

Respiratory Syncytial Virus Induces Indoleamine 2,3-Dioxygenase Activity: A Potential Novel Role in the Development of Allergic Disease

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Running title: RSV induces IDO activity.

Abbreviations: IDO, indoleamine 2,3-dioxygenase; moDC, monocyte derived dendritic cells; MOI, multiplicity of infection; PRRs, pattern recognition receptors; RIG-I, retinoic acid-inducible gene-I; RSV, respiratory syncytial virus; rgRSV, recombinant green fluorescent protein-expressing RSV.

Keywords: Human respiratory syncytial virus, tryptophan, Indoleamine 2,3-Dioxygenase, kynurenine, allergy, asthma, airway hyperresponsiveness, viral bronchiolitis.

Abstract

Background: Infants that develop severe bronchiolitis due to Respiratory Syncytial Virus (RSV) are at increased risk of developing asthma later in life. We investigated a potential immunological mechanism for the association between RSV and the development of allergic inflammation. The enzyme indoleamine 2,3-dioxygenase (IDO) has been reported to induce selective apoptosis of T helper 1 (Th1) cells, and contribute to Th2-biased immune responses. Objective: To determine whether RSV infection *in vitro* could induce IDO expression and bioactivity in human dendritic cells, leading to a Th2-biased immune response. **Methods:** Human peripheral blood monocytes from healthy adult donors were isolated, differentiated to dendritic cells (moDC), *in vitro*. We studied RSV infection and mechanisms of IDO activation in moDC with subsequent effect on T-bet expression. **Results:** We found that moDC were infected

by RSV and that this induced IDO activation. RSV-induced IDO activity was inhibited by Palivizumab, UV-inactivation, TL4 inhibition, and Ribavarin. However, blocking endosomal TLR function with chloroquine did not inhibit IDO activity. Selective inhibitors suggested that RSV-induced IDO activity was dependent on the retinoic acid inducible gene-I-(RIG-I) related pathway via NF- κ B and p38 MAPK. Co-culture of RSV-infected moDC with activated T-cells, in a transwell system, suppressed expression of T-bet (a Th1-associated factor) but not GATA3 (a Th2 regulator). Inhibition of IDO activity with the competitive inhibitor, 1-methyl tryptophan, blocked the effect on T-bet expression. **Conclusion and Clinical Relevance:** Our data show for the first time that RSV can induce the expression and bioactivity of IDO in human moDC, in a virus replication-dependant fashion. We suggest that RSV activation of IDO could be a potential mechanism for the development of allergic diseases.

Introduction

Respiratory Syncytial Virus (RSV) is one of the leading causes of lower respiratory tract (LRT) disease during infancy and early childhood. Despite high levels of maternally transferred specific serum antibodies [1], 50% of infants acquire RSV infection in the first year of life and all children by age three. RSV usually manifests as mild cold-like symptoms in young children and adults, but in 30-40% of infants it can present with a more severe breathing disorder called bronchiolitis [2, 3]. Severe RSV bronchiolitis in infancy is considered a risk factor for the development of allergic asthma in childhood lasting into adulthood [4-8].

To determine potential mechanisms involved in this link between RSV and the development of allergic asthma, we investigated role of the enzyme indoleamine 2,3-dioxygenase (IDO) in promoting an allergic-type, Th2-biased immune responses. IDO is the rate-limiting enzyme in the extrahepatic oxidative catabolism of tryptophan [9], one of the least available essential amino acids. IDO is expressed in many extra-hepatic organs, with a major presence in the placenta and lungs [10]. IDO activation both *in vivo* and *in vitro* has been reported to modulate the immune response, including Th1-Th2 imbalance in favor of a Th2-like cytokine profile [11, 12]. IDO is associated with apoptosis of proliferating T cells, especially in Th1 compared with Th2 cells [11, 12]. The reason why Th1 cells, but not Th2 cells are selectively targeted is currently under investigation. This immune modulation can be the result of either tryptophan depletion itself or production of the tryptophan catabolites, kynurenines, within this milieu, or both [13].

Human dendritic cells (DC) are critical in antigen presentation [14]. While DC can be induced to express IDO activity [15-17], this has not been reported as a direct consequence of RSV. It is conceivable that an increase in IDO activity in DC during RSV infection could cause a skewed immune response toward Th2. If this IDO activity occurs during a window of opportunity in infancy, when the infant's immune system is developing the capacity to respond to antigens/allergens, IDO may form the basis for the Th2 polarization described in infants that develop allergic asthma later in life.

Human dendritic cells (DC) are important players in viral immunity [14]. Classically, induction of IDO in monocyte-derived dendritic cells (moDCs) occurs following stimulation by interferons, especially IFN- γ [18]. We hypothesized that RSV would infect moDC and induce

IDO expression and bioactivity, *in vitro*, which would then contribute to a Th2-type response in co-cultured human T cells. For the first time, we demonstrate that RSV infection induces IDO activity in human moDC. This activation appears to be RSV-replication dependent, but IFN- γ -independent. RSV induction of IDO suppressed Th1-expression in activated human T cells, *in vitro*.

Methods

Culture media and reagents: Dendritic cells were cultured in RPMI-1640 (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 2 mM l-glutamine, 10% FBS, 100 U/L penicillin, and 100 μ g/L streptomycin (complete medium). For culture of the THP-1 monocytic cell line (TIB-202TM-ATCC[®]), complete medium was supplemented with 4.5 g/L glucose, 1 mM sodium pyruvate and 0.05 mM 2-mercaptoethanol. For culture of HEp-2 epithelial cell line (CCL-23TM-ATCC[®]), RPMI-1640 in complete medium was replaced with DMEM (Sigma-Aldrich, Oakville, Ontario, Canada) and supplemented with 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. In experiments with virus, media were supplemented with 2% FBS in place of 10% for the first two hours of the incubation time. Recombinant human IL-4, IFN- γ , IFN- α , TNF and GM-CSF were purchased from R&D Systems (Minneapolis, MN). Ligands of TLR3 and TLR7/8 (poly I:C and R848 respectively) and antagonist of TLR4 receptor, LPS-RS, were purchased from Invivogen (San Diego, CA). Anti-human IFN- γ Ab (Cat. No. AF-285-NA) and monoclonal anti-human TNF (Cat. No. MAB2101) were purchased from R&D Systems (Minneapolis, MN). Mouse anti-human Indoleamine 2,3-dioxygenase monoclonal Ab (clone 10.1, Cat. No. MAB5412) was purchased from Millipore (Billerica, MA) and mouse monoclonal anti-human IFN- α (Cat. No. 21105-1) was purchased from PBL interferonsource (Piscataway, NJ). *L-*

tryptophan (Cat. No. T8941), 1-Methyl-*DL*-tryptophan (1MT)(Cat. No. 860646), Menadione (Cat. No. M5625), Ribavirin (Cat. No. R9644), Chloroquine diphosphate (Cat. No. C6628), 4-(Dimethylamino) benzaldehyde (Ehrlich reagent)(Cat. No. D2004) and *L*-Kynurenine (Cat. No. K8625) were obtained from Sigma-Aldrich. Stock solution of menadione (100mM) was prepared in DMSO and kept at -20°C and in the dark. Stock solution of *L*-Kynurenine (50mM) was prepared in 0.5M HCL and kept in dark. Palivizumab (Synagis®) was provided by MedImmune (Gaithersburg, MD). WST-1 (Cell Proliferation/cytotoxicity reagent) was purchased from Roche Applied Science (Laval, Quebec, Canada).

Cell lines: THP-1 and/or HEp-2 cells were used as a positive control for IDO induction in some experiments. We also used HEp-2 for viral titration (plaque assay), preparation of RSV stock and as a prototype of epithelial cells in the inducibility of IDO by RSV in such cells (see below).

Preparation of primary human DC: Peripheral blood was collected into heparinized tubes by venipuncture from healthy adult donors. All subjects gave written informed consent approved by the University of Alberta, Health Research Ethics Board. After sedimentation of red blood cells in 6% dextran for 30 min at RT, the leukocyte-rich supernatant was collected and subjected to density gradient centrifugation on Ficoll-Paque™ PLUS (Cat. 17-1440-02, GE healthcare, Quebec, Canada). The buffy coat layer containing mononuclear cells was collected on a Ficoll gradient, washed twice and incubated in 6 well plates (about 10^7 cells/well in RPMI-10%FBS) for two hours at 37°C and 5% CO₂. Non-adherent cells were collected and, in some experiments, were enriched using a Human T Cell Enrichment Kit (Cat. No. 19051, Stemcell Technologies, Vancouver, BC, Canada). Remaining adherent cells in 6-well plates from the previous step were

washed vigorously with RPMI and incubated for 6 days (37°C, 5% CO₂) with GM-CSF (20ng/ml) and IL-4 (12.5 ng/ml) [19]. This regimen differentiated monocytes into myeloid dendritic cells (moDC) with a purity of >95% confirmed by flow cytometry [20].

Preparation of RSV stock and measurement of RSV titre: Briefly, monolayers of mycoplasma-free HEp-2 cell were grown up to 60-70% confluence in T-225 flasks. Cells were infected with either human Respiratory Syncytial Virus (hRSV)(strain A2, ATCC VR-1540) or a GFP-expressing Respiratory Syncytial Virus (rgRSV) [21] (a generous gift from Drs. Mark E. Peebles and Peter L. Collins, also based on strain A2 of RSV) each with MOI of 0.1 for 1 hour incubation at RT and 1 hour at incubator (37°C and 5% CO₂). After inoculation, cells were nourished with fresh complete media (FBS 10%). After 24 hrs, media were changed and infected HEp-2 cells were grown in medium containing 2% FBS. RSV cultures were harvested when more than 60-70% of the HEp-2 monolayer showed signs of cytopathy and syncytia formation. After one step concentration with polyethylene glycol, the harvested material was subjected to two steps of sucrose discontinuous gradient ultracentrifugation. The opaque band formed between 37% and 47% of sucrose (w/w) were collected and kept frozen at -80°C for future use in experiments.

Quantification of RSV by plaque assay: HEp-2 cells were seeded in 24-well plates (80,000 cells per well, 37°C, 5% CO₂) the day before, resulting in the desired 70% confluent monolayer for the day of experiment. Serial dilutions of samples were prepared in DMEM with 2% FBS and distributed in triplicate in wells with 70% confluent monolayer. Inoculated cells were covered with complete DMEM containing 2% Carboxy Methyl Cellulose. After 24 to 48 hrs (37°C, 5%

CO₂), the presence of bright green spots were counted by fluorescent microscopy; each spot representing one Plaque Forming Unit (PFU). In the case of non fluorescent RSV (hRSV), plates were kept for up to seven days (37°C, 5% CO₂) allowing the typical syncytial plaques to mature. Cells were fixed (1% Gluteraldehyde, 1 hour at RT). Monolayers were stained with crystal violet (0.0375% w/v in water, 5 minutes). Syncytial plaques (seen as clearings without violet staining) were counted using 5× magnification.

Infection of moDC with RSV: rgRSV or hRSV (MOI 0.1-20) were added to moDC in RPMI containing 2% FBS (1 hour at RT on a slow rocker; then 1 hour at 37°C, 5% CO₂). For some experiments evaluating productivity of infection, moDCs were washed twice following incubation with rgRSV. In addition, media from uninfected Hep-2 cells was used as a sham control for some experiments. Complete medium with 10% FBS was then added to reach the final concentration of 10⁶ cells/ml. Following various measurements of different multiplicity of infection (MOI)(0.1-20) we determined the optimized condition of the experiments in terms of infection, IDO induction, and viability of cells in culture. MOI of 1 was chosen. RSV infectivity (confirmed by plaque assay) was blocked with UV-inactivation (UV light for 20 min on ice, 8-watt lamp, located 5 cm above the tube containing the virus), with Palivizumab (a humanized monoclonal Ab directed against an epitope in the F protein of RSV; 200 ug for 10⁶ PFU of RSV, 15 min on ice), or with Ribavirin (a competitive inhibitor of viral RNA polymerase, 220 uM).

Measurement of IDO bioactivity: To measure IDO bioactivity, supplemental *L*-tryptophan was added to the medium to a final concentration of 150 µM (conditioned medium). Culture supernatants of moDCs were collected five days post infection (stored at -20 °C). Five days was

chosen based on data that IFN- γ induces IDO expression in fibroblasts up to 192 hrs [22]. Supernatants were de-proteinized with the same volume of 30% trichloroacetic acid in ddH₂O (w/v). Samples were vortexed and centrifuged at 14,000 G for 10 min at 4°C. Samples were thawed and the kynurenine component of the supernatant was measured by a spectrophotometric method using the Ehrlich reagent (absorption of 490 nM). Kynurenine concentration values were extrapolated from a standard curve using commercial L-kynurenine [23]. All experiments were carried out in duplicates or triplicates.

Flowcytometry: To determine infection with rgRSV, GFP expression was measured in moDC, or HEp-2 cells after fixation with 4% paraformaldehyde on ice for 10 minutes. Cells were washed with cold PBS and resuspended in flow buffer. For cells infected by hRSV, fixed cells were incubated with mouse anti-RSV Fusion protein (Cat. No. MAB8599, Millipore, Billerica, MA, USA). To evaluate intracellular expression of IDO, cells were fixed with 2% paraformaldehyde and permeabilized using 0.5% saponin in flow buffer. Mouse IgG3 isotype control (R&D, MAB007) and mouse anti-human IDO (Chemicon, MAB5412) were used as primary antibodies. APC-labeled Goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-135-209) was used as secondary Ab (5ug/ml). Samples were read on BD FACSCalibur™ flow cytometer machine. Intact moDC were gated and distinguished from debris and impurities based on size and granularity characteristics, as measured by forward scatter and side scatter, respectively. Results were analysed using WinMDI (V2.8).

Assessment of infection by live-cell confocal microscopy: We observed moDCs alone (i.e., without rgRSV) or moDCs incubated with rgRSV for morphology (differential interference contrast-DIC images) and expression of GFP (excitation and emission wavelengths: 488nm and 520nm, respectively) using a laser-scanning microscope (Olympus FLUOVIEW FV1000 Confocal Microscope and FV10-ASW viewer V1.3b, Olympus, Markham, Ontario, Canada).

Cytokine measurement: Cell supernatants collected one day post infection were kept frozen at -80°C for subsequent measurement of TNF, IFN- γ , IFN- α , CCL-5 (RANTES), IL-12p70, IL-4 and IL-13 levels using a commercially available “Searchlight® Protein Array Analysis” service provided by Pierce Biotechnology (Woburn, MA).

Inhibitors of IDO: 1-Methyl-DL-tryptophan (1MT) is an analogue of tryptophan and a competitive inhibitor of IDO [24]. Stock solutions (3.2 mM) of 1-MT were prepared in RPMI (the maximum level of solubility for 1-MT in water is 6 mM [24]). Menadione (2-methyl-1,4-naphthoquinone otherwise known as Vitamin K₃) is an alternate inhibitor of IDO with a low micromolar potency (with *in vitro* IC₅₀ and LD₅₀ values of 28.9 and 126 μ M, respectively) [25, 26].

Cell signaling pathways in RSV-induced IDO activation: To evaluate the mechanism of RSV-induced IDO activation, we studied the potential intracellular signaling pathways reported for IDO activation. We used a variety of specific inhibitors from Calbiochem (a Brand of EMD Chemicals Inc., an affiliate of Merck KGaA, Darmstadt, Germany) including MEK inhibitor I (IC₅₀=12nM, Cat. No. 444937), MEK inhibitor II (IC₅₀=380nM, Cat. No. 444938), a potent cell-

permeable inhibitor of p38 MAP kinase, which has no effect on the activity of the ERK or JNK-MAP kinase subgroups (SB202190, IC_{50} =350nM; Cat. No. 559397), JNK inhibitor II (SP600125 [27], IC_{50} =40~90nM; Cat. No. 420128), an inhibitor of IKK β (IKK inhibitor III, BMS-345541, IC_{50} =300nM) and appropriate negative controls. To investigate the role of TLR-4 signaling we used an anti-TLR-4 monoclonal antibody (Cat. No. MCA2061EL, Serotec, NC, USA) and an antagonist of TLR-4 receptors, rsLPS (Cat. tlr1-rslp, Invivogen, San Diego, California). To study RIG-I-like receptors we used an inhibitor of TBK1/IKK ϵ , which are downstream signaling kinases in RIG-I/MDA5 induction (BX795; Invivogen, Cat. tlr1-bx7). All inhibitors and vehicles were measured in a dose-response manner for their cytotoxicity using WST-1 cell cytotoxicity reagent. Working concentrations of the inhibitors were kept well below cytotoxic values.

Transwell assays to measure T cell responses: To determine if RSV can alter T cell responses, we employed a transwell system. A purified population (>95%) of T cells was prepared using the EasySep® Human T Cell Enrichment Kit (Cat. No. Vancouver, BC, Canada). T cells were initially activated in a separate plate using anti-CD2/CD3/CD28-loaded microbeads (T Cell Activation/Expansion kit; Cat. No. 130-091-441, Miltenyi Biotech, Auburn, CA). On day 6, some moDCs were infected with rgRSV in the lower chamber of a 12-well transwell plate (10^6 cells/1.2ml) with or without 1MT. After 24 hrs, 8×10^5 of the previously activated T cells were placed in inserts (membrane pore size 0.4 μ m; Cat. No. 3460, Corning Incorporated, Corning, NY); the inserts were placed in wells with moDC from different experimental pretreatments. The IDO inhibitor, 1MT, was added (final concentration of 320 μ M) to relevant wells. In some experiments, 8×10^5 of the T cells along with 4×10^5 of anti-CD2/CD3/CD28-

loaded microbeads were placed in inserts with or without addition of 1MT (320 μ M). After four days, the T cells in inserts were collected. We stained 10^6 cells per condition for CD3 (Cat. No. 17-4724-42, ebiosciences), permeabilized for 18-24 hrs in a fixation and permeabilizing buffer (Cat. No. 00-5521-00, ebiosciences) prior to staining to determine the expression of the intranuclear transcription factors, T-bet and GATA3 (Cat. No. 561266 and cat. No. 560077 respectively, BD Biosciences, Mississauga, ON, Canada). Corresponding isotype controls were used according to the manufacture's inter-chromatin (IC) staining protocol originally optimized for the Foxp3 transcription factor. T cells were then assessed by flow cytometry on a FACSCalibur machine. Results were analyzed using WinMDI (V2.8) for the detection of transcription factor expression.

Statistical analysis: Both GraphPad prism[®] (V.4) and Microsoft Office Excel[®] (2003) were used for statistical analysis. Paired *t*-test was used to compare two group experiments. Repeated Measures ANOVA with Bonferroni's Multiple Comparison Test were employed to compare multiple groups. Data are presented as mean $\pm$ 1 SEM, unless otherwise specified. A *p* value of >0.05 was considered non-significant.

Results

Respiratory Syncytial Virus infects moDC: Primary human moDC were incubated with rgRSV (MOI 0.1 to 20). Confocal microscopy and flow cytometry of moDC incubated with rgRSV showed that GFP is expressed in the cells, confirming infection and virus replication (figure 1A and 1B respectively). Incubation of moDC with viruses that were pre-treated with Palivizumab or UV light did not show infection in Hep-2 cells (figure 1C). Infection with hRSV was also

confirmed using flowcytometry (see efigure 1, online). To evaluate the productivity of the infection, moDCs were washed twice following incubation with rgRSV. The supernatants were collected (days 1, 2 and 3) for RSV titration by the plaque assay to investigate presence of progeny viruses. HEp-2 cells treated with the supernatant of infected moDC became infected even at the first time point. The level of infection did not rise over days 2-3 (figure 1D).

RSV Infection induces IDO activity in moDCs: Five days following infection of moDC with rgRSV, cell supernatants were collected for evaluation of IDO activation by measuring kynurenine production. IFN- γ was used as a positive control (1000 IU/ml). Compared to uninfected moDC, incubation of moDC with rgRSV (MOI ≥ 0.1) induced IDO activity to levels comparable to IFN- γ (figure 2A, $P < 0.001$). Both Palivizumab-treatment and UV-inactivation of rgRSV (MOI=1) blocked kynurenine release compared with untreated rgRSV (figure 2B, $P < 0.01$ for both conditions). Infection of moDCs with hRSV (MOI 0.1-5) generated comparable values of kynurenines in supernatant (data not shown). Considering the importance of TLR4 in the innate immune response to RSV [28-30], we pretreated moDC with rsLPS or anti-TLR-4 before RSV infection. While pretreatment of rgRSV-infected moDC with anti-TLR-4 (10 μ g/ml, n=3) and rsLPS (10 μ g/ml, n=2) inhibited RSV-induced kynurenine release by over 42 and 59 percent respectively, the inhibition was likely due to blockade of infection. The fluorescence of treated cells was greatly inhibited compared to untreated moDC. This visual impression was confirmed by flow cytometry where rsLPS-treated moDC showed reduced expression of RSV GFP in comparison to untreated moDC (n=1, MFI=5.1 vs. 11.3). Thus, we suggest that the inhibitory role of TLR-4 on RSV-induced kynurenine release is likely secondary to blockade of RSV entry and infection of moDC.

To confirm the IDO specificity of the kynurenine release, we used the IDO inhibitors, 1MT (800 μ M) and Menadione (10 μ M). Both inhibitors suppressed kynurenine release in response to RSV (figure 2C and 2D). To further confirm that RSV induces IDO expression, we used flow cytometry to evaluate intracellular IDO expression. Infected cells showed IDO expression compared to isotype control (figure 2E).

We also determined whether IDO activity could be induced in an epithelial (HEp-2) and monocyte cell line (THP-1). Infection was confirmed with flow cytometry (data not shown). While IFN- γ induced kynurinine release in HEp-2 (140 $\pm$ 6 μ M), neither rgRSV nor sham treatment showed significant release (28.5 $\pm$ 11.8 and 10.8 $\pm$ 2.7 respectively, n=3)(eFigure 2A).

Similarly, in THP-1 cells, IFN- γ induced kynurenine release (94.98 $\pm$ 16.54, n=4) while neither rgRSV (even at a high MOI of 10) nor sham treatment had an effect (4.46 $\pm$ 0.03 and 4.38 $\pm$ 0.01, respectively; n=4)(eFigure 2B).

Induction of IDO requires viral replication: To investigate the role of viral replication in IDO activation, we used Ribavirin, a nucleoside antimetabolite that blocks RSV nucleic acid synthesis through the inhibition of the enzyme “RNA dependent RNA polymerase”. Ribavirin (>220 μ M) suppressed kynurenine release (72.1 $\pm$ 9.1 vs. 41.6 $\pm$ 11.2, figure 3A), but did not inhibit IFN- γ -induced kynurenine release compared to IFN- γ alone (78.7 $\pm$ 12 vs. 72.3 $\pm$ 15, respectively; n=3). This suggested that the Ribavirin effect on IDO was not due to moDC toxicity. Further, this dose of Ribavirin did not appear to have cytotoxic effects on moDC as their ability to metabolize WST-1 was unaffected (data not shown, n=3). We also incubated moDC with higher doses of UV-inactivated rgRSV to investigate whether loading moDC culture with more RSV antigenic

proteins initiated the IDO induction. Adding 20 times more UV-inactivated rgRSV in comparison to live rgRSV did not induce IDO, further confirming that RSV antigenic epitopes had no role in IDO activation (figure 3B; n=3).

The role of cytokines or chemokines in IDO induction: It is possible that RSV replication induces a factor from the moDC, which may act in an autocrine fashion to induce IDO activation. IFN- γ is one of the most potent inducers of IDO [31]. IFN- α is also able to induce IDO but modestly [32]. There was a small but not statistically significant increase in the presence of IFN- α and IFN- γ in the supernatant of RSV treated moDC compared to uninfected controls (2.00 ± 0.73 vs. 14.10 ± 4.94 pg/ml and 2.52 ± 0.39 vs. 13.15 ± 3.67 pg/ml, respectively; n=5). In keeping with these data, neutralizing monoclonal antibodies against human IFN- α and IFN- γ did not block RSV-induced IDO activation (n=6). Anti-IFN- γ (10 μ g/ml) inhibited the positive control of exogenous IFN- γ (eFigure 3; n=4). In contrast, TNF release was seen in rgRSV infected moDC compared to controls (194 ± 58 vs. 9.9 ± 2.0 pg/ml, $P < 0.05$; n=7). While the addition of TNF to moDC cultures did not induce kynurenine release (even at high doses up to 200 ng/ml; n=3; figure 4A), TNF appeared to augment RSV-induced IDO activity (n=6; $P < 0.05$). Further, anti-TNF treated moDC (1 or 10 μ g/ml) produced lower concentrations of kynurenines in response to rgRSV compared to untreated moDC (15.3 ± 1.9 vs. 89.6 ± 8.9 μ M; $P < 0.01$) or matching isotype controls (figure 4B; n=7).

We did measure release of other cytokines and chemokines (CCL5/ RANTES, IL12p17, IL4, IL13 and IL10) in the rgRSV infected moDC. Only CCL-5 showed significant release compared to uninfected supernatants (87 ± 38 vs. 7074 ± 2426 , respectively; n=8; $p < 0.05$).

RSV-induced IDO activation appears to be independent of intracellular TLR3: Since virus replication appeared essential in IDO induction, we determined the potential intracellular sites where this stimulation might occur. During RSV replication, double stranded RNA is formed inside the host cell. TLR3 is an intracellular endosomal receptor for double-stranded RNA. Incubation of human moDC with the ligand for TLR3, poly I:C (10 µg/ml), induced kynurenine release (figure 5). Chloroquine accumulates in the endosomes where TLR3 is located and blocks TLR3 function [33-35]. While chloroquine treatment of moDC (5 µM) inhibited poly I:C-induced kynurenine release, it did not inhibit kynurenine release from IFN-γ-treated or rgRSV-infected moDC (figure 5, n=5).

Potential cytoplasmic signaling pathways involved in RSV-induced IDO activity:

To better elucidate the mechanism of IDO induction by RSV, we studied potential intracellular pathways known for their role in RSV infection or IDO activation. RIG-I is a cytoplasmic receptor for single stranded RNA [36], which would make it a potential receptor for RSV replicating intracellularly. RIG-I signals through IKKε/TBK1, which can be inhibited by BX795 [37]. Addition of BX795 (10 µM), to cultures of rgRSV-infected moDCs inhibited kynurenine release compared to untreated rgRSV infected moDC (24.0± 2.7 vs. 50.4 ±6.8, respectively; figure 6).

NFκB and its associated pathways are required for IDO activation [38]. To determine if RSV infection is signaling through this key pathway, we used BMS-345541, an inhibitor of IKKβ. IKKβ is a kinase in the canonical pathway of NFκB activation. Addition of BMS-345541 to

cultures of rgRSV-infected moDCs inhibited kynurenine release compared to untreated rgRSV infected moDC (40.4 ± 8.8 vs. 73.9 ± 11.3 , respectively, $n=8$; figure 7).

p38-MAPK is important for IDO activation. SB202190, the inhibitor of p38-MAPK, (300nM and 3 μ M) also blocked kynurenine release (54.7 ± 8.7 and 15.5 ± 1.5 , respectively) when compared with untreated rgRSV-infected moDC (103.3 ± 10.3).

Other potential signaling pathways that we considered for their role in IDO activation included JNK-MAPK (SP600125, 1 μ M) and MEK/ERK-MAPK (MEK inhibitor I, 120 nM; MEK inhibitor II, 4 μ M). These did not appear to be involved in rgRSV-mediated induction of IDO as no inhibition was observed (efigure 4). A negative control for each inhibitor was either a specific control provided by the manufacturer or based on the concentration of diluent used for that inhibitor. The signaling pathways studied in our experiments are shown as a schematic diagram (Figure 8).

Co-culture of rgRSV-infected moDC with activated T cells suppresses T-bet expression through an IDO-dependent mechanism: We determined whether RSV infection of moDC can influence the bias of the immune response towards favoring a Th2 response via IDO. We measured the changes in T cell expression of two important transcription factors in Th1 and Th2 immune responses, T-bet and GATA3, respectively. We separated moDC and T cells using a transwell system. To simulate a T cell immune response, T cells were first stimulated by anti CD2/CD3/CD28 beads. They were then added to the inserts in the presence or absence of infected moDC in the lower compartment of the wells. Expression of T-bet and GATA3 were

assessed by flow cytometry in CD3+ cells. In contrast to uninfected controls, rgRSV infection of moDC diminished T-bet expression ($12.3\% \pm 2.9$ vs. $4.8\% \pm 1.8$, $p < 0.05$). This effect was not seen in the presence of IDO inhibitor, 1MT. Expression of GATA3 was not affected by the infection (Figure 9).

Discussion

Epidemiological studies have suggested a strong association between RSV bronchiolitis during early infancy and the later development of allergic asthma [4, 5]. There has been extensive work looking at the immune response to RSV. Reports show that various strains of RSV can induce different response in host with some inducing more allergic-like response *in vivo* [39, 40]. We and others have hypothesized that RSV infection could be a factor altering a child's future response to allergen exposure [41-43]. It is important to recognize that this illness is occurring at a unique time point when a child's immune response to the environment is still developing. In mouse models, allergen exposure and early-life viral infection can interact to induce a Th2-phenotype profile in adult mice [42]. The mechanisms regulating these synergistic interactions remain poorly understood [44]. IDO has the capacity to contribute to Th2-biased immune responses [11, 12, 45, 46]. In this report, we show for the first time that RSV infection can induce IDO activation in human moDC, *in vitro*. We also determined that co-culture of RSV-infected moDC with stimulated T cells could inhibit Tbet-expression.

To determine the mechanisms involved, we first studied moDC infection by RSV. We confirmed that RSV infection (and replication) occurred in moDC and was required for IDO activation. Both UV-inactivation and Palivizumab blocked infection and IDO bioactivity. Blocking RSV

nucleic acid synthesis with Ribavarin suppressed IDO activation, while Ribavirin had no effect on IFN- γ -mediated IDO induction. Thus, we concluded that both active infection and viral replication are required for RSV-induced IDO bioactivity.

The F-protein of RSV interacts and signals through TLR4/CD14 in human monocytes and is important for RSV infection [28-30]. LPS also binds to the CD14/TLR4/MD2 receptor complex and induces IDO activation independent of IFN- γ [47-49] in moDC and macrophages [50, 51]. We blocked TLR4 using rsLPS and anti-TLR4 blocking antibodies, respectively. While rsLPS and anti-TLR4 antibodies inhibited RSV-induced IDO activation, moDC incubated with these inhibitors also showed much lower RSV infection. In addition, we used high concentrations of UV-inactivated RSV, which also did not induce IDO activity. Overall, we feel that the inhibition of IDO activation was due to blockade of RSV infection rather than blockade of a TLR4-based stimulation pathway. .

Within the cell, there are pattern recognition receptors (PRRs), which recognize molecular patterns associated with microbial pathogens. RSV infection is reported to induce phosphorylation and translocation of NF- κ B to the nucleus [52, 53], the critical signaling pathway for IDO activation. The mechanism of this induction remains unclear. For the purpose of this study, we focused on the role of intracellular PRRs previously reported to play a role in RSV immunity. RSV replication produces dsRNA, recognized in turn by TLR3 in lysosomes. TLR3 stimulation is reported to induce NF- κ B activity [54] and IDO activation [38]. Similar to a previous report, we observed that the ligand for TLR3, poly I:C, could induce release of kynurenine. We used the lysosomotropic agent, chloroquine, to block the function of lysosomal

TLRs, including TLR3 [35]. The inability of chloroquine to inhibit RSV-induced IDO activity was somewhat surprising suggesting that RSV may not utilize TLR3 to induce IDO activity. We excluded TLR7 or TLR8 in our model as a previous report had already demonstrated that they lacked the ability to induce IDO in DCs [55]. Nonetheless, the lack of inhibition by chloroquine may also indicate the improbability of a role for endosomal TLR7 and TLR8.

RIG-I is another intracellular PRRs involved in RSV pathogenesis [56, 57] that recognizes dsRNA in the cytosol of infected cells [36, 58, 59]. This recognition signals activation of the IKK-related protein kinases, IKK ϵ and TBK1, leading to the phosphorylation of interferon regulatory factors 3 and 7 (IRF3/7) [60]. Ultimately, these IRFs upregulate the transcription of IFN α and β and other IFN-stimulated genes [60]. BX795 is an inhibitor of the catalytic activity of TBK1/IKK ϵ [37, 61]. Incubation of rgRSV-infected moDC with the inhibitor of TBK1/IKK ϵ suppressed IDO activity, suggesting a potential role for RIG-I in cytoplasmic detection of RSV-derived nucleic acid. It further suggests that the RIG-I receptor and TBK1/IKK ϵ may play a role in IDO induction. Unfortunately, blocking RIG-I in human moDC directly was beyond our technical capability.

We also considered whether replicating RSV could induce IDO activation through the autocrine action of inflammatory cytokines. The release of IFN- α and IFN- γ were low and blockade of these cytokines had no effect on RSV-induced kynurenine release. In contrast, TNF was present in the supernatant of rgRSV-infected moDC. TNF is reported to increase synergistically IFN- γ -induced IDO activity [62-65]. In line with previous studies [50], TNF alone did not induce IDO in our model. Interestingly, blocking TNF decreased RSV-induced IDO activity and adding

exogenous TNF enhanced RSV-induced IDO activity. Thus, blocking TNF did not completely abrogate RSV-induced IDO activation. We suggest that TNF may play a role in RSV-induced IDO activation in moDCs, although the precise mechanisms involved are yet to be determined. One possible mechanism is that the initial infection of moDCs with RSV could induce release of TNF. The released TNF may bind to its cell membrane receptor resulting in amplification of intracellular signals already in play (e.g. RIG-I).

To further define potential targets for a role in RSV-induced IDO activity, we inhibited other intracellular signaling pathways. LPS, TLRs and viral infection are stimuli of p38 MAP kinase [66, 67]. Our data suggest that p38 MAPK has a definitive role in the induction of IDO. It remains to be determined whether RSV replication (and its ssRNA/dsRNA products) can directly stimulate p38 MAPK pathway, or if the effect is mediated by up-stream stimulation of p38 MAPK through PRRs (i.e. RIG-I). Inhibition of p38 MAPK also inhibits TNF accumulation in IFN- γ -stimulated macrophages [68]. Our data allow us to speculate that p38 MAPK may mediate IDO activity in response to RSV through TNF production in moDC.

Expression of IDO is regulated by NF- κ B-associated transcription factors [64]. IKK- α and IKK- β are part of the I κ B kinase complex that plays an important role in regulating the NF- κ B transcription factor. Upon cell stimulation, IKK- α or IKK- β induces I κ B degradation, allowing NF- κ B-associated transcription factors to translocate to the nucleus to express the target genes [60]. BMS-345541 (IKK Inhibitor III) is an inhibitor of IKK β (IKK-2) and significantly more selective for IKK β over the other three members of the IKK subfamily (IKK α , IKK ϵ and TBK1) [61]. IKK- β inhibition reduced RSV-induced IDO activation, confirming a role for canonical

pathway of NF- κ B in the activation. Interestingly, IKK- β is also reported as essential for the signaling cascade of TNF [60]. Thus, IKK- β may play a double role in IDO induction and as a mediator for RIG-I and TNF signaling.

For RSV to cause bronchiolitis, the virus must infect epithelial cells. It is interesting that while an epithelial cell line (HEp-2 cells) released kynurenine in response to IFN- γ , this did not occur after RSV infection. IDO is not constitutively bioactive in immune/inflammatory cells, with the exception of eosinophils [45]. Under inflammatory conditions, IDO becomes expressed and activated in dendritic cells [16, 17, 69-71]. Since moDCs are critical cells that link innate and adaptive immune responses, including a central role in immune response to RSV [72, 73], we focused on moDCs, instead of epithelial cells.

IDO is associated with apoptosis in proliferating T cells, especially in Th1, compared with Th2 cells [11, 12, 74]. The mechanism underlying the induction of anti-inflammatory effect may result from tryptophan depletion itself and/or production of the “immunocytotoxic or immunoactive” tryptophan catabolites, kynurenines [13, 75]. IDO converts tryptophan into *N*-formylkynurenine, which is converted subsequently to L-kynurenine by the enzyme formamidase [23, 76]. Unlike other amino acids, the concentration of free serum tryptophan is less than 100 μ mol/l [77], lower than most “non-essential” amino acids in serum (50 μ M to 1 mM) [78]. In a milieu where immune cells express IDO activity, the concentration of tryptophan becomes depleted to nanomolar levels [79] imposing a condition of starvation on susceptible cells and pathogens [80]. It appears that dendritic cells can survive this condition by a specific low affinity transporter system that takes membrane-impermeable tryptophan to the intracellular

space [79]. This is not the case for other immune cells including CD4+ cells [22]. Whether Th1 or Th2 cells have differences in this response to low tryptophan or the kynurenines is not yet reported.

There is some controversy regarding the role of IDO in Th2 responses as some groups have shown that IDO activity can suppress Th2 type inflammation *in vivo* [74, 81-83]. IDO activity appears to suppress many cell types including Th1 and Th2 cells. Thus, as suggested by Xu et al., it is not unexpected to see that imposing IDO activity on an already primed and disproportionately "pure" Th2 cell environment could result in suppression of Th2 function. In our *in vitro* model, we are exposing a mixture of Th cells to IDO activity, which is closer to what is happening naturally in immune system. If IDO activity leads to greater suppression of Th1 than Th2 responses as has been reported [45, 74] then our data are not in opposition with the current understanding.

Our findings show that co-culture of activated T cells with rgRSV-infected moDCs reduces the number of cells that express T-bet, a transcription factor specific to Th1 cells. We suggest that this may decrease Th1 cells available to respond to an antigenic stimulus thereby reducing the Th1/Th2 ratio compared with uninfected control groups. The inhibiting factor is likely to be a soluble agent in the medium, able to pass through 0.4 μ M pores in a transwell membrane with the potential to reach T cells in the upper chamber of the inserts. Only negligible levels of IFN- γ , IL-12, IL-4 and IL-13 were measured in rgRSV-treated moDC, rendering them unlikely candidates. A limitation of our study is the lack of cytokine data showing that the supernatant from IDO stimulated cells contains more Th2-like cytokines (e.g. IL13). While these data would

be supportive, they still would not be definitive. An *in vivo* experiment, with IDO inhibition in infant animals would be the better next experiment.

While there has been interest in IL-17/Th17 in RSV related immune responses [84], its role in our model seems less likely. IDO activity appears to inhibit IL17-mediated effects [85, 86]. Further, given our *in vitro* model's design, the induction of IL17 producing T cells would be unlikely. Finally, the inhibitory effect on T-bet and the change in Th1/Th2 ratio was reversed in the presence of 1-MT, the inhibitor of IDO function. Whether the effect on T cells was through kynurenine-induced cytotoxicity or tryptophan depletion remains undetermined.

In conclusion, our study shows for the first time that that RSV infection can induce IDO activation in primary human moDC through a mechanism that is dependent on RSV replication. Co-culture of RSV-infected moDC inhibited T-bet expression in T cells. We suggest that having a lower Th1-like response to environmental antigens during infancy as the immune system is being educated could be a factor in promoting the development of allergic disease.

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Conflict of interest:

The authors declare no conflict of interest.

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Legends to Figures

Figure 1- RSV (rgRSV) infects human moDC. **A-** Live-cell confocal microscopy images of moDCs incubated with rgRSV at MOI=5 (2 days post infection) show GFP expression (DIC images on the left, fluorescence in the middle, and merged on the right). **B-** Flow cytometry of moDC 24 hours following incubation with rgRSV (MOI=1) showed that $35.8 \pm 4\%$ of cells have GFP expression (M1 gated). **C-** By flow cytometry, Δ Mean Fluorescence Intensity (MFI) of moDCs incubated with UV-inactivated rgRSV or rgRSV blocked with Palivizumab are shown in comparison with un-treated rgRSV (n=3). **D-** Time-course plaque assays in Hep-2 cells using the supernatant of infected moDC with RSV (n=2, each line is a separate experiment). Uninfected cells showed no plaques. A and B are representative of three independent experiments.

Figure 2- RSV induces IDO activation. **A-** Infection of moDC with rgRSV (at different MOIs) induced kynurenine release in culture media 5 days post infection (n=9). IFN- γ was used as a positive control in all experiments (1000 IU/ml). **B-** UV-inactivation or Palivizumab blockade of rgRSV suppressed virus-induced IDO induction in moDCs (n=4). **C and D-** Inhibition of IDO with the specific competitive inhibitor, 1-MT (800 μ M, n=3), and menadione (10 μ M, n=2). **E-**

Flow cytometry of rgRSV-infected moDCs with an anti-IDO monoclonal Ab confirmed induction of the enzyme one-day post infection (histograms are representative of two independent experiments). Error bars show SEM.

Figure 3- Induction of IDO 5 days post-infection by RSV is dependent on viral replication and needs active infection with the virus. **A-** Blocking replication of RSV by Ribavirin suppresses RSV-induced kynurenine release (n=5). **B-** Incubating moDC with doses up to MOI=20 of UV-inactivated RSV did not result in kynurenine release (n=3). Error bars show SEM.

Figure 4- Role of TNF in RSV induced IDO activity. **A-** TNF did not induce IDO in human moDC regardless of the doses used (n=3). RSV-induced IDO activity in human moDC was increased by TNF (n=6) **B-** Addition of a neutralizing monoclonal Ab against TNF to RSV-infected moDC culture medium (10µg/ml) suppressed kynurenine release compared to isotype control (n=7). Error bars indicate SEM.

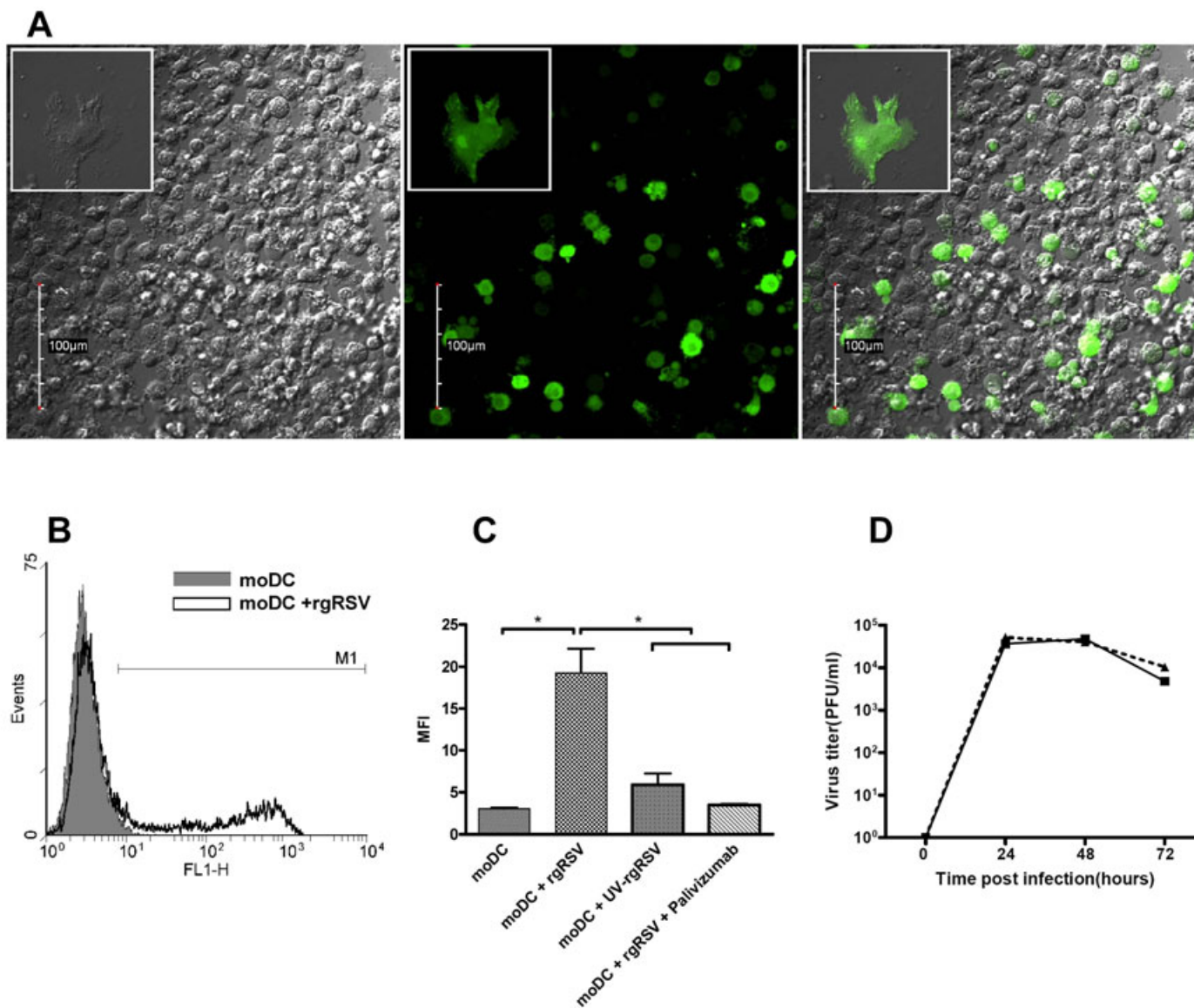
Figure 5- IDO induction by RSV is not mediated through TLR-3. Incubation of moDC with Poly I:C (10 µg/ml), the ligand for TLR-3, induced kynurenine generation that was blocked with Chloroquine (Chlq)(5µg/ml). In contrast, addition of Chlq to RSV-infected moDC did not inhibit RSV-induced IDO activity (n=5). Error bars indicate SEM.

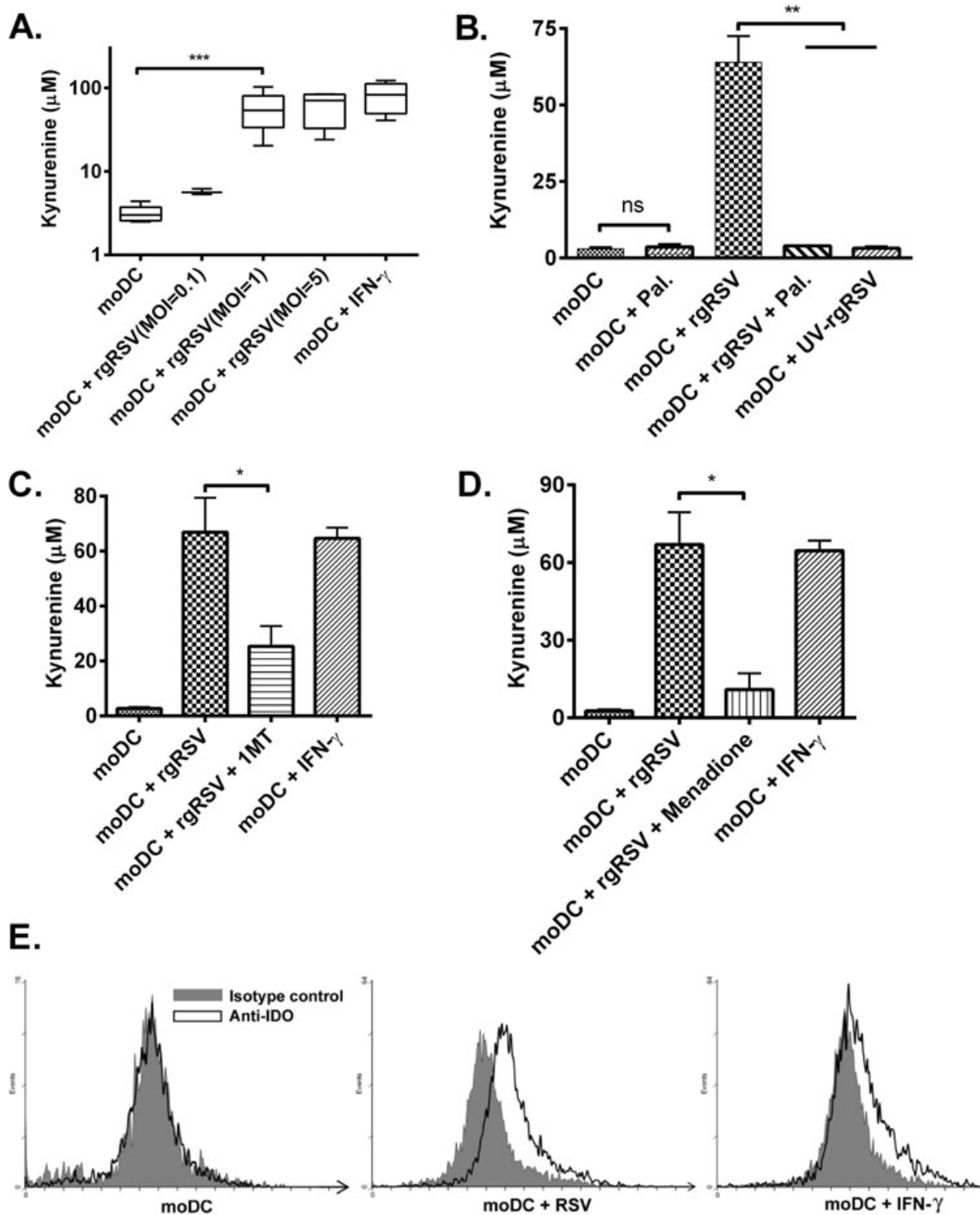
Figure 6- Inhibition of downstream signaling pathway of RIG-I-like receptors (TBK1/IKKε) suppressed IDO induction by RSV in moDCs. Addition of BX795 (10 µg) to RSV-infected moDC did not inhibit RSV-induced IDO activity (n=4). Error bars indicate SEM.

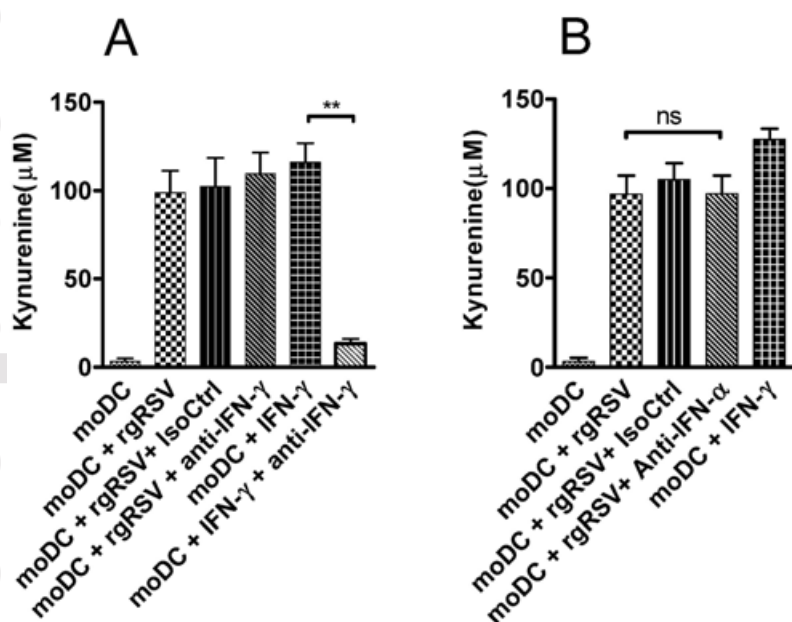
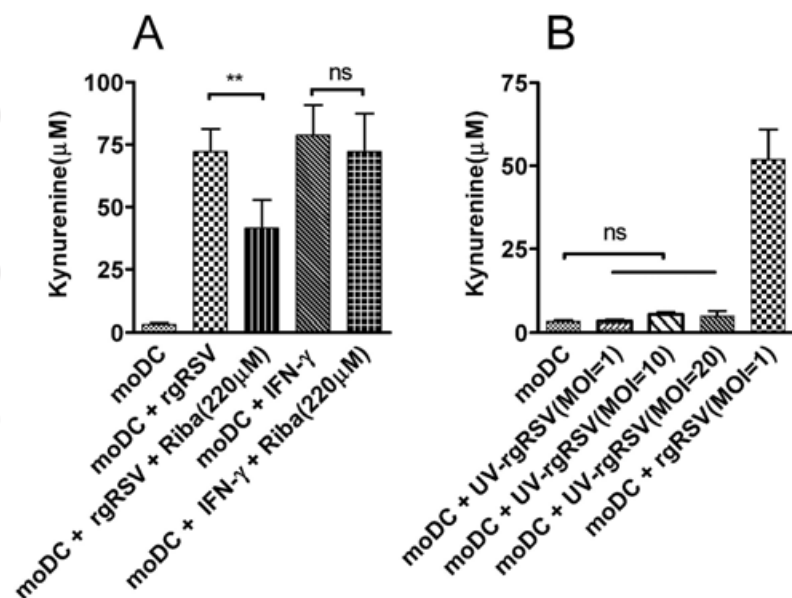
Figure 7- Role of down-stream signaling pathways in RSV-induced IDO activity. **A-** IKK Inhibitor III (BMS-345541), the inhibitor of IKK β of the NF κ B signaling pathway, suppressed RSV-induced IDO activity when compared with controls (n=9). **B-** Inhibition of MAPK-P38 of the MAPK family with SB202190 suppressed IDO activation by RSV in a dose response pattern compared to negative control (SB202474)(n=4).

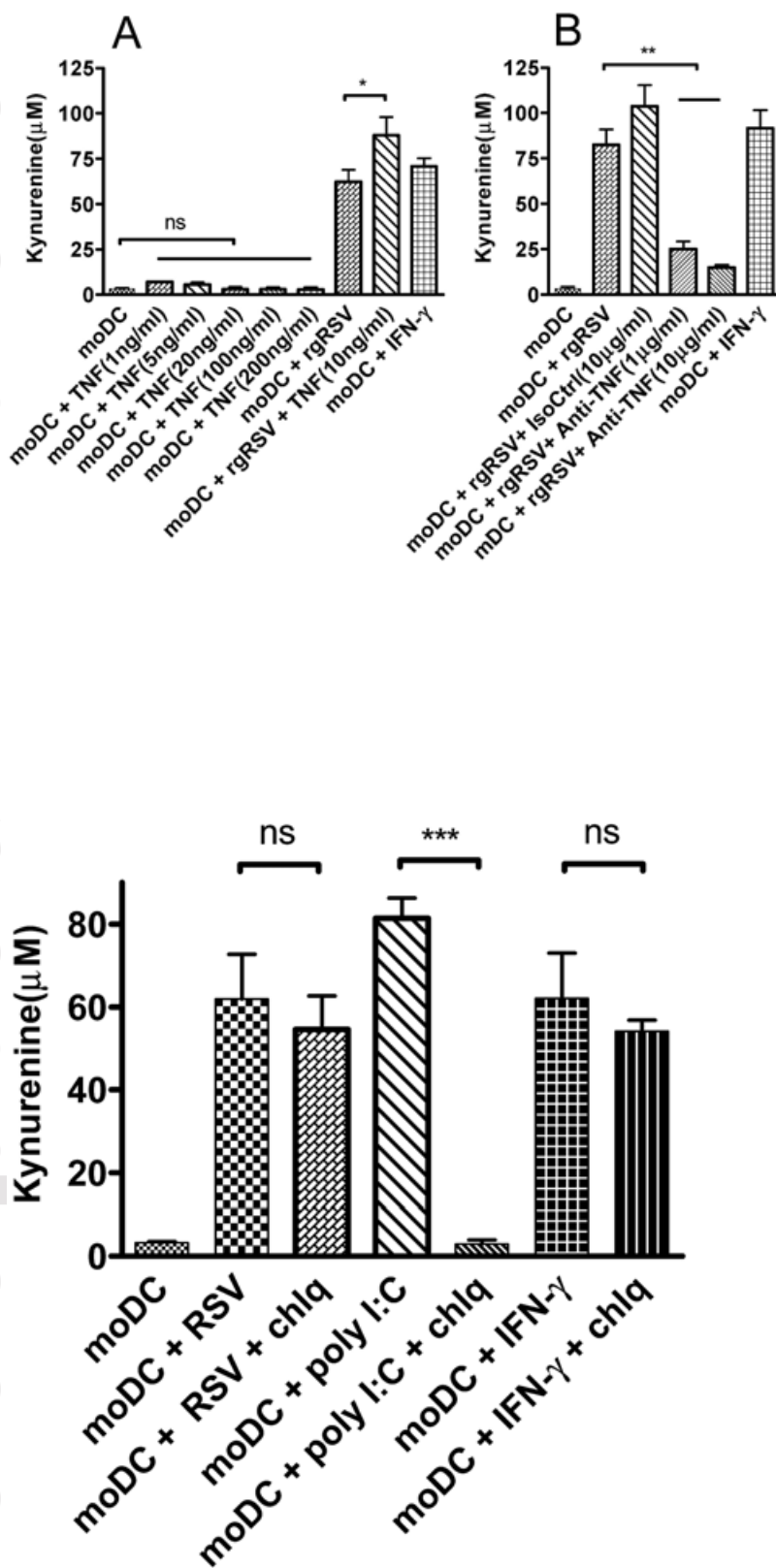
Figure 8- A schematic diagram showing signalling pathways that may be involved in RSV-induced IDO induction in moDC. The inhibitors that induced measurable effects are placed in boxes along with the site of their action.

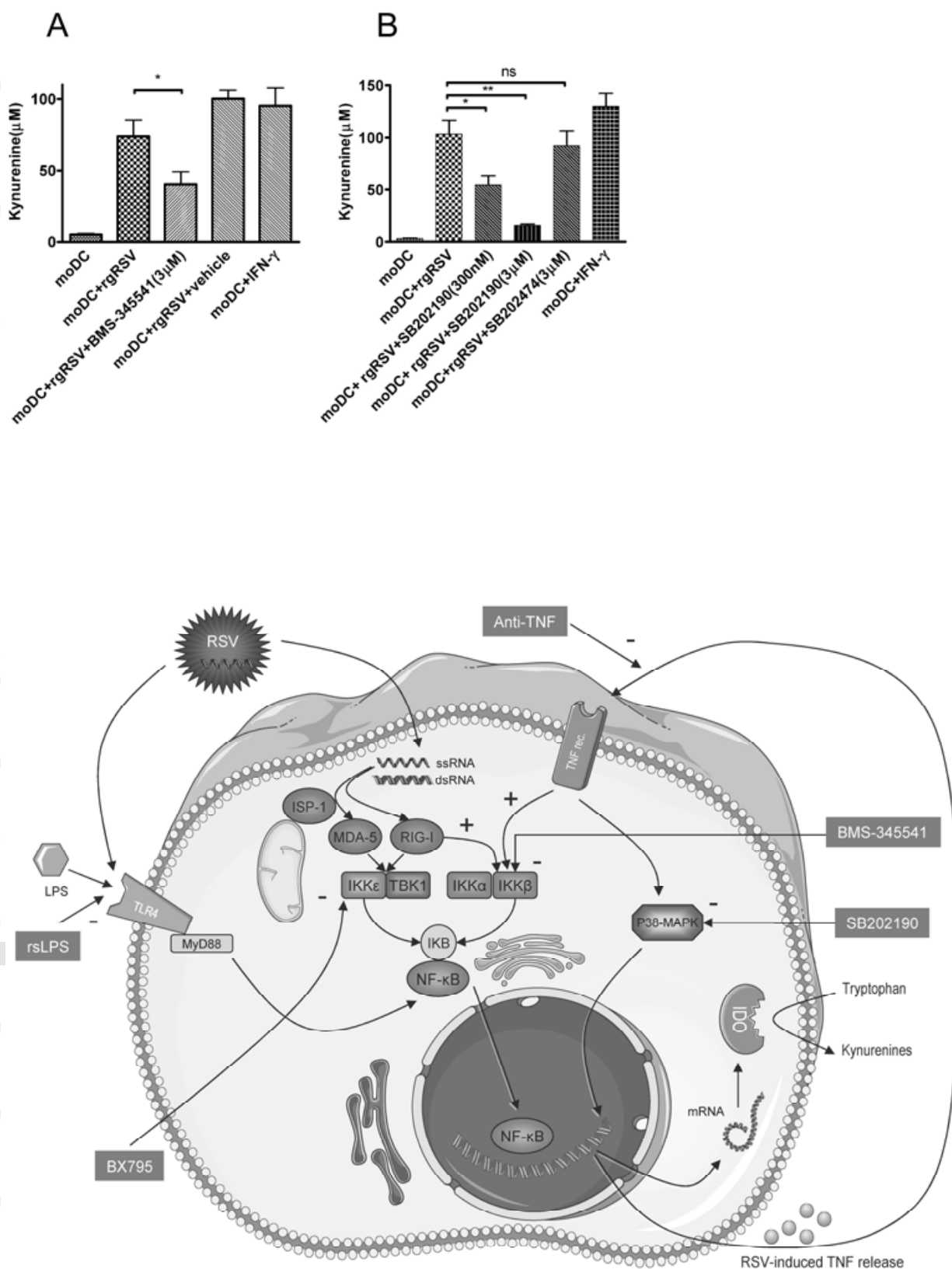
Figure 9- RSV infection of moDC decreases T-bet but not GATA3 expression in co-cultured T cells: **A-** Co-culture of activated T-cells in a transwell system with RSV-infected moDCs suppressed T-bet expression. This suppression was blocked in the presence of the inhibitor of IDO, 1MT (n=5). **B-** Expression of GATA3 was not affected in this co-culture model (n=5). Error bars indicate SEM. **C-** Representative dot plots in one of the experiments. Y axis in all plots represents CD3 staining.

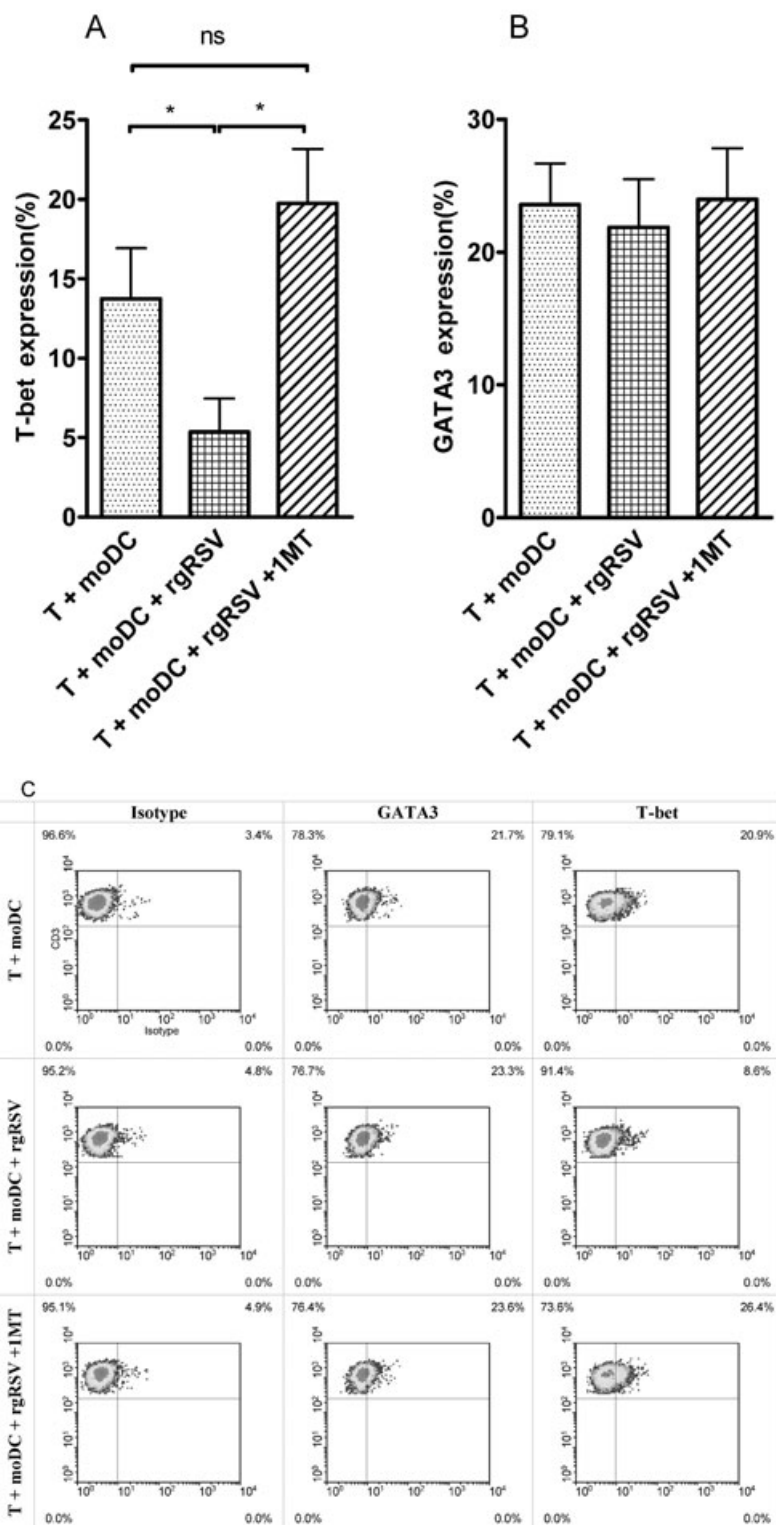


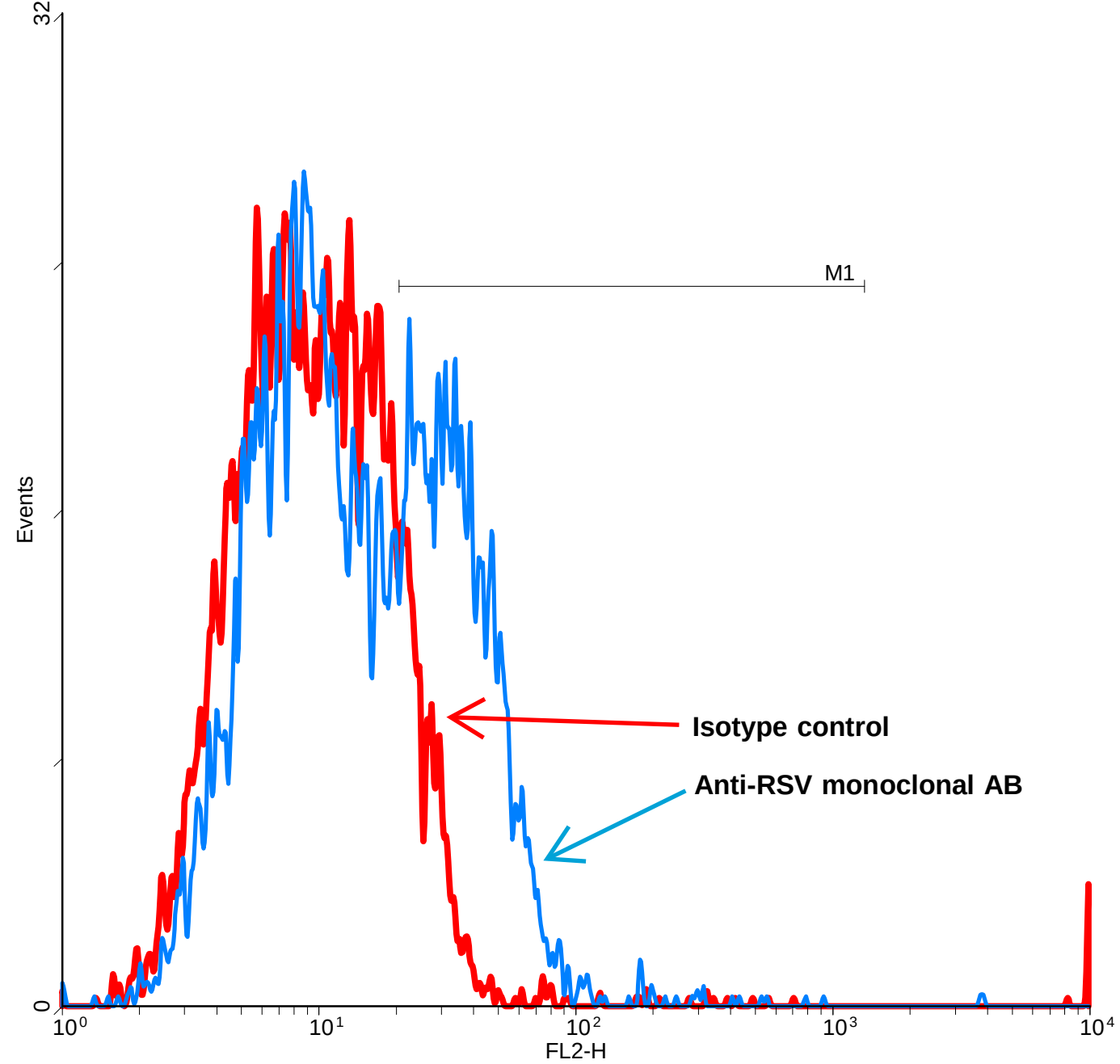


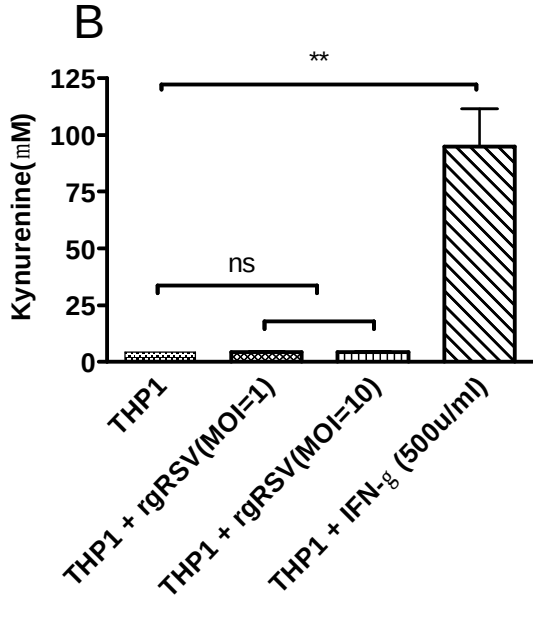
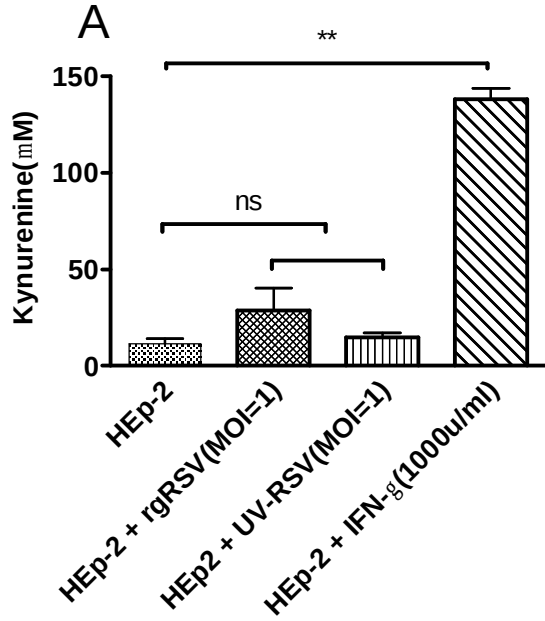


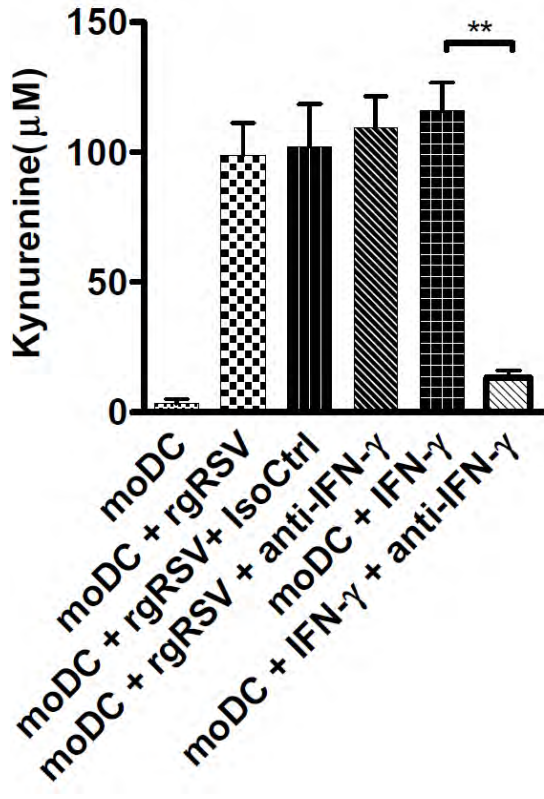
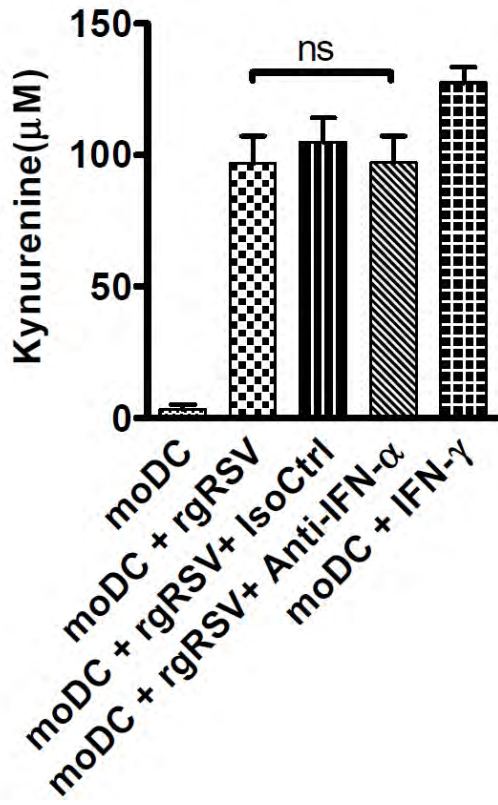




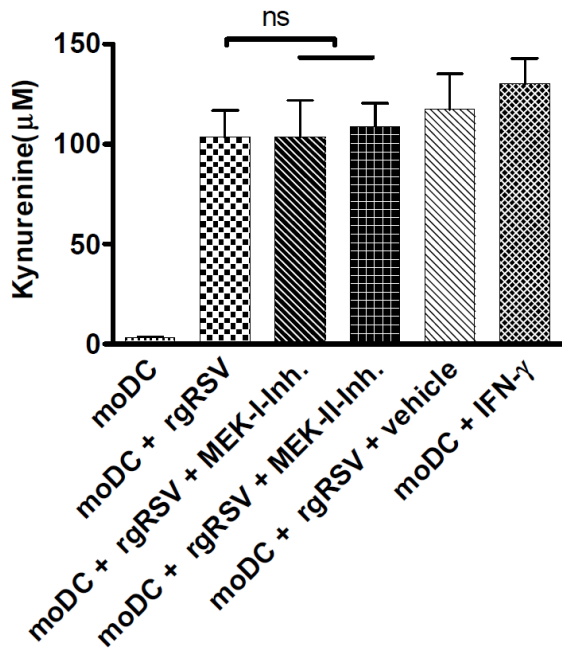




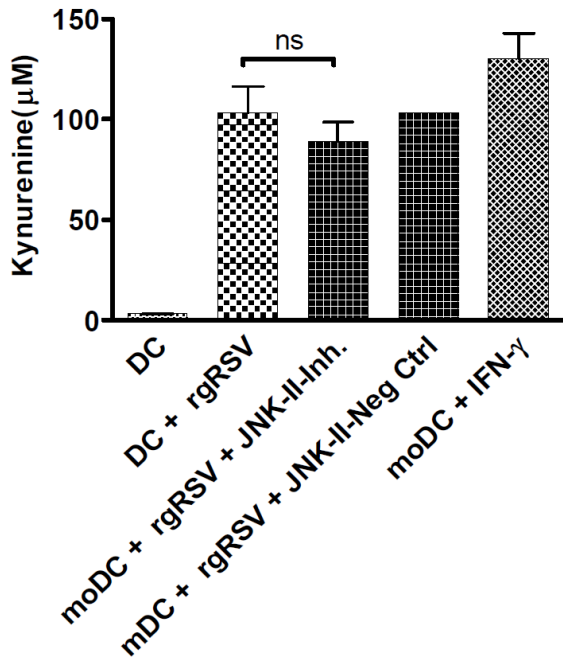


A**B**

A



B



Legends for eFigures

eFigure 1- Flow cytometry of moDC 24 hrs following incubation with hRSV strain A2 (MOI=1) and stained with either mouse anti-human RSV F protein (please refer to Materials and methods) or relevant isotype control. M1 gated cells represent 24.1% shift in cells infected with RSV. This figure is representative of three independent experiments.

eFigure 2- RSV does not induce IDO in HEp-2 and THP-1 cell lines. **A**- While IFN- γ induced kynurenine release in HEp-2 cells, neither rgRSV nor sham treatment showed significant release (n=3). **B**- While IFN- γ induced kynurenine release, neither rgRSV nor sham treatment had an effect (n=4).

eFigure 3- **A** and **B**, Neutralizing monoclonal antibodies against IFN- α and IFN- γ in supernatant (10 μ g/ml) did not inhibit the RSV-induced IDO activity in moDC following RSV infection, suggesting lack of a role in this induction (n=6 each). Anti-IFN- γ was able to counteract the effect of exogenous IFN- γ in a control experiment (10 μ g/ml) (n=4). Error bars indicate SEM.

eFigure 4- **A** and **B**- Down-stream signaling pathways in RSV-induced IDO activity. Inhibition of JNK, MEK-I and MEK-II of the MAPK family signaling pathway did not affect RSV-induced IDO activity (n=4).