Herpes Simplex Virus Induces the Early Activation of NK Cell *via* MyD88-Dependent Signal

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Abstract: In the course of a Herpes Simples Virus type-1 (HSV-1) infection, the bidirectional cross-talk between Natural Killer (NK) cells and Dendritic Cells (DCs) is imperative in mounting an efficient immunological response. Enhanced DC maturation by Toll-Like Receptor (TLR) ligands is considered as a pivotal link between innate and adaptive immune responses, yet the impact of direct TLR signaling on NK:DC interaction during an HSV infection has yet to be examined. We demonstrate that HSV mediates the activation of NK cells and the induction of the cooperative relationship between NK cells and DCs *via* TLR. During the course of an HSV infection, the initial response required both types of innate cells and demonstrated phenotypic and functional activation much like that of TLR-stimulated cells. Using a MyD88 knock-out system diminished the early interaction between DCs and NK after HSV exposure. Our results indicate that HSV TLR-ligands modulate the early NK: DC interaction and may be a mechanism used by these innate immune cells to overcome virus mediated immune evasion and the initiation of a potent acquired immune response.

Keywords: NK cell, dendritic cells, HSV, TLR.

1. INTRODUCTION

Interaction of innate immune cells is essential for inducing and sustaining an immunological response to both pathogen and tumor, especially dendritic cells (DCs) and Natural Killer (NK) cells. In lymphoid organs, DCs exist in two functionally and phenotypically distinct states: immature and mature. Immature DCs (iDCs) are proficient at endocytosis and antigen processing; however, MHC class I surface levels and expression of co-stimulatory molecules are low on iDCs. Therefore, iDCs are proficient in sampling their environment and taking up antigen, yet their ability to present antigens to T cells is deficient [1]. Upon the detection of microbial products *via* Toll-like Receptors (TLR) or in response to pro-inflammatory cytokines (IL-12, IL-15, IL-18, Type I interferons), iDCs undergo a maturation process creating efficient T cell priming DCs [2].

NK cells have long been considered important in initiating an immune response [3]. While NK cell activation occurs due to lack of surface MHC class I on targets, target lysis requires a multitude of signals that include stress proteins, cytokines and involvement of other sentinel cells. Although NK cells were originally thought to act independently, accumulating evidence indicates that NK cells also respond to stimuli from other immune effectors, especially DC. Kassim *et al.* have demonstrated that *in vivo* ablation of CD11c-positive DCs increase the susceptibility to HSV-1 infection, likely through decreased NK and T cell response [4]. Furthermore, DCs are required for most advantageous activation of NK cell function post primary infection [5].

DCs are present in tissues that HSV initially infects (skin or mucosa). HSV has not only evolved to infect DCs but has also acquired efficient means to prevent DC activation [6]. Although, this process has been characterized as an HSV immune avoidance mechanism, we hypothesize that HSV infected DCs are now capable of productive interaction with NK cells, due to reduced expression of MHC class I molecules combined with the production of IFN-α and IL-12, which stimulates NK cells [6].

In a recent report by our group, NK cells were demonstrated to be novel helpers in an anti-HSV adaptive immune response [7]. The quantity and quality of anti-HSV CD8+ T cells were diminished in the absence of NK cells. Consequently, mice that lacked NK cells were more susceptible to HSV induced lesions. NK cell supplementation enhanced the function of CD8+ T cells; compensated for the loss of CD4+ helper T cells; and were able to partially rescue dysfunctional CTLs that were generated in the absence of CD4+ helper T cells. However, the exact reason for such an influence by NK cells on immune induction is not completely understood. The mechanism of HSV's ability to directly activate NK cells and influence NK:DC interaction remains unknown.

In the following study, we demonstrate that HSV PAMPS influence the bidirectional cross-talk between NK cells and DCs. With both NK cells and DCs expressing TLRs, it is our hypothesis that HSV mediates the early interaction and signaling events of NK cells and DCs *via* direct TLR engagement. To address HSV PAMP mediated cross-talk initiation, DCs and NK cells were stimulated with UV-inactivated HSV or TLR-ligands to examine function

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and activation. Despite the decreased expression of co-stimulatory molecules, HSV stimulated dendritic cells significantly augmented the production of DC derived IL-12 and increased NK activation and cytokine production. To specifically address the role of direct activation by HSV through TLR, DCs and NK cells from MyD88$^{-/-}$ were used to examine whether or not activation is dependent upon MyD88 signaling. Our results indicate the possibility that viral TLR-ligands modulate NK:DC interaction resulting in the initiation of a potent acquired immune response.

2. MATERIALS AND METHODS

2.1. Mice

Female C57BL/6 (7-8 wk old), purchased from Harlan Sprague Dawley, Indianapolis, Indiana, and female transgenic PK136, in which transgenic B cells make PK136 monoclonal antibody to deplete NK cells, from Dr. Yuan, UT Southwest Medical Center (previously described [8]; and HSV-gB transgenic mice, with TCR specific for HSV immunodominant peptide, SSEIFARL, were received from Dr. Francis Carbone, University of Melbourne, Australia [9]. All mice were housed under standard conditions with procedures approved by Institutional Division of Laboratory Animal Research. Animals were kept in specific pathogen-free conditions in the Division of Animal Resources, College of Medicine, East Tennessee State University, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

2.2. Cell Isolation

Natural Killer cells were isolated from wild-type fresh splenocytes using IMag NK cell enrichment (negative selection) magnetic bead separation techniques. Dendritic Cells were isolated from wild-type splenocytes *via* DC IMag enrichment selection post collagenase digestion. HSV-gB CD8+ T cells were isolated from HSV-gB transgenic splenocytes *via* a CD8 enrichment kit. All cell populations were isolate as per manufacturer's protocols (BD Biosciences) and purity verified *via* flow cytometry, (>95%).

2.3. Generation of Bone-Marrow Derived Dendritic Cells (DCs)

C57BL/6 mice and MyD88$^{-/-}$ mice were sacrificed and femur and tibia were extracted. Extraneous tissue was removed, and using a 1ml syringe, the marrow was expelled and placed in a 1:1 solution of Hanks Balance Salt Solution (HBSS-HyClone) and RPMI (HyClone) supplemented with10% FBS. The marrow was teased through a cell strainer and a single cell suspension was prepared in 10% FBS supplemented RPMI. Cells were cultured at 1x10^7 cell/ml in 24-well plate and supplemented with10ng/ml Granulocytic Macrophage-Colony Stimulating Factor (GM-SFC) + IL-4 in RMPI. The GM-CSF/IL-4/RPMI was replaced every 48 hours for 5 days.

2.4. Generation of Bone-Marrow Derived Natural Killer Cells (NKs)

Using the protocol in 2.3, NK cells were generated from C57BL/6 mice and MyD88$^{-/-}$ mice bone marrow. Cells were cultured at 1x10^7 cell/ml in a 24-well plate and supplemented with 50 µl/ml with T-STIM (BD bioscience) culture supernatant, in 500 µl of fresh RMPI 10% FBS. The T-Stim/RPMI was replaced every 48 hours for 5 days [10, 11].

2.5. Virus

HSV-1 KOS and HSV-1 17 strains of viruses were titrated after growth on Vero (CCL81; American Type Culture Collection, Manassas, VA) cell lines. The virus was stored in aliquots at -80 until required.

2.6. HSV Infection and Stimulation

In vitro: NK cells and DCs were incubated with UV-inactivated HSV-kos (3 MOI before inactivation) for 18 hours at 37°C for stimulation experiments. Mock stimulation with diluent (endotoxin free buffer) was used as control.

In vivo: 20µl of HSV-1 17 containing 1x10^5 PFU of virus was administered *via* the intra-peritoneal route of infection. All animal experiments were performed in agreement with American Association for Accreditation of Laboratory Animal Care.

2.7. TLR Ligand Stimulation

Pre-titrated synthetic ligands for TLR2 (palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH) (10µg/ µl); TLR3 (Polyriboinosinic polyribocytidylic acid [poly(I:C)]) (5µg/ µl); and TLR9 (Cytosine-phosphate-guanine containing oligodeoxynucleotides (CpG ODN) (10µg/ µl); were added as a cocktail to cell suspensions seeded at 1x10^6 cells/dose for 18hours at 37°C @ 5% CO_2. (TLR Ligands were purchased from Invivogen, SD, CA).

2.8. Co-Culturing Experiments

Isolated and purified DCs and NK cells were infected or cultured with appropriate TLR ligand stimulation for 18 hours and then co-cultured in 1:1 ratio for 18hours at 37°C in 2ml of RPMI containing 10%FBS-RPMI to examine phenotypic and functional changes.

2.9. Cell Surface Staining

One million splenocytes were incubated with appropriate cell surface markers for 20 minutes. Dendritic cells were analyzed using: anti-CD11c (PE, APC), anti-CD11b (FITC), anti-MHC-I (FITC), anti-MHC-II (PE), anti-CD86 (PE), and anti-CD80 (FITC). NK cells were stained with anti- NK1.1 (FITC, PE), anti-CD3 (FITC, PE, PE-Cy-7, APC), CD69 (FITC). Appropriate isotype antibodies were used as control. All antibodies were purchased from BD biosciences.

2.10. Intracellular Cytokine Staining

One million splenocytes were incubated with brefeldin A for 4-5 hours. After incubation, cells were stained with cell surface markers, fixed and permeabilized using perm-fix and perm wash, and, subsequently, stained for intracellular markers with anti-IFN-γ(PE-Cy7) or IL-12 (APC). All antibodies were purchased from BD Bioscience.

2.11. *In Vitro* Proliferation Assay

NK cells and HSV-gB CD8+ T cells in independent experiments were resuspended to 1x10^6 cells/ml in 1xPhospate-Buffered Saline, pH7.2 (PBS). To measure induced proliferation, the cells were labeled with 5µM carboxyfluorescein diacetate, succinimidyl ester (CFSE)

(Molecular Probes). Post labeling, cells were incubated for 8 minutes at 37°C and the reaction was quenched by adding equal volumes of cold fetal bovine serum. The cells were thoroughly washed twice with 1xPBS avoiding light. Proliferation was measured by examining CFSE dilutions using a flow cytometer.

2.12. Flow Cytometry

The staining of cells was performed according to standard protocols and flow cytometric analysis was performed using a four color FACSCalibur4™ cytometer. Approximately 100,000 live events were collected. The data were analyzed using Cellquest or FCS Express software.

2.13. Statistical Analysis

Graph Pad Prism 4 software was used. Student t-tests were used to determine the statistical significance. To compare samples in co-culture experiments, an analysis of variance (ANOVA) was used to determine significance followed by a Tukey's post-hoc t test. Significance, indicated by (*), represent $p<0.05$.

3. RESULTS

NK cells play a role in shaping the acquired immune response in part by their interaction with DCs. Since, HSV is known to impact the function of both DC and NK cells [6, 12]; we examined the activation of NK cells and induction of NK-DC interaction in response to HSV exposure. This study explores the early events that occur immediately post virus exposure and the involvement of MyD88-dependent (and likely TLR-dependent) signals.

3.1. HSV Effects on DCs Results in NK Activation

HSV infection induces the accumulation of DCs and NK cells in the DLN, but the molecular mechanisms subsequent to this process are still not understood [13]. We examined HSV induced changes in DCs that could possibly affect the NK cell population. DCs isolated from C57BL/6 (WT) mice were stimulated with either a cocktail of TLR ligands (TLR-L) (PamCys, Poly I:C, and CpG), serving as positive control, or UV-inactivated HSV and then analyzed for co-stimulatory molecule, CD80 (B7.1) and CD86 (B7.2), and MHC-Class I expression. Flow cytometric analysis of $CD11c^{+}CD11b^{+}$ DC revealed a two-fold decreased expression of both co-stimulatory molecules and surface expression of MHC-I when exposed to HSV in comparison to TLR-L stimulation (Fig. **1A**). When compared to control, HSV exposed DC showed increased levels of CD80, CD86, and MHC-Class I, yet HSV has been demonstrated to inhibit DC maturation [6]. To investigate the role of HSV-DCs in NK cell activation, DCs and NK cells were enriched from C57BL/6 splenocytes and co-cultured in 1:1 ratio. DCs were exposed to UV-HSV or stimulated with TLR-L cocktail or unstimulated as described in previous experiments. NK cells were labeled with 5μM CFSE to observe the proliferation induced by co-cultured TLR-DCs or HSV-DCs. Unstimulated DCs and NK cells alone were used as control. Simultaneously, NK cells were also assessed for the capacity to produce IFNγ *via* intracellular staining for flow cytometry. Positive results were recorded for cells expressing CD3-NK1.1+ CFSE+. As indicated in Fig. (**1B**), HSV-DC's ability to activate NK cells was two times greater than the level seen with TLR ligand stimulated DC (TLR-DC) and 12 more than the control (unstimulated DCs). Activation was measured in terms of IFNγ production. The percentage of $IFN\gamma^{+}$ NK cells were 87±2.5% in HSV-DCs, while it was 35.8±2.6% in TLR-DCs. The control unstimulated DCs were able to induce IFNγ in 6.8±1% of NK cells. The difference in NK cell stimulating ability of various DCs were statistically significant (HSV-DCs *vs* TLR-DCs = $P<0.05$; HSV-DCs *vs* Control DCs= $P<0.0001$). Induction of at least four rounds of NK cell divisions by HSV-DCs was measured. This was significantly greater compared to the NK cells stimulated by TLR-DC which underwent only two rounds of cell divisions. Despite the previously reported negative effects of HSV on DCs, the data presented here demonstrated that as a consequence of HSV exposure, DCs probably because of their lower levels of MHC-I induced NK cell activation and proliferation indicating a possible host mechanism to overcome HSV mediated immune evasion.

3.2. NK Cells Contribute to Efficient HSV-gB T Cell Priming

The requirement of DCs for T cell activation has been demonstrated by many groups; however, the contribution of NK cells in DC mediated activation of T cell has not been appreciated in the context of HSV infection. DCs were isolated from previously infected ($1x10^{6}$pfu/mouse) WT and PK136 transgenic mice (which lack NK cells), and subsequently co-cultured with CFSE (5μM) labeled HSV-gB CD8+ T cells, obtained from HSV-gB transgenic mice [8, 9]. DCs and T cells (0.5:1.0) were co-cultured and then analyzed for HSV specific T cell proliferation. DCs obtained from infected WT mice increase proliferation of T cells two log fold ($p<0.05$); however, DCs isolated from infected mice lacking NK cells (PK136) were inefficient stimulators of T cell proliferation (Fig. **2**). Taken together, these results reveal that NK cells are required for efficient T cell priming by DCs with lasting impact on the adaptive immune response. This could very well explain the reason for inferior anti-HSV response in the absence of NK cells as observed by us earlier [7].

3.3. Concomitant Infection of DCs and NK Cells Leads to Increased Phenotypic and Functional Activation

3.3.1. Effects on DCs

The individual response of DCs to treatment with TLR-L or HSV has been previously examined, yet the impact on DCs and NK cells interaction and bidirectional cooperation has yet to be explored. DCs and NK cells were isolated from WT splenocytes and purified *via* magnetic negative selection; subsequently, NK cells were stimulated with pre-titrated synthetic TLR-L or exposed to UV-HSV and co-cultured with DCs to analyze phenotypic and functional variations.

Phenotypic activation of DCs was determined by measuring the surface expression of co-stimulatory molecule, CD80, and expression of MHC class II; functional activation was determined by measuring the production of IL-12. HSV infection of DCs alone increased the expression of activation markers, CD80 and MHCII; however, the

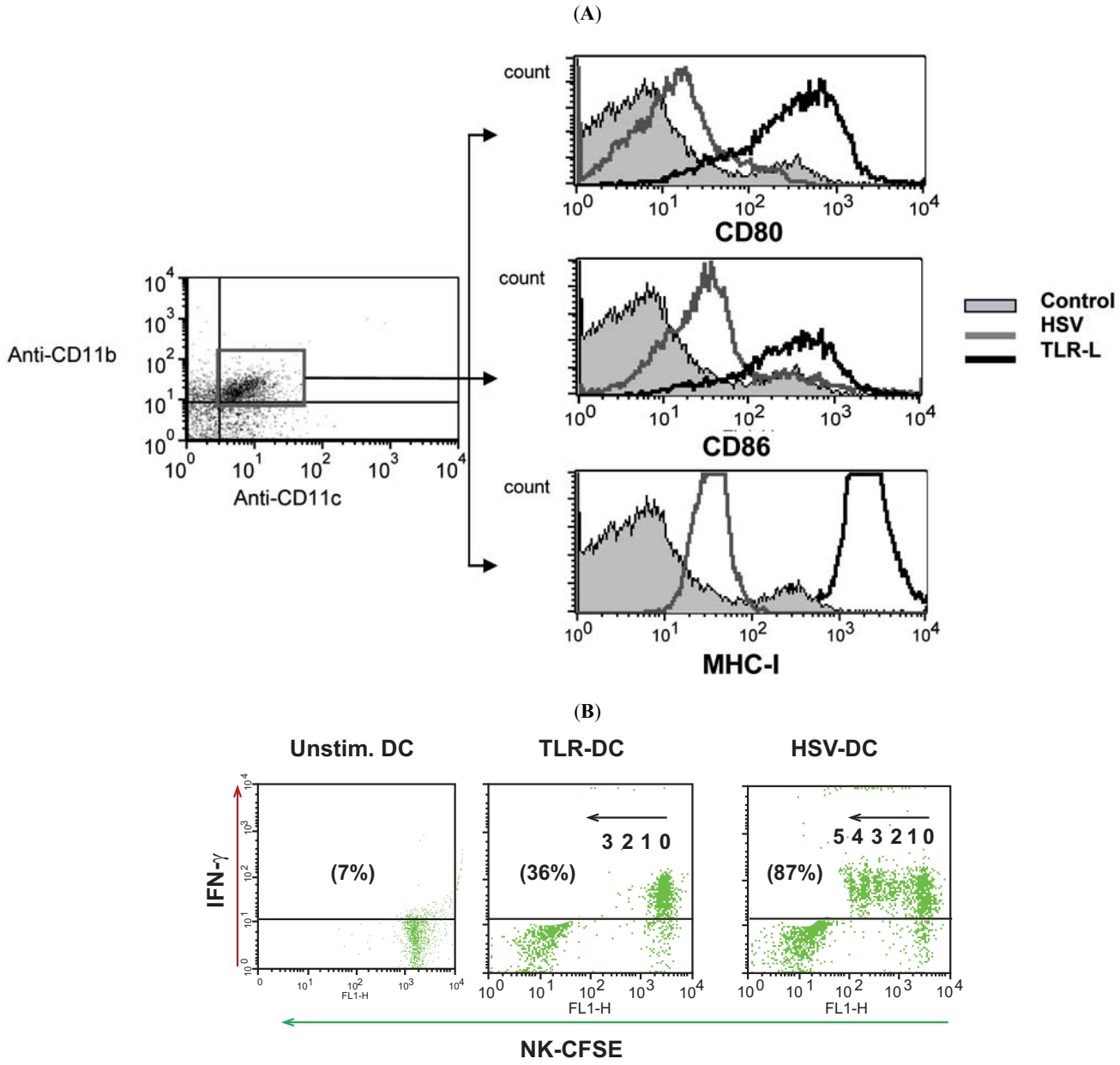

Fig. (1). HSV induced negative effect on DCs increases its NK stimulatory ability. (A): HSV Exposed Dendritic Cells show diminished co-stimulatory molecules and MHC- I expression: Dendritic Cells were isolated from C57BL/6 mice and enriched. They were then stimulated with a cocktail of TLR-ligand or 1µl of HSV-kos (3 MOI before UV-inactivation) for 24 hours. Co-stimulatory molecules (CD80-FITC & CD86-PE) and MHC-I (PE) expression was analyzed using flow cytometry by gating on CD11c+ population. The histogram plot shows the data collected from one of the three experiments. The filled area depicts mock stimulated DCs. **(B): HSV stimulation enables DCs to induce NK cell proliferation and cytokine production:** NK cells and DCs were isolated and purified using appropriate magnetic beads from age and sex-matched naive C57BL/6 splenocytes. NK cells were labeled with 5µM of CFSE and co-cultured with stimulated (TLR ligand [TLR-Dc] or HSV [HSV-DC]) DCs for 24 hours to examine the impact of HSV- DC on NK cell proliferation by CFSE dilution and cytokine production (IFN-γ-PECy7). IFN-γ production was assayed through standard intracellular staining. Analysis is a representation of $CFSE^{+}IFN\text{-}\gamma^{+}$ NK cells from one of the experiment. The experiment was repeated at least three times with similar outcome. The number in parenthesis is the percentage of NK cells positive for IFN-γ and the numbers below the arrow indicate the rounds of NK cell division. The difference in NK cell stimulating ability of various DCs were statistically significant (HSV-DCs *vs* TLR-DCs = $P<0.05$; HSV-DCs *vs* Control DCs= $P<0.0001$).

co-infection and co-culture of DCs and NK cells resulted in more efficient upregulation of phenotypic activation markers (results not shown). The pattern was consistent with many other studies. In contrast to phenotypic activation, a significant increase in IL-12 production by DCs was observed in the presence of HSV exposed NK cells. DC function was measured in terms of IL-12 (p70) production, since IL-12 is a critical component in NK cell stimulation. HSV exposure of DCs alone and in co-cultures with NK cells resulted in an increased secretion of IL-12 (Fig. **3**). To

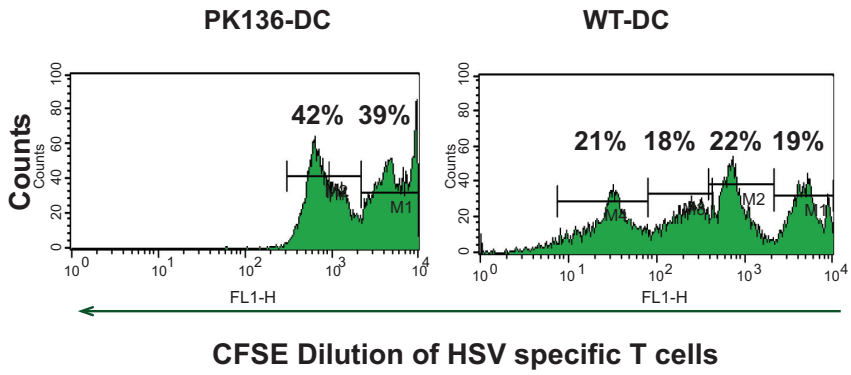

Fig. (2). DCs in the absence of NK cells are not potent stimulators of T cells. DCs were isolated from HSV infected ($1x10^6$pfu) WT C57BL/6 and transgenic PK136 splenocytes *via* magnetic separation technique. DCs were co-cultured with purified CFSE (5μM) labeled HSV specific CD8+ T from HSV-gB transgenic mice in 0.5:1 ratio. Dilution of CFSE suggests impact of NK cells on the DC's ability to stimulate anti-HSV CD8+ T cells. This experiment was carried out three times with similar outcome. The above result represents one such experiment. The numbers indicate percentage of cells in each division.

address whether or not the activation of DCs in the UnDC/HSVNK resulted from contact with HSV infected NK cells, we compared HSV-DCs alone with UnDC/HSV-NK. HSV-DCs exhibited a significant increased production of IL-12 when compared to UnDC/HSV-NK ($p=0.0436$). No significant difference in phenotypic activation (CD80 or MHCII) was detected when untreated DCs were co-cultured with TLR-L stimulated or HSV-NK cells (data not shown). The concern of carryover virus has been addressed by us in our previous investigation into the mechanism of HSV induced IL-12 production [14]. Briefly, anti-HSV mouse serum was included in some control wells in media to neutralize any free virus and besides an aliquot was also tested for any plaque formation. Based, on these results the chances for free virus particle precluding the results is minimal.

3.3.2. Effect on NK Cells

CD69, the early activation marker, was used to measure the NK cells early response to UV-HSV treatment. A significant increase in NK activation (CD3-NK1.1+CD69+) was observed in response to virus exposure. In Fig. (**4A**), co-culturing HSV-DCs with unstimulated NK demonstrated the largest percentage increase of NK cell activation (UnDC/HSV-NK: 10.3±1.6, HSV-DC/UnNK: 25.1±6.2; $p\leq0.01$). In Fig. (**4B**), analysis of NK IFNγ production highlighted the variations of NK function in response to stimulation with a dramatic nine fold increase in cytokine production in HSV infected co-cultures when compared to the negative control (Control: 0.30±0.11; HSV: 9.39±1.62). HSV-DCs co-culture resulted in increased IFNγ production in resting NK cells compared to direct exposure of the NK cells suggesting that reduction of the MHC-I on virally infected DCs (ligand for the inhibitory receptor on NK cells) and/or increase in an unknown activating ligand, serves as a stronger inducers of NK cell function than probable direct virus engagement.

3.4. Absence of MyD88 Adaptor Protein Decreases NK Cell Function

The most well known pathway of TLR signaling involves the myeloid differentiation factor 88 (MyD88) adaptor protein; absence of MyD88 hampers TLR signaling pathways; and mice deficient in MyD88 quickly succumb to lethal encephalitis post HSV-1 infection [15]. We hypothesized that HSV mediated NK cell activation (CD69 expression and IFNγ production) will be negatively influenced if MyD88 (and TLR) signaling was negated. To test this, immature DCs and NK cells were generated from C57BL/6 (WT) and MyD88$^{-/-}$ bone marrow to yield a

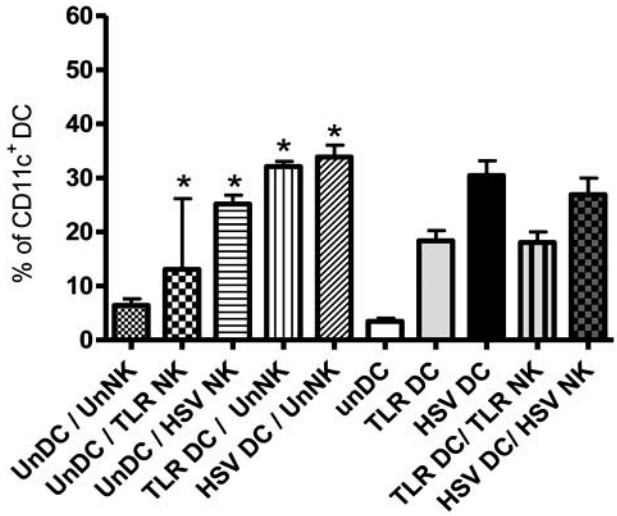

Fig. (3). Concomitant Infection of DCs and NK cells leads to increased DC derived IL-12 Production. One million enriched DCs and NK cells isolated C57BL/6 mice were stimulated with pre-titrated synthetic TLR ligands (as described in methods) or UV-inactivated HSV for 18 hours. Control groups received mock stimulation. Cell populations were then co-cultured in a 1:1 (DC:NK) ratio for 18 hours. IL-12 production was measured using standard intracellular staining protocol. The experiment was repeated three times and the result presented is the average of three separate experiments. The difference between HSV stimulated and TLR stimulated co-cultures were significant. When both DC-NK were exposed to UV-HSV, the expression was significantly higher compared to one of them being stimulated. Significance (*) is $p<0.05$. Groups labeled with (#) were significantly different from control group for that particular co-culturing condition. The values shown are mean±SD of three independent experiments.

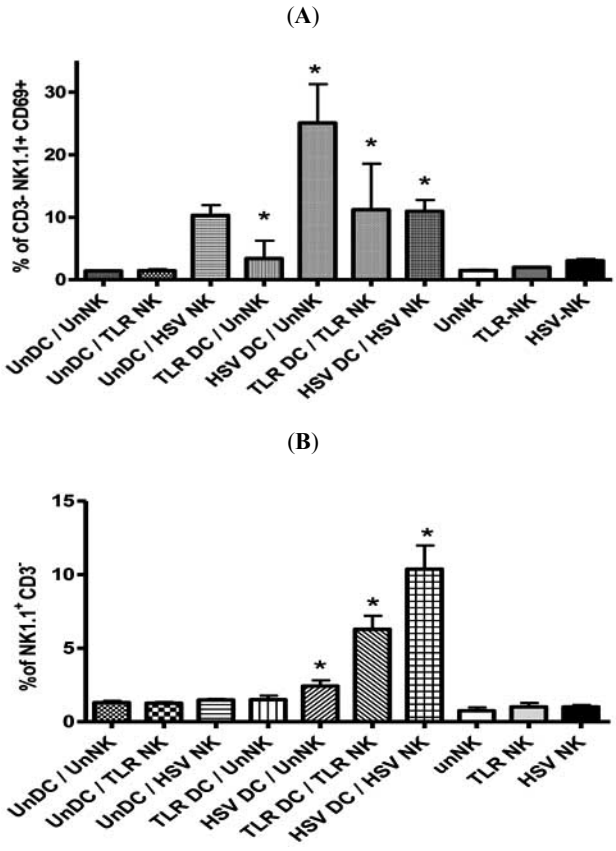

Fig. (4). Increased NK Cell Activation and IFNγ production in response to HSV Infection. In a similar co-culture experimental set up as described in Fig. (**3**), NK cell activation was assessed. Phenotypic changes were examined by measuring CD69, an early activation marker, expression on gated $CD3^{-}NK1.1^{+}$ lymphocytes. Cytokine production was determined by intracellular staining for $CD3^{-}NK1.1^{+}IFN\gamma^{+}$ in brefeldin A treated cells. The experiment was repeated multiple times and the figures shows data (mean±SD) collected from one of the experiment. *p<0.05.

homogenous population of cells post *in vitro* expansion. Maturity (DC: CD80, CD86, and MHC-II; NK: CD69) was continually measured *via* flow cytometry. DCs and NK cells were co-cultured in various combinations and stimulated with UV-HSV or TLR ligands and analyzed for phenotypic and functional changes. Mock stimulation with diluent (endotoxin free buffer) was used as control. Disrupting the TLR signaling ($MyD88^{-/-}$ DC) prevented the up-regulation of co-stimulatory markers on HSV exposed DCs (data not shown). NK cell activation was impacted significantly with CD69 expression being reduced by half (WT-NK: 8.87±0.49; MyD88-NK: 4.82±0.18) in the absence of MyD88. Infected Wt DCs were able to induce activation of Wt NK cell (Fig. **5A**); however, $MyD88^{-/-}$ NK cells activation was impinged even in the presence of Wt DCs. Direct activation in HSV-NK cells, CD69 expression, was reduced when compared to NK cells co-cultured with HSV-DCs. Further examination into the contribution of DC-mediated cytokines to NK cell activation is needed. However, NK cell activation was reduced by 25% with MyD88 DCs when used in the co-culture. Minimal NK activation was observed in MyD88 deficient NK and DC co-cultures (4 fold decrease; WT: 8.87±0.49; MyD88: 2.83±0.29). Taken together, the results highlight the importance of DC and TLR signaling in HSV mediated activation of NK cells.

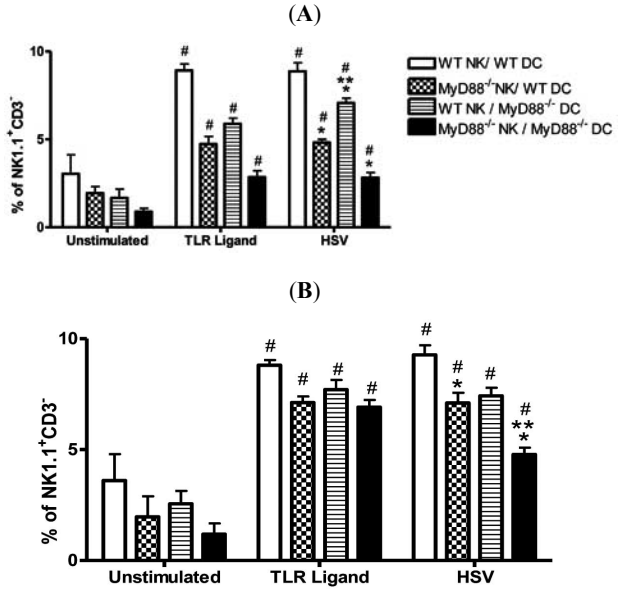

Fig. (5). (**A**, **B**) **Diminished response to HSV in $MyD88^{-/-}$ NK cells.** Dendritic cells and Natural Killer Cells were generated from C57BL/6 and $MyD88^{-/-}$ long bone marrow. Cells were supplemented with appropriate growth factors alternate days for 5 days. DCs were stimulated with UV-HSV-KOS strain (MOI of 3 before UV inactivation); stimulated with pre-titrated synthetic TLR ligands (for TLR 2,3,9); or mock stimulated for 18 hours. To examine the impact on NK cells, various groups of stimulated DCs were co-cultured together with NK cells for 18 hours. Cells were labeled with anti-CD3 and anti-NK1.1 to discriminate between NK cells and DC populations. FITC-CD69 was used as an indicator of phenotypic activation of the NK cells (NK1.1+CD3-CD69+). Using the same cell identification markers, the cytokine production of NK cells was examined by measuring IFN-γ (NK1.1+CD3-IFNγ+) production using standardized intracellular staining procedure. These experiments were carried out three times with similar results and the data shows one such experiment. Data is mean±SD of three independent experiments. Experiments were also carried out in unstimulated cells to serve as a negative control. Significance is p<0.05. *Significance between wild-type DC/wild-type NK and experimental groups. **Significance from TLR ligand stimulation *vs* HSV stimulation.

Unlike phenotypic activation (CD69 expression), IFNγproduction seem to be more dependent on the MyD88 adaptor protein presence in the DC population (Fig. **5B**). Accordingly, Wt DCs were able to compensate for the lost of MyD88 in NK cells (MyD88 NK/WT DC 7.6±0.32 *vs* MyD88 NK/MyD88 DC: 4.8±0.17). The difference was even more pronounced when IFNγ was assessed 48 hours after co-culture. IFNγ production was reduced significantly in the absence of MyD88 in both populations in comparison to other experimental groups. However, the reduction was not as significant as observed with early activation (CD69) of NK cell suggesting that early NK cell activation involves direct virus engagement of TLRs, while continued IFNγ production may be mediated by cytokines like IL-12 or IL-18 produced by viral PAMPs stimulated DCs.

4. DISCUSSION

In the course of pathogen-induced inflammation, NK cells and DCs are recruited to inflammatory tissues in response to infection [16]. The successive reciprocal interaction results in a potent crosstalk that regulates both the quality and the intensity of innate immune responses [17]. Pathogen-primed NK cells, in the presence of cytokines released by DCs, become activated [13]. At this stage NKs favor DC maturation and also select the most suitable DCs for subsequent migration to lymph nodes for priming of T cells. Historically the principal function of DCs is the activation of naive T cells [18] and as well as memory T cells [19]. However, DCs are capable of priming natural killer (NK) cells, resulting in enhanced lysis of target cells, IFN-γ production and tumor clearance [20]. DC TNF-α production cooperates with IL-12 to influence NK cell IFN-γ production [17]. The activated NK cells are capable of enhancing DC maturation and IL-12 production. NK generated IFN-γ also increases IL-12 production by DCs thus shaping the ensuing adaptive immune responses towards a T helper type 1 phenotype [21]. These described interactions are cell contact and TNF-α dependent, and are further controlled by cell ratios, with a low NK: DC ratio (1:5) required for DC maturation and a high NK: DC ratio resulting in inhibition of DC function [22]. We hypothesized that in the case of HSV infection, the activation of NK cells during early stages is initiated through engagement of TLR by viral PAMP with a long term consequence on adaptive immune memory.

Although the engagement of TLRs by HSV is not novel, the role of HSV inducing the interaction NK and DC and subsequent impact the early immunological response remains unclear [23-27]. This study shows the effect of MyD88 signaling on NK:DC reciprocal interaction and its importance for the initiation of an anti-HSV immune response. Using bone marrow derived immature DCs and NK cells from MyD88$^{-/-}$ and WT mice, we addressed the question whether HSV engages TLR on either DCs or NK cells, individually, or concomitantly to induce the initiation of a cooperative relationship. TLR stimulated DCs underwent maturation and up-regulated co-stimulatory molecules (CD80 and CD86) and MHC molecules. HSV perturbs this upregulation and renders the DCs immature [6]. However, HSV DCs significantly enhanced activation and proliferation of resting NK cells compared to direct TLR stimulated NK cells. Concomitant stimulation of both NK cells and DC through MyD88 had the most significant impact on phenotypic changes and functional activation.

HSV-1 activates both TLR2 and TLR9 in a MyD88 dependent manner, suggesting the importance of TLRs in lesion development and host, as well as TLR3 in a MyD88 –dependent and –independent mechanism [26-28]. Mansur's studies further suggest that innate resistance to HSV-1 is mediated by MyD88 and may rely on activation of multiple TLRs, indicating a critical role of MyD88 in anti-viral defense [15]. Further studies are needed to examine TRIF-dependent signaling not only in the context of NK cells but as well as IFNγ production. Multiple studies have examined TRIF signaling in Type I IFN production, yet no evidence has been reported in HSV infection/stimulation model or NK cell activation [29-31].

There are a few hundred TLRs on immature DCs with the number of TLRs expressed by NK cells being unclear [2]. HSV has the potential to engage multiple TLRs on both DCs and NK cells simultaneously or in a multistep process. Sato *et al*. reported that both TLR2 and 9 can be engaged in sequence within the same DC, a probable mechanism that allows virions to engage TLR2 on the cell surface to induce maturation of endosomes that have taken up the virions to facilitate the ensuing recognition of viral genomic dsDNA by TLR9 [24]. HSV's ability to engage multiple TLRs simultaneously may enable DCs to initiate immune induction more than other cells of the early response, indicating their importance in this process. Our data indicates that HSV PAMPs activate DCs to initiate NK cell activation and initiates a productive interaction.

Our results correlate with numerous other studies in that HSV has not only evolved to infect the professional APC; the virus has acquired efficient means to prevent DC activation following infection and may actually be largely responsible for the phenomenon [6, 32, 33]. Down-regulation of co-stimulatory molecules and MHC-I molecules has been characterized as an HSV immune avoidance mechanism [6]. Consequently, these HSV infected DC are now capable of a productive interaction with resting NK cells according to the "missing-self" theory [34]. Likewise, DCs stimulated with TLR ligand(s) results in the production of key cytokines such as IL-12, IL-15, IL-18 and Type I IFNs, which induce and promote NK cell proliferation, survival, and cytotoxicity (as reviewed in [35]). The cooperative relationship of NK cells with DC (plasmacytoid and myeloid) was effectively compounded suggesting that the role of TLRs in NK cells might be indirect [36]. Yet, no studies have focused on the MyD88 dependent NK cell activation and the ability to augment the NK:DC interaction in the context of HSV.

A MyD88 knock-out system was employed to examine the impact of negating TLR signaling in HSV infection. Bone marrow DCs and NK cells derived from MyD88 knock-out mice were mixed and matched with WT DCs or NK cells and analyzed for the response to HSV or TLR ligands. Ablation of MyD88 mediated signaling resulted in defective production of DC derived IL-12 as also shown by Hou *et al*. [37]. NK activation and IFNγproduction was decreased in the absence of MyD88 signaling. However, unlike phenotypic activation, IFNγproduction seem to be more dependent on the MyD88 adaptor protein presence in the DC population, as WT DCs were able to compensate for the loss of MyD88 in NK cells. IFNγ production was reduced significantly in the absence of MyD88 in both populations compared to other combinations suggesting that early NK cell activation involves direct engagement of TLRs by viral PAMPs, while continued IFNγ production by NK cells may be mediated by virus activated DC produced cytokines like IL-12 or IL-18. Barr *et al.* describe the role of DC subpopulations and IL-18 dependent activation of NK cells results following HSV-1 infection [38]. Future studies are needed to examine the contributions of various DCs subpopulations to NK cell immunobiology like has been addressed in context of T cell priming and activation [39].

However, controversy exists over the precise viral structure or glycoprotein(s) that engage the TLR to initiate

the cooperation between cells such as NK cells and DCs. HSV infected monocyte derived DC were recently found to respond to viral glycoproteins gB, gD, gH and gL, independently of other viral proteins or nucleic acids leading to up-regulation of costimulatory molecules and HLA-DR. Unexpectedly, all four glycoproteins were necessary and sufficient for the induction of DC maturation [25].

Numerous studies reveal the beneficial impact of administering TLR agonists to enhance the resistance against an infection. Yet, the exact component of HSV or specific glycoprotein(s) that serve as TLR ligands remains to be elucidated. If addressed, therapies could be designed to not necessarily to prevent infection but to decrease the severity of sequelae such as keratitis or encephalitis. In addition, search for HSV proteins that directly engage NK receptors (inhibitory or activating) may yield clues as to the role NK cells play at different stages of infection. Such information will be useful to manipulate NK cells to suit adaptive immune response.

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