sarafend strain) was propagated as described previously [32]. JEV (Nakayama strain), kindly provided by Dr. Mario Lobigs (The John Curtin School of Medical Research, Canberra, ACT, Australia), was similarly propagated. Aliquots were stored at −80°C. Viral titers were determined by plaque assay.

Plaque assay

BHK cells were infected with serially diluted culture supernatants. Virus was removed after 1 h, and cells were overlaid with MEM containing 5% FCS/1.5% (w/v) agarose II (Amresco, Solon, OH, USA) and incubated for 3 days. Cells were fixed and stained with 3% (w/v) crystal violet in 20% methanol (Sigma-Aldrich) for plaque enumeration. Titers were expressed as PFU/mL.

Virus infection and cell treatments

MDM were infected with 0.035–35 PFU/cell WNV or 3.5 PFU/cell JEV. HeLa cells were infected with 0.1 PFU/cell WNV. For some experiments, WNV was UV-inactivated. After 1 h of virus exposure, cells were washed twice vigorously and the medium replaced. Some cells were stimulated with IFN-γ (500 U/mL; R&D Systems, Minneapolis, MN, USA). For cytokine inhibition, 10–20 μg/mL anti-IFN-α2, anti-IFN-γ, and anti-TNF (eBioscience, San Diego, CA, USA) or anti-IFN-β antibody (or the mouse IgG_1 isotype; Millipore, Billerica, MA, USA) were added to the culture medium. In certain experiments, medium was supplemented with L-Trp (100–350 μM; Sigma-Aldrich) or the IDO inhibitor L-1-MT (500 μM; Sigma-Aldrich). For phosphorylated IκBα Western blotting, cells were exposed to the proteasome inhibitor MG-132 (25 μM; Merck, Darmstadt, Germany) for 3 h before cell harvest. To inhibit NF-κB activation, cells were treated with BMS-345541 (5 μM; Sigma-Aldrich) for 1 h prior to infection and for the 24- to 48-h incubation period postviral infection.

Plasmid transfection of HeLa cells

HeLa cells were transfected with pcDNA3 encoding human IDO cDNA or the empty control vector (1 μg/well) with Lipofectamine 2000 (Invitrogen), as described previously [33].

Protein and cell viability assays

Protein concentration and cell viability were measured using a BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) and a cytotoxicity detection kit (Roche Applied Science, Penzberg, Germany), respectively.

RT and PCR

RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg RNA using 0.2 μg random primers (New England Biolabs, Ipswich, MA, USA) and MMLV RT (Epicenter Biotechnologies, Madison, WI, USA). WNV RNA was quantified by TaqMan PCR using WNV primer and probe sequences that detect the WNV 3′-noncoding region [34]. IDO and 18S expression were quantified by TaqMan PCR (assay IDs: Hs00984148_m1 and 4319413E, respectively; Applied Biosystems, Carlsbad, CA, USA). Data were normalized to 18S and analyzed using the ΔΔcomparative threshold method.

Immunofluorescence

Cells were fixed and permeabilized with 4% paraformaldehyde and 0.1% Triton X-100. Cells were incubated with FITC anti-WNV NS1 antibody (1:100; gift from Dr. Roy Hall, University of Queensland, QLD, Australia) or for NF-κB staining, rabbit anti-p65 (1:50; eBioscience) or anti-RelB (1:50; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), followed by anti-rabbit IgG-FITC (1:200; Invitrogen). For IDO and WNV-NS1 double-staining, cells were incubated with mouse antihuman IDO mAb (1:1000; a generous gift from Dr. Osamu Takikawa, Hokkaido University, Sapporo, Japan), followed by biotinylated anti-mouse IgG_1 (1:100; BioLegend, San Diego, CA, USA). After washing, anti-WNV-NS1-FITC and streptavidin-Alexa Fluor 594 (1:200; Invitrogen) were added. Coverslips were mounted with ProLong Gold antifade with DAPI (Invitrogen).

ELISA

Human IFN-α and IFN-β (PBL Interferon Source, Piscataway, NJ, USA), IFN-γ (BioLegend), and TNF (eBioscience) ELISA kits were used as directed by the manufacturers. Detection limits were 12.5, 25, 4, and 8 pg/mL, respectively.

Western blotting

Western blotting was performed as described previously [31], using a mouse antihuman IDO mAb (a generous gift from O. Takikawa, Hokkaido University) or a rabbit polyclonal antiphosphorylated IκBα (1:1000; Cell Signaling Technology, Danvers, MA, USA), although in this study, NuPAGE Bis-Tris gels and the iBlot dry-blotting system (Invitrogen) were used for protein electrophoresis and transfer, respectively. Membranes were reprobed with mouse anti-β-tubulin antibody (1:5000; Cell Signaling Technol-

ogy) as a protein-loading control. Band density was quantified using ImageJ (NIH, Bethesda, MD, USA).

HPLC

The extent of conversion of L-Trp into Kyn in the culture medium was used as an index of cellular IDO activity [31, 33]. L-Trp and Kyn levels were measured by HPLC (Agilent Technologies 1200; Agilent Technologies, Santa Clara, CA, USA), as detailed previously [35].

Statistics

Data were analyzed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA). Student's *t* test, one-way or two-way ANOVA, was applied as appropriate, and a *P* value of ≤ 0.05 was considered the threshold for significance.

Online supplemental material

The supplemental data provided in this study include immunofluorescent staining, demonstrating nuclear translocation of NF-κB subunits in WNV-exposed WNV^+ and WNV^- MDM (Supplemental Fig. 1) and quantification of WNV infection in MDM infected with high or low viral doses ± L-Trp ± L-1-MT, infected with the normal virus dose ± L-1-MT ± anti-IFN-α/β antibody, and in MDM pretreated with IFN-γ prior to virus inoculation (Supplemental Fig. 2).

RESULTS

WNV infection of human MDM

Human and mouse macrophages are susceptible to infection with various strains of WNV [27–30]. In MDM infected with 0.035 or 3.5 PFU/cell, WNV RNA and the WNV-NS1 protein were detected at 24 h p.i., with a decline in viral RNA and protein in MDM infected at 3.5 PFU/cell by 48–72 h p.i. (**Fig. 1A–C**). Thus, although $WNV\text{-}NS1^+$ staining indicated that the virus underwent at least one round of replication in infected MDM, infectious virion production decreased ~100-fold after 72 h (Fig. 1D). Infection of MDM with 0.035–3.5 PFU/cell WNV was accompanied by minimal cytotoxicity (<10%); >20% cell death was observed only following infection with 35 PFU/cell of WNV for 72 h (Fig. 1E). Thus, human MDM

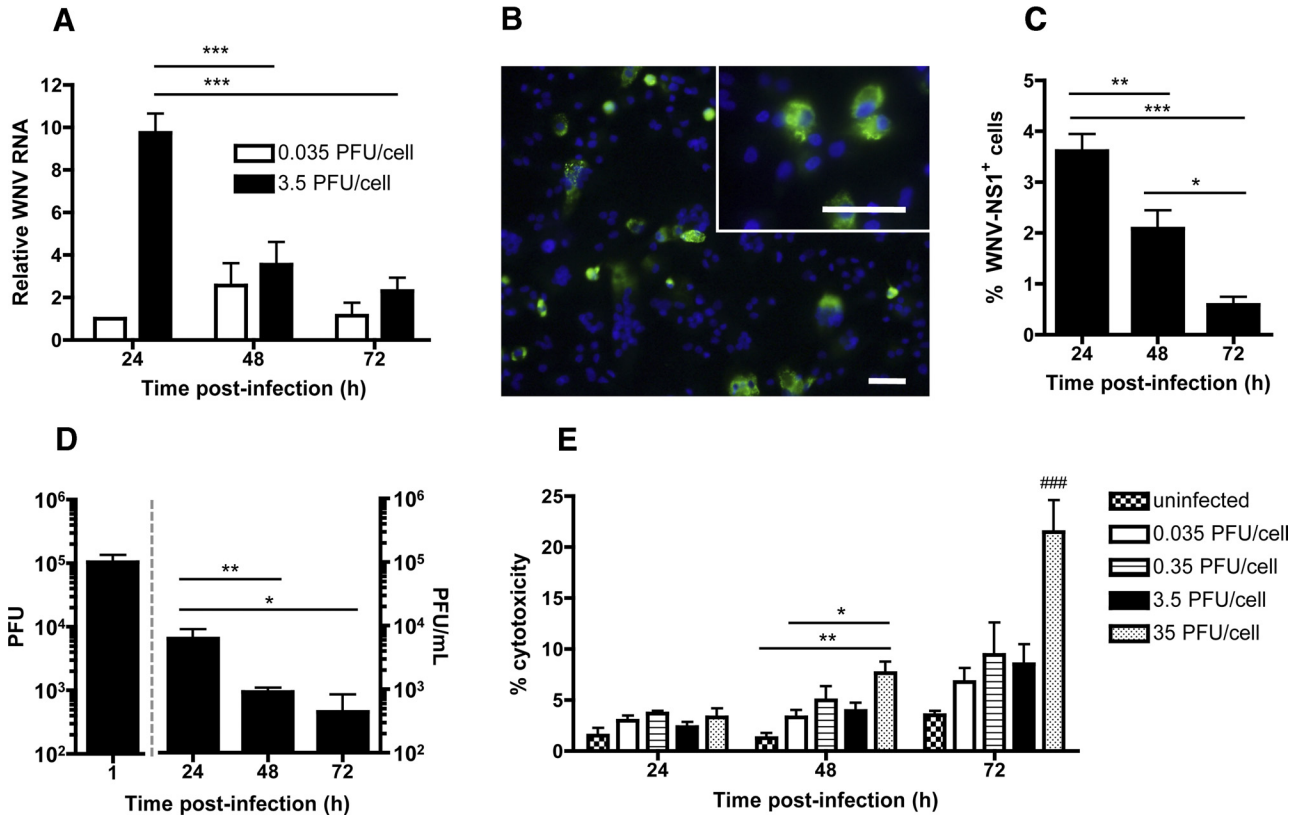


Figure 1. WNV infection of MDM. (A) WNV RNA expression in MDM following infection with WNV at 0.035 or 3.5 PFU/cell for 24, 48, and 72 h. Expression levels of WNV RNA are shown relative to the levels measured in MDM infected at 0.035 PFU/cell for 24 h. (B) Representative immunofluorescent staining for WNV-NS1 antigen (green, FITC; indicative of replicating virus) and cell nuclei (blue; DAPI) in MDM infected with WNV at 3.5 PFU/cell for 24 h. No $WNV\text{-}NS1^+$ cells were detected in uninfected MDM or isotype-stained cells (not shown). Original bars, 50 μm. (C) Percentage of $WNV\text{-}NS1^+$ cells in MDM cultures infected with 3.5 PFU/cell of WNV for 24, 48, and 72 h. (D) Infectious virus in the supernatant of MDM infected with WNV at 3.5 PFU/cell for 24, 48, and 72 h and expressed as PFU/mL (right *y*-axis). Data at each time-point are cumulative. The 1-h time-point represents the quantity of virus (PFU; left *y*-axis) remaining on the cells after 1 h of viral absorption and vigorous washing. (E) Percentage cytotoxicity in uninfected MDM or MDM infected with 0.035—35 PFU/cell of WNV for 24, 48, and 72 h, as measured by lactate dehydrogenase release into the culture supernatant relative to 100% cell death representing total lysis of MDM in one well. All data represent the mean ± SEM of three to ten independent experiments. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{\#\#\#}P \leq 0.001$ compared with all other viral doses at 72 h p.i.

possess effective defense mechanisms against WNV, despite initial viral replication within the cells.

WNV induces IDO in MDM

IDO expression was not detected in uninfected, control MDM. However, WNV infection significantly induced the expression of IDO mRNA and protein in a time- and dose-dependent manner (**Fig. 2A** and **B**). This correlated with increased IDO activity, as reflected by increased L-Trp consumption and concomitant accumulation of Kyn in the culture medium by 48 h p.i. and onward (Fig. 2C–E). Replicating virus was required for the induction of IDO, as UV-inactivated WNV did not induce IDO activity (Fig. 2C and D). IFN-γ treatment also up-regulated IDO expression in MDM, with enzyme protein and activity detectable by 24 h after stimulation (Fig. 2B–D). Thus, relative to IFN-γ-treated MDM, there was a 24- to 48-h delay in active IDO expression in WNV-infected MDM, and the overall level of IDO protein was significantly less than that achieved with IFN-γ (Fig. 2B), the principle inducer of IDO in vitro and in vivo [5]. Nonetheless, infection with WNV at 3.5 PFU/cell depleted supraphysiological levels of L-Trp (>200 μM) from the medium by 72 h p.i. (Fig. 2C). Immunofluorescent staining for IDO and WNV-NS1 in WNV-infected MDM cultures demonstrated IDO to be expressed predominantly in noninfected cells, with colocalization observed occasionally and only in cells that stained at low intensity for WNV-NS1 (Fig. 2F and G). Of note, although ~5% of MDM were positive for WNV-NS1 at 48 h p.i., >70% of noninfected MDM expressed IDO protein (Fig. 2F).

Cytokine involvement in the induction of IDO by WNV

The localization of IDO primarily in uninfected bystander cells, in addition to the ~24-h delay in IDO expression in WNV-infected MDM compared with IFN-γ-stimulated MDM, suggested that de novo synthesis of soluble factors is required for IDO induction in response to WNV infection. High quantities of IFN-α, IFN-β, and TNF were detected in the supernatant of WNV-infected MDM at 48 h p.i., peaking at 2384, 484, and 2067 pg/mL, respectively (**Fig. 3A–C**).

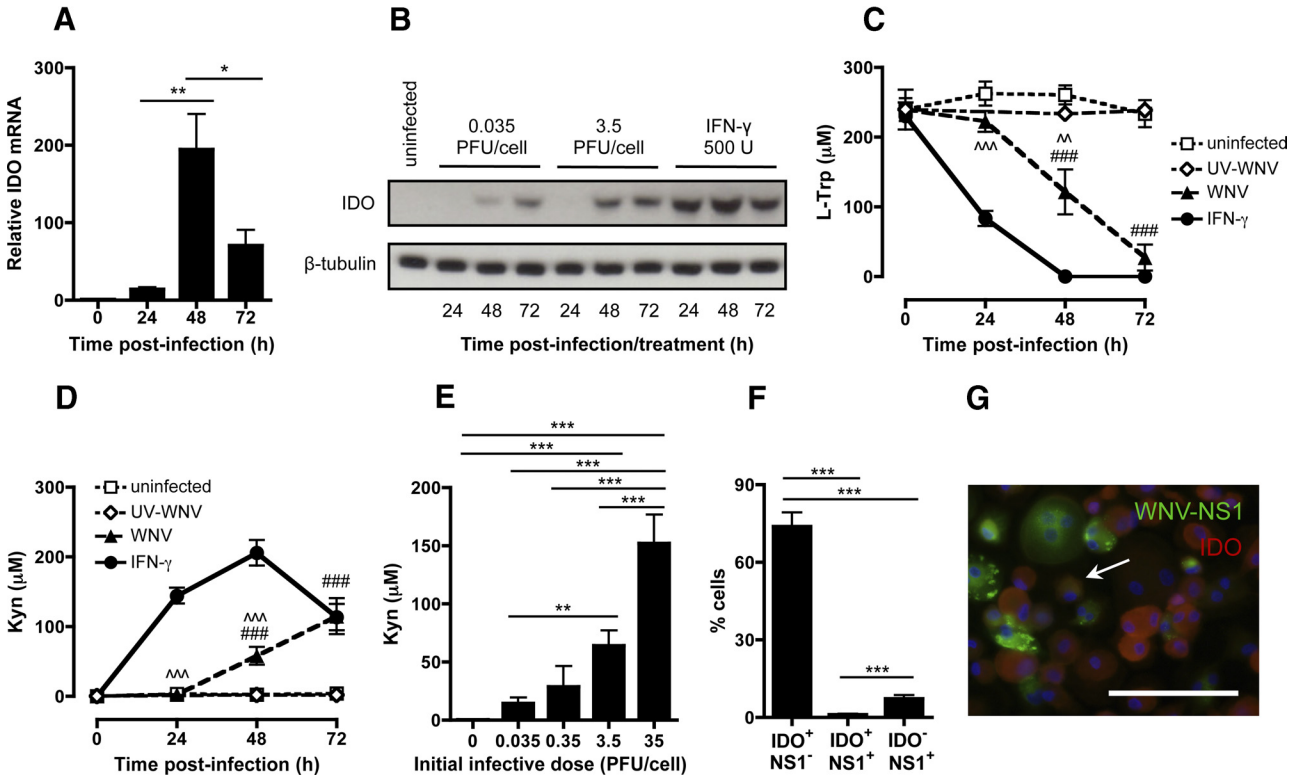


Figure 2. IDO expression and activity in WNV-infected MDM. (A) IDO mRNA expression in MDM infected with WNV at 3.5 PFU/cell for 0, 24, 48, and 72 h. Expression is relative to the levels measured in uninfected (0 h) cells. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. (B) Representative Western blot for IDO protein in uninfected MDM and MDM infected with WNV at 0.035 or 3.5 PFU/cell or treated with 500 U/mL IFN-γ for the indicated time-points. β-Tubulin was used as a protein-loading control. (C) L-Trp and (D) Kyn concentration present in the culture medium of uninfected MDM, and MDM infected with WNV or UV-inactivated WNV at 3.5 PFU/cell or MDM stimulated with IFN-γ (500 U/mL) for 0, 24, 48, or 72 h, as indicated. $^{\#\#\#}P \leq 0.001$ compared with uninfected cells; $^{\wedge\wedge}P \leq 0.01$; $^{\wedge\wedge\wedge}P \leq 0.001$, compared with IFN-γ-stimulated cells. (E) Kyn concentration in the supernatant of MDM infected with WNV at various PFU/cell for 48 h, demonstrating dose-dependent induction of IDO activity. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. (F) Percentage of IDO^{+} and/or $WNV\text{-}NS1^{+}$ cells in MDM cultures infected with 3.5 PFU/cell of WNV for 48 h, as determined by immunofluorescent staining. $^{***}P \leq 0.001$. (G) Representative immunofluorescent staining for IDO (red, Alexa Fluor 594), WNV-NS1 (green, FITC), and nuclei (blue, DAPI) in MDM, infected as in F. No positive staining for IDO or WNV-NS1 was observed in uninfected MDM or isotype-stained cells (not shown). Arrow indicates the occasional cellular colocalization of IDO with low-intensity WNV-NS1 staining. Original bar, 50 μm. All data shown represent the mean ± SEM of three to nine independent experiments.

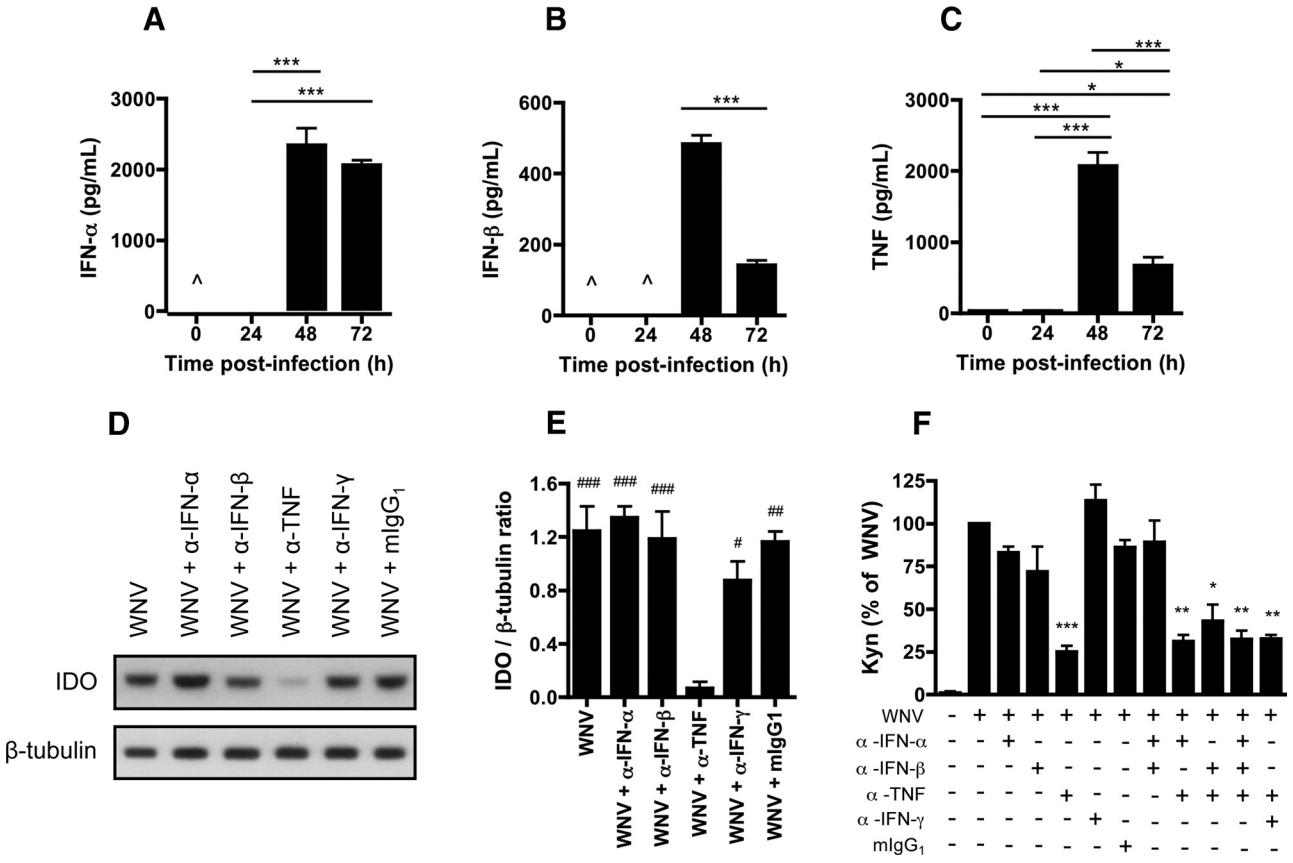


Figure 3. Involvement of TNF in the induction of IDO by WNV infection. (A) IFN-α, (B) IFN-β, and (C) TNF concentration in the supernatant of MDM infected with 3.5 PFU/cell of WNV, as measured by ELISA. Data at each time-point are cumulative, expressed as the mean ± SEM of triplicate determinations and are representative of two independent experiments; ^below the detection limit; $^{*}P \leq 0.05$; $^{***}P \leq 0.001$. (D) Representative Western blot for IDO and β-tubulin in cell lysates of MDM infected with WNV at 3.5 PFU/cell and treated with neutralizing antibodies against IFN-α, IFN-β, TNF, or IFN-γ or the mouse (m)IgG_1 isotype for 48 h. (E) Relative IDO protein band density from three independent experiments performed as in D, expressed as an IDO/β-tubulin ratio. $^{\#}P \leq 0.05$; $^{\#\#}P \leq 0.01$; $^{\#\#\#}P \leq 0.001$ compared with WNV + α-TNF. (F) Corresponding Kyn concentration in the supernatant of WNV-infected cells treated with neutralizing antibodies singly, as in E, or in the combinations indicated. Data are expressed as the percentage of the values for MDM infected with WNV alone and are the mean ± SEM of three independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ compared with the isotype control.

Antibody neutralization of TNF, but not IFN-α or IFN-β, significantly inhibited IDO expression and activity (Fig. 3D–F). Although treatment of MDM with up to 500 U/mL TNF alone did not induce IDO (not shown), TNF can synergize with IFN-γ to stimulate IDO activity in MDM [36]. A role for IFN-γ was, however, ruled out, as the cytokine was undetectable in the supernatant of WNV-infected MDM (ELISA detection limit 8 pg/mL), and neutralization of IFN-γ, alone or in combination with TNF neutralization, did not abrogate IDO protein expression or activity compared with isotype-treated cells or TNF neutralization alone, respectively (Fig. 3D–F). Together, this indicates that induction of IDO in MDM by WNV does not involve type I or II IFN and instead, is predominantly TNF-mediated.

Activation of the NF-κB pathway is required for WNV-induced IDO expression

NF-κB signaling is necessary for the induction of IDO by certain stimuli [37]. Furthermore, WNV-induced up-regulation of MHC class I is mediated by NF-κB [38]. Indeed, the NF-κB pathway is activated in WNV-infected MDM, as indicated by the twofold-higher levels of phosphorylated IκBα in WNV-infected versus control cultures at 24 h p.i. (**Fig. 4A** and **B**). Furthermore, staining for p65 and RelB, subunits activated in the canonical and noncanonical NF-κB pathway, respectively, showed that these subunits are localized predominantly in the cytoplasm in uninfected, control MDM cultures but translocate to the nucleus upon infection (Fig. 4C). This demonstrates that WNV infection activates canonical and noncanonical NF-κB signaling in MDM. In WNV-infected wells, nuclear translocation of p65 and RelB was observed in virus-infected and noninfected MDM (Supplemental Fig. 1).

Blockade of NF-κB signaling in WNV-infected MDM with BMS-345541, a specific allosteric site inhibitor of the IκB kinase complex (required for the activation of the NF-κB pathways) [39, 40], significantly inhibited IDO expression and activity at 48 h p.i. (Fig. 4D and E). This reduction in IDO was not a consequence of reduced TNF levels, as BMS-345541 did not abrogate the WNV-mediated increase in TNF (Fig. 4F). Together, these findings establish a central role for NF-κB in the induction of IDO by WNV.

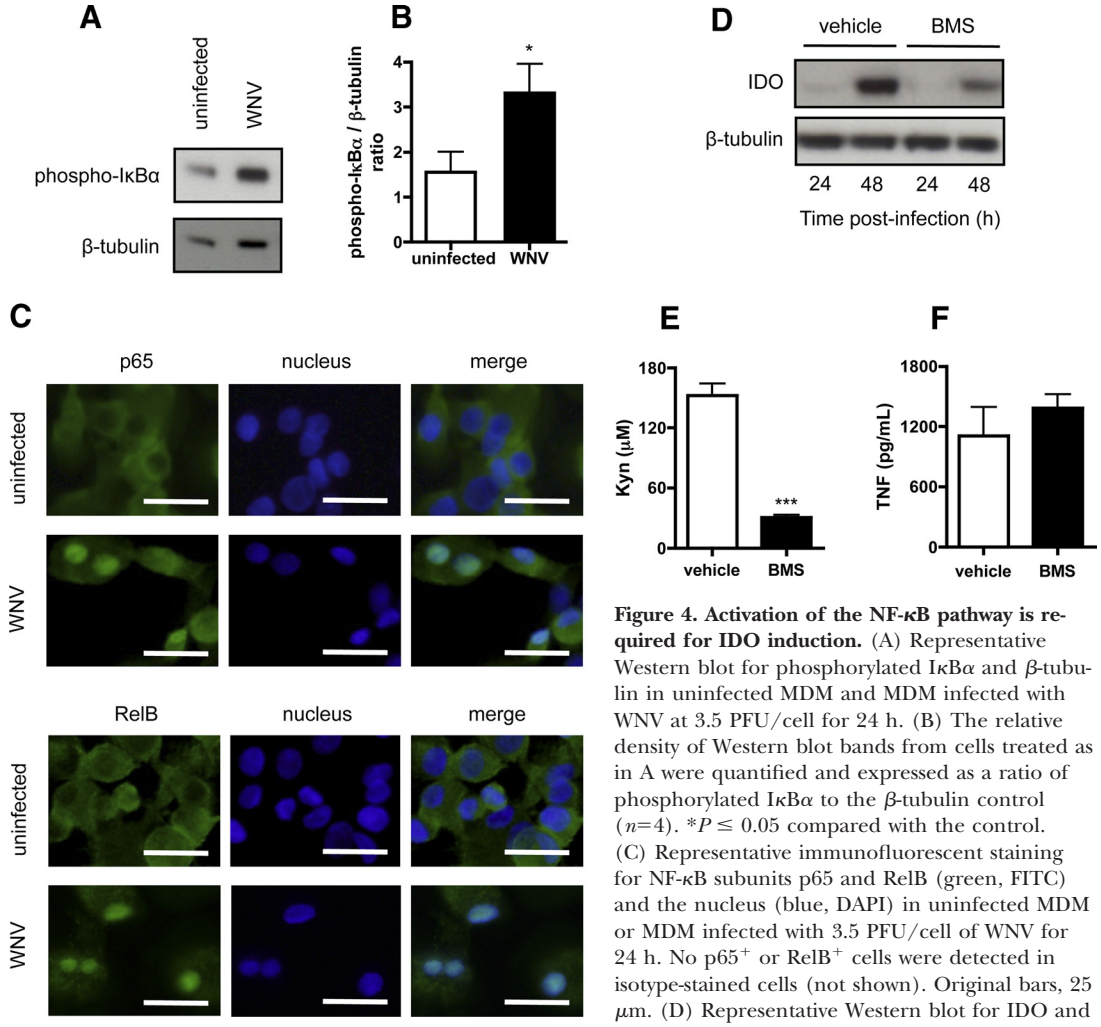


Figure 4. Activation of the NF-κB pathway is required for IDO induction. (A) Representative Western blot for phosphorylated IκBα and β-tubulin in uninfected MDM and MDM infected with WNV at 3.5 PFU/cell for 24 h. (B) The relative density of Western blot bands from cells treated as in A were quantified and expressed as a ratio of phosphorylated IκBα to the β-tubulin control ($n=4$). $*P \leq 0.05$ compared with the control. (C) Representative immunofluorescent staining for NF-κB subunits p65 and RelB (green, FITC) and the nucleus (blue, DAPI) in uninfected MDM or MDM infected with 3.5 PFU/cell of WNV for 24 h. No $p65^+$ or $RelB^+$ cells were detected in isotype-stained cells (not shown). Original bars, 25 μm. (D) Representative Western blot for IDO and β-tubulin in MDM infected with 3.5 PFU/cell and treated with BMS-345541 (5 μM) or the vehicle (DMSO) for 24 or 48 h. (E) Kyn and (F) TNF concentration in the supernatant of MDM, infected and treated as in D for 48 h. Results are the mean ± SEM of three to four independent experiments. $***P \leq 0.001$ compared with the control.

IDO is not required by MDM to control flavivirus replication

To investigate the role of IDO for the control of WNV infection by MDM, IDO-mediated L-Trp depletion was nullified by supplementation of WNV-infected cultures with excess L-Trp (200 μM) or inhibition of IDO activity with the competitive substrate inhibitor 1-methyltryptophan (1-MT). Initial studies established that the L- but not the D-isomer of 1-MT inhibited IDO activity in IFN-γ-stimulated MDM, as indicated by the dose-dependent attenuation of L-Trp consumption (not shown) and Kyn production (**Fig. 5A**). As 1 mM L-1-MT caused significant cytotoxicity, 500 μM L-1-MT was used in subsequent experiments. Importantly, this nontoxic L-1-MT concentration prevented the depletion of L-Trp in the medium of WNV-infected cultures; i.e., the concentration of L-Trp in the medium before and after 72 h p.i. remained >30 μM (Fig. 5B). Supplementation with 200 μM L-Trp also ensured that ~160 and 30 μM L-Trp was present in culture at 48 and 72 h p.i., respectively, i.e., at time-points when viral replication was controlled by MDM.

Despite abrogating IDO-mediated L-Trp depletion, neither L-1-MT treatment nor L-Trp supplementation significantly altered viral RNA expression or replication in MDM, compared with WNV infection alone at 3.5 PFU/mL (Fig. 5C and D), indicating that IDO was not essential for the control of WNV infection in MDM. Similar results were obtained with 0.035 and 35 PFU/cell of WNV (Supplemental Fig. 2A and B). Antibody neutralization of IFN-α/β concurrently with 1-MT treatment also did not alter viral titers in the supernatant of infected MDM (Supplemental Fig. 2C), showing that even in the absence of IFN-α/β, WNV-induced IDO was not required for the control of virus by MDM.

To determine whether IDO up-regulation and the lack of effect of L-Trp starvation on viral replication were specific to WNV, MDM were also infected with another flavivirus, JEV. JEV infection also induced IDO activity in MDM in a TNF- and NF-κB-dependent manner, with kinetics comparable with that of WNV-infected cells (Fig. 5E and F). Furthermore, addition of excess L-Trp or L-1-MT did not significantly affect JEV replication in MDM (Fig. 5G).

The lack of a role for IDO-mediated L-Trp depletion for control of flavivirus replication in MDM likely reflects the delayed expression of the enzyme, when infection is already being controlled by other first-line, antiviral mechanisms. To investigate whether prior IDO

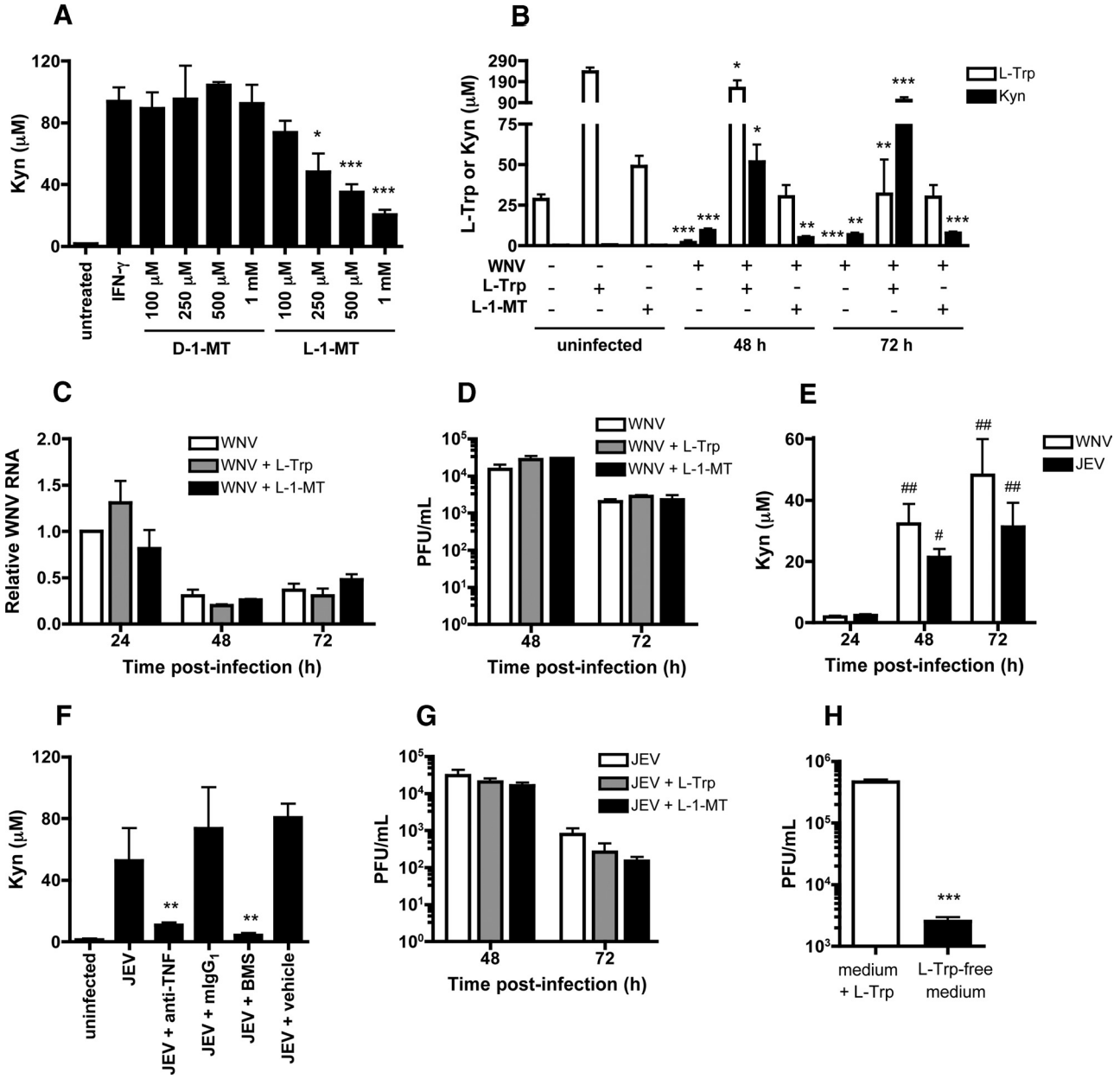


Figure 5. Effect of IDO activity on flavivirus replication in MDM. (A) Kyn in the culture medium of uninfected MDM or MDM stimulated with IFN-γ (500 U/mL) and treated with the indicated concentration of D- or L-1-MT for 24 h. Data represent the mean ± SEM of three independent experiments. (B) L-Trp and Kyn concentration in the supernatant of uninfected MDM or MDM infected with WNV at 3.5 PFU/cell for 48 or 72 h and left untreated, supplemented with 200 μM L-Trp or treated with 500 μM L-1-MT for the duration of infection. Data are the mean ± SEM of four to nine experiments. $*P \leq 0.05$; $**P \leq 0.01$; and $***P \leq 0.001$ compared with the respective controls. Note that the *y*-axis is discontinuous. (C) WNV RNA expression in MDM following WNV infection at 3.5 PFU/cell and exposed to 200 μM L-Trp or 500 μM L-1-MT for 24, 48, or 72 h. WNV RNA levels are expressed relative to the level apparent in cells infected with WNV alone for 24 h. Results are the mean ± SEM of triplicate determinations and are representative of five independent experiments. (D) Plaque assay for infectious WNV virions in the supernatant of MDM infected with WNV and treated as in C for 48 and 72 h. Results are the mean ± SEM of triplicate determinations and are representative of five independent experiments. (E) Kyn concentration in the supernatant of MDM infected with 3.5 PFU/cell of JEV or WNV for 24–72 h. Results are the mean ± SEM of triplicate determinations and are representative of two independent experiments. $^{\#}P \leq 0.05$; $^{\#\#}P \leq 0.01$ relative to data at 24 h p.i. (F) Kyn concentration in the supernatant of uninfected MDM, JEV-infected MDM, and JEV-infected MDM incubated with anti-TNF antibody (or the mouse IgG_1 isotype) or BMS-345541 (or the DMSO vehicle control) for 72 h. Data are shown as the mean ± SEM of $n = 3$ from one experiment. $**P \leq 0.01$ relative to control-treated cells. (G) Infectious JEV virions in the supernatant of MDM infected with JEV at 3.5 PFU/cell in medium supplemented with 200 μM L-Trp or 500 μM L-1-MT. Data are shown as the mean ± SEM of $n = 3$ from one experiment. (H) Infectious WNV virions in the supernatant of MDM infected with 3.5 PFU/cell of WNV for 24 h in the presence of complete medium supplemented with 200 μM L-Trp or in L-Trp-deficient medium. Data are expressed as the mean ± SEM of triplicate determinations and are representative of two independent experiments. $***P \leq 0.001$ relative to cells cultured in L-Trp supplemented medium.

activation and resultant L-Trp depletion could impact on WNV infectivity, MDM were stimulated with IFN-γ for 24 h before WNV infection. Whereas virus infection was suppressed significantly in IFN-γ-treated cells, neither excess L-Trp nor L-1-MT treatment affected the extent of viral inhibition by IFN-γ pre-treatment (Supplemental Fig. 2D). This supports that IFN-γ inhibits WNV infection independently of IDO. To more directly investigate the effect of early IDO-mediated L-Trp deprivation in the absence of IFN-γ-induced, antiviral mechanisms, MDM were cultured in L-Trp-deficient medium prior to and during WNV infection. Under L-Trp-deprived conditions, viral replication was attenuated significantly compared with MDM cultured in L-Trp-containing medium at 24 h p.i. (Fig. 5H), with no increase in cell death (not shown). Therefore, although WNV is sensitive to L-Trp depletion early during infection, supporting a role for IDO in preventing the establishment of infection, MDM evidently use antiviral mechanisms other than IDO and IFN-α/β to control WNV replication.

IDO expression prior to virus exposure inhibits WNV infection

To examine whether IDO is antiviral for WNV when expressed prior to infection, we transfected the human IDO gene into cells before virus infection to determine its impact on viral replication. As the transfection procedure itself abrogated WNV infection in MDM, to approach this question, we instead used the well-characterized, transfectable human epithelial HeLa cell line as an approximate representative of a barrier cell type, as epithelial cells are potentially relevant to initial infection in vivo. These cells afforded productive viral infection when infected at 0.1 PFU/cell with WNV (**Fig. 6A**). Moreover, in contrast to MDM, WNV infection alone (Fig. 6B and C) or together with 100 U/mL TNF (not shown) did not induce IDO in HeLa cells. However, in HeLa cells transfected with pcDNA3-IDO, but not the empty pcDNA3 vector, active IDO protein was expressed, with substantial depletion of L-Trp and accumulation of Kyn in the supernatant detected within 48 h after transfection (Fig. 6B and C). Furthermore, compared with cells transfected with the empty vector, WNV replication was reduced by ~100-fold in cells expressing active IDO at 48 h p.i. (Fig. 6D). This was reflected in the lack of WNV-NS1$^+$ staining in WNV-infected, IDO-expressing HeLa cells (Fig. 6E). Viral replication was restored to normal levels in IDO-expressing HeLa cells upon supplementation of the culture medium with 200 μM L-Trp (Fig. 6D), demonstrating that IDO-mediated L-Trp starvation was responsible for the antiviral activity of IDO in HeLa cells.

DISCUSSION

The present study shows that flavivirus infection of MDM stimulates significant expression of active IDO protein in a TNF- and NF-κB-dependent manner. Despite its robust induction, WNV-induced IDO was not required for MDM to inhibit viral replication, likely as a result of the delay in its expression. Indeed, culturing MDM in L-Trp-deficient medium to mimic early IDO induction or overexpression of IDO in human epithelial cells prior to virus infection both significantly inhibited viral replication via L-Trp starvation. Thus, although IDO-mediated L-Trp depletion is an effective antiflaviviral mechanism, this is likely to be dependent on the timing of IDO expression relative to viral infection.

Although dengue, a related flavivirus, up-regulates IDO mRNA in human DCs [7], this study is the first to demonstrate dose-dependent, flavivirus-induced expression of active IDO protein capable of depleting supraphysiological levels of L-Trp (i.e., >100 μM) from the culture medium by 72 h p.i. Of note, TNF was required for this robust induction of IDO, rather than the principal and most potent inducers of the enzyme, types I and II IFN. It should be noted that although we neutralized the most prominent type I IFNs (i.e., IFN-α2 and IFN-β), at least 12 IFN-α subtypes are known in humans [41]. Accordingly, a role for these different IFN-α subtypes in IDO expression cannot be excluded. Hence, more detailed studies are necessary to address this issue. Of interest, flavivirus-induced IDO induction occurred almost solely in uninfected, bystander MDM. As stimulation of MDM with TNF alone does not up-regulate IDO, this suggests that TNF acts in conjunction with other yet-to-be-defined factors to induce IDO. For example, TNF can synergize with IL-6 and IL-1β to induce IDO in a human monocyte cell line [37].

NF-κB is involved in the induction of IDO in response to stimuli other than IFN-γ [37, 42]. Indeed, in our study, the canonical and noncanonical NF-κB pathways are activated in WNV-infected MDM, and NF-κB signaling is a critical, intermediary mediator of WNV-induced IDO expression. Of the two pathways, recent reports support a role for the noncanonical pathway for the up-regulation of IDO in human and murine DCs [43, 44]. We tested the effects of the NEMO-binding domain peptide and also siRNA to target specific components of each NF-κB activation pathway on IDO expression in WNV-infected MDM, as described in these studies. However, in MDM, the NEMO-binding domain peptide caused substantial cytotoxicity, whereas siRNA transfection reagents alone abrogated WNV infection, confounding these avenues of investigation.

Notwithstanding, our study identifies, for the first time, the importance of TNF and NF-κB signaling for the induction of IDO by viral infection. Importantly, this is not restricted to WNV infection, as JEV also up-regulates IDO in MDM via TNF and NF-κB. It will be of interest to test whether viruses other than flaviviruses also induce IDO in macrophages via similar signaling pathways.

The role of IDO as an antiviral mechanism in vitro has, to date, only been investigated as an antiviral effector mechanism of IFN-β or IFN-γ [7, 13–18]. The current study shows that although induced within 48 h p.i. in a TNF-dependent manner, IDO is not required by MDM to effectively control the replication of established WNV or JEV infections. However, WNV replication was attenuated significantly in MDM cultured in L-Trp-deficient medium. Furthermore, up-regulation of IDO in HeLa cells prior to WNV infection also significantly inhibited WNV replication via L-Trp depletion. Therefore, the timing of IDO expression relative to viral infection evidently determines whether the antiviral properties of the enzyme are revealed. This suggests that although induced too late to help the control of virus in infected MDM, the delayed induction of IDO in uninfected cells may act to limit the spread of WNV by promoting local L-Trp depletion and thus, an antiviral milieu in uninfected cells surrounding the infective focus. As an antiviral mechanism operating through TNF, this may be a useful alternative to type I IFN, as many flaviviruses interfere with the potent antiviral actions of IFN-α and -β [45]. It should be noted that the lack of WNV-NS1 staining (i.e., an indi-

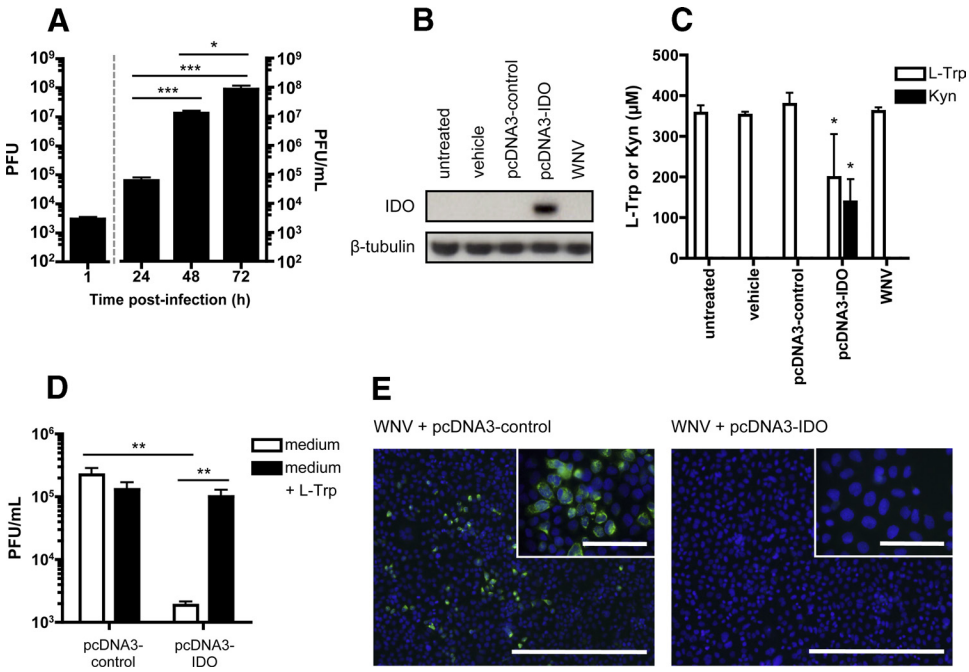


Figure 6. IDO expression in HeLa cells suppresses WNV infection. (A) Infectious WNV virions in the supernatant of HeLa cells infected with WNV at 0.1 PFU/cell for 24, 48, or 72 h, expressed as PFU/mL (right *y*-axis). Data at each time-point are cumulative. The 1-h time-point represents the quantity of virus (PFU; left *y*-axis) on the cells following 1 h of viral adsorption and vigorous washing. Data are shown as the mean ± SEM of triplicate determinations and are representative of two experiments. (B) Representative Western blot for IDO and β-tubulin in HeLa cells transfected with pcDNA3, encoding the human IDO gene or an empty control vector; the Lipofectamine 2000 vehicle control; or infected with 0.1 PFU/cell of WNV for 48 h. (C) Corresponding L-Trp and Kyn concentration in the supernatant of cells treated as in B. Data are the mean ± SEM from three independent experiments. (D) Infectious WNV virions in the supernatant of HeLa cells that were transfected with the empty vector or pcDNA3-IDO for 48 h prior to infection with 0.1 PFU/cell of WNV and culturing for an additional 48 h in the presence of complete medium or complete medium supplemented with 200 μM L-Trp. Data are the mean ± SEM of triplicate determinations and are representative of three independent experiments. (E) Immunofluorescent staining for WNV-NS1 (green, FITC) and nuclei (blue, DAPI) in HeLa cells transfected with the pcDNA3-control or pcDNA3-IDO vector for 48 h and infected with 0.1 PFU/cell of WNV for an additional 48 h. No positive WNV-NS1 staining was detected in uninfected HeLa cells or isotype-stained cells (not shown). Original bars, 500 μm; original inset bars, 100 μm. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

cator of replicating virus) in IDO$^+$ MDM does not exclude the possibility that the virus enters the cell but is prevented from replicating as a result of insufficient available L-Trp.

Also of note was the relative absence of IDO in WNV-NS1$^+$ cells, suggesting that replicating virus itself may inhibit the up-regulation of IDO in infected cells. This blockade of IDO up-regulation may explain why induction of IDO only inhibits the establishment of viral infection rather than control of virus in already infected cells.

IDO expression can potently inhibit the immune response [46, 47]. It is therefore plausible that active IDO, expressed in MDM after virus is controlled by other first line innate mechanisms, acts to suppress a potentially damaging cellular immune response. Alternatively, IDO induction may represent a mechanism by which WNV evades the host immune response. For example, IDO up-regulation in HIV-exposed plasmacytoid DCs prevents conventional DC maturation [19], whereas in a mouse model of HIV-induced encephalitis, IDO inhibits the trafficking of HIV-specific CD8$^+$ T cells to the brain to resolve infection [20].

Taken together, the findings of this study indicate that IDO induction is a major macrophage response to flavivirus infection, and depending on the timing of its expression relative to initial infection, IDO may be strongly antiviral. However, the implications of IDO induction in flavivirus-infected individuals are likely to be complex, given the significant immunopathology accompanying infection. Ongoing research will determine the extent to which IDO is antiviral or is used by flaviviruses to subvert antiviral immune responses in vivo.

AUTHORSHIP

A.W.S.Y. designed and performed experiments, analyzed results, and wrote the manuscript. W.W. and M.F. performed experiments. R.S. was involved in early experimental design and discussions and provided intellectual input. N.J.C.K. and S.R.T. conceptualized the project, performed preliminary experiments, designed the research, discussed the results, and wrote the manuscript.

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West Nile virus · Japanese encephalitis virus · tryptophan · kynurenine