Dendritic Cells Are Required for Optimal Activation of Natural Killer Functions following Primary Infection with Herpes Simplex Virus Type 1[∇]

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Natural killer (NK) cells play an important role in the optimal clearance of herpes simplex virus type 1 (HSV-1) infection in mice. Activated NK cells function via cytokine secretion or direct cytolysis of target cells; dendritic cells (DCs) are thought to make critical contributions in the activation of both of these functions. Yet, the magnitude and physiological relevance of DC-mediated NK cell activation in vivo is not completely understood. To examine the contribution of DC help in regulating NK cell functions after infection with HSV-1, we utilized a transgenic mouse model that allows the transient ablation of DCs. Using this approach, it was found that the gamma interferon (IFN- γ) expression potential of NK cells is quantitatively and qualitatively impaired in the absence of DCs. With regard to priming of NK cytolytic functions, the ablation of DCs did not significantly affect cytotoxic protein expression by NK cells. An in vivo cytolytic assay did, however, reveal impairments in the magnitude of NK cell cytotoxicity. Overall, this study provides direct evidence that functional DCs are required for optimal IFN- γ expression and cytolytic function by NK cells following infection with HSV-1.

Natural killer (NK) cells are powerful innate effectors of the immune system that play an especially important role in the development of optimal antiviral, antibacterial, and antitumor immunity. Activated NK cells operate through direct cytolysis of target cells and through cytokine secretion (16). Dendritic cells (DCs) make crucial contributions affecting the overall magnitude of NK cell activation (13). Recent studies using intravital microscopy show that DCs interact with NK cells in strategic areas of secondary lymphoid tissues (7). The functional DC/NK interactions that take place in these tissues are thought to be particularly important in the regulation of both innate resistance (3, 19, 31) and adaptive immunity to pathogens (4) and tumors (17).

DCs activate NK cells via a combination of direct cell contact and the secretion of inflammatory cytokines (23, 62). In vitro coculture studies have shown gamma interferon (IFN- γ)

production by NK cells to be dependent on the synergistic interaction of DC-derived interleukin-12 (IL-12) and IL-18 following stimulation with lipopolysaccharide (18) and poly(I:C) (19). Barr et al. (9), by contrast, have shown herpes simplex virus type 1 (HSV-1)-induced IFN- γ expression by NK cells in vitro to be dependent on IL-18 but not IL-12. Type I IFN production by DCs plays a central role in the induction of in vitro NK cell cytotoxicity following activation by viruses or by Toll-like receptor (TLR) ligands (19, 23).

In vivo studies have demonstrated that the activation of NK cell responses to viral and bacterial pathogens requires the presence of CD11c^{high} DCs (31, 39, 48). In the absence of DCs, HSV-1-infected mice were found to have significantly lower levels of activated NK cells and succumbed to mortality within 4 days of infection (31). Although it was speculated that DC/NK interactions were important for innate resistance to HSV-1 infection, the nature of this interaction was not examined (31). With regard to the protozoan parasite *Leishmania infantum*, the induction of NK cell responses was found to be dependent on DCs via a TLR-9- and IL-12-linked mechanism (48). Lucas et al., by contrast, found that NK cell responses to viral and bacterial pathogens in vivo were dependent on DCs via a type I IFN- and IL-15-dependent mechanism and that IL-12 played a limited role in DC priming of NK cells (39).

NK cell functions are highly regulated by a balance of activating and inhibitory receptors (38). Many of these inhibitory receptors are specific for major histocompatibility complex class I (MHC-I) molecules—making NK cell lysis, in large part, a function of MHC-I expression on target cells (42), with target

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cells expressing low levels of MHC being more susceptible to NK lysis than cells expressing high levels of MHC. Cells lacking the beta-2 microglobulin ($\beta 2 m^{-/-}$) gene, which encodes the small subunit of the MHC-I molecule and is generally required for the transport of class I heavy chains from the endoplasmic reticulum to the cell surface, are particularly sensitive to NK cell lysis both in vitro (42) and in vivo (29). Most studies of the role of DCs in regulating NK cell cytotoxicity have been performed using the traditional chromium-51 (^{51}Cr) release assay, a method limited to the in vitro and ex vivo analysis of end-stage lysis of target cells; thus, the in vivo magnitude of DC-mediated activation of NK cell cytotoxicity and the physiological relevance of this phenomenon following viral infection remain unknown (5, 39, 48).

In this study, we took advantage of a transgenic mouse model in which DCs can be transiently ablated (30) to examine the in vivo contribution of DCs to the induction of NK cell activation following subcutaneous foot pad (FP) infection with HSV-1. It was found that the depletion of DCs leads to a profound impediment in the acquisition of important activation markers but does not affect the granule-forming abilities of NK cells. Functionally, intracellular staining revealed that only a specific subset of NK cells, limited to the popliteal lymph nodes (PLN) of mice, had the ability to produce IFN- γ and that the absence of DCs led to a marked decrease in IFN- γ production by NK cells. These observations indicate that DC help is essential for IFN- γ expression. An in vivo flow cytometric carboxyfluorescein succinimidyl ester (CFSE)-based assay was used to study NK cell-mediated cytolysis following infection. Using this approach, the highest levels of NK cytolysis were found in non-DC-depleted HSV-1-infected mice. The absence of DCs, however, did not lead to a complete abolishment of NK cell-mediated cytolysis; similar levels of activity were observed in naïve, DC-depleted, and DC-depleted infected mice. Collectively, these results suggest that DC help is required for the optimal priming of NK cell cytotoxicity. Moreover, they indicate the potential existence of two NK cell populations in terms of having a DC requirement for the activation of cytotoxicity, with one population being dependent on DC priming and the other being independent of DC priming following viral infection. Finally, the role of NK cells in innate resistance against HSV-1 was examined. Depletion of NK cells did not significantly affect viral titers or viral spread. Likewise, NK cell depletion had no marked effect on HSV-1-specific CD8 $^{+}$ T-cell responses. Collectively, the data presented herein provide new insights into the relevance of DC-mediated regulation of NK cell functions in vivo within the context of HSV-1 infections.

MATERIALS AND METHODS

Mice. All studies have been reviewed and approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee. A breeding pair of B6.FVB-Tg Itgax-DTR/EGFP 57Lan/J (DTR) mice was purchased from Jackson Laboratories (Bar Harbor, ME) at 4 to 5 weeks of age (15th-generation backcross to C57BL/6) (30). Mice were bred in house and were genotyped from tail DNA using published primer sequences (30). Male B6 (H-2b) and B6.129P2-B2mtm1Unc/J ($\beta 2 m^{-/-}$) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were used between 6 and 12 weeks of age.

DT treatment and HSV-1 immunization of DTR mice. DTR transgenic mice were injected intraperitoneally (i.p.) with 100 ng diphtheria toxin (DT) (Sigma-Aldrich, St. Louis, MO) suspended in phosphate-buffered saline (PBS) or given

PBS alone (36). Sixteen to 24 h posttreatment with DT or PBS, mice were anesthetized by i.p. injection of 60 mg/kg of bodyweight of sodium pentobarbital (Butler, Columbus, OH). Mice were then given 5×10^6 PFU HSV-1 in 50 μl of Iscove's modified Dulbecco's medium (IMDM) in each hind FP. Twenty-four to 30 h postinfection (p.i.), mice were killed. The spleen, draining PLN, and inguinal lymph nodes (ILN) were harvested for phenotypic and cytotoxicity analyses using fluorescence-activated cell sorting (FACS).

NK cell depletion. Male B6 mice were depleted of NK cells by the administration of an anti-NK1.1 immunoglobulin G (IgG) antibody (PK136) (32, 33). Mice were given i.p. injections of 600 μg of antibody diluted to 0.5 ml in normal saline (32, 33). Control mice were injected with saline alone.

Virus. HSV-1 strain KOS, obtained from S. L. Wechsler, University of California, Irvine, previously plaque purified three times on Vero cell monolayers, was established as a stock by infection of CV-1 cells at a multiplicity of infection of 0.01.

Poly(I:C) treatment. B6 mice were injected i.p. with poly(I:C) (0.1 mg/mouse) (Sigma Chemical Co., St. Louis, MO) diluted to 0.5 ml in normal saline. Control mice were injected with saline alone.

CFSE-based in vivo cytolytic assay. A 1:1 mixture of target cells labeled with differential concentrations of CFSE was used to detect in vivo cytotoxic activity. Briefly, spleens were harvested from $\beta 2 m^{-/-}$ and B6 mice, the latter group serving as a control target cell population. Erythrocytes were removed from the generated splenocyte suspensions by osmotic lysis. The cells were then washed and split into two equal populations. The $\beta 2 m^{-/-}$ target cell population was incubated at 37°C for 45 min and labeled with 5.0 μM CFSE (CFSE $^{\text{hi}}$ cells). The B6 control target population was pulsed and labeled with 0.5 μM CFSE (CFSE $^{\text{lo}}$ cells). For intravenous (i.v.) injection, an equal number of cells from each population was mixed together such that each mouse received a total of 8 to 10 million cells in 300 μl of saline solution. Cells were injected into mice that had previously been infected with HSV-1 or treated with poly(I:C). After 5 h, mice were killed for their lymph nodes and spleens. Harvested organs were homogenized into single-cell suspensions, washed, and suspended in PBS containing 1% fetal calf serum. A FACSCalibur instrument (Becton-Dickinson [BD], San Jose, CA) was used for event acquisition. Up to 3×10^4 CFSE-positive cells were collected for analysis, which was performed using Flow Jo software (Tree Star, Ashland, OR) and Cell Quest (BD). To calculate specific lysis, the following formula was used: ratio = (percent CFSE $^{\text{lo}}$ /percent CFSE $^{\text{hi}}$). Percent specific lysis = $[1 - (\text{ratio unprimed}/\text{ratio primed})] \times 100$.

Antibodies and reagents. The following reagents were used in this study: phycoerythrin (PE) or biotin-conjugated anti-NK1.1 (clone PK136; eBiosciences), allophycocyanin (APC) anti-B220 (clone RA3-6B2; eBiosciences), APC anti-CD16 (clone 93), and biotin anti-CD11c (clone N418; eBiosciences). To analyze biotinylated reagents, streptavidin-PerCP (BD) was used for phenotypic analysis of cell populations in the spleen. For the intracellular staining of perforin and granzyme B, cells were fixed and permeabilized after surface staining by using a fixation and permeabilization kit (eBiosciences). Cells were intracellularly stained with a PE-conjugated anti-mouse perforin antibody (clone eBioOMAK-D; eBiosciences) and fluorescein isothiocyanate-conjugated anti-mouse granzyme B antibody (clone 16G6; eBiosciences) or the appropriate isotype controls (PE rat IgG2a, catalog no. 12-4321, and fluorescein isothiocyanate rat IgG2b, catalog no. 11-4031; eBiosciences). For the intracellular analysis of ex vivo IFN- γ production by NK cells, single-cell splenocyte suspensions were cultured for 5 h in a 96-well U-bottom microtiter plate (Costar, Cambridge, MA) at a concentration of 1×10^6 cells/well in 0.2 ml IMDM containing 10% fetal calf serum, 20 mM HEPES, 2 mM L-glutamine, 50 μM β -mercaptoethanol, and 20 μg gentamicin sulfate (complete medium) with 1 $\mu\text{l}/\text{ml}$ brefeldin A (GolgiPlug; BD Biosciences). With respect to the positive control group, cells were cultured in the presence of 500 ng/ml ionomycin (Sigma) and 5 ng/ml phorbol myristate acetate (PMA). At the end of the culture period, cells were surface stained with PE anti-NK1.1 (clone PK136; eBiosciences, San Diego, CA), fixed and permeabilized by using a fixation and permeabilization kit (eBiosciences), and intracellularly stained with APC anti-IFN- γ (clone XMG1.2; eBiosciences). Cells were collected for analysis by using a FACSCalibur instrument (BD, San Jose, CA). The data were analyzed using Flow Jo software (Tree Star, Ashland, OR) and CellQuest (BD). The same protocol was utilized for intracellular IFN- γ and tumor necrosis factor alpha (TNF- α) detection of HSV-1-specific CD8 $^{+}$ T cells from the PLN of mice, with the following modifications. Lymph node cells were cultured at a concentration of 1×10^6 cells/well in 0.2 ml complete medium with 1 $\mu\text{l}/\text{ml}$ GolgiPlug, HSV-1 glycoprotein B ($^{498}\text{SSIEFARL}^{505}$) peptide, or vesicular stomatitis virus nucleoprotein ($^{52}\text{RGYGYOGL}^{59}$) as a control (31).

IFN bioassay. Mice were bled at the times indicated, and serum was separated by high-speed centrifugation. Total IFN bioactivity from the serum was measured by inhibition of the cytopathic effect of encephalomyocarditis (EMC) virus

FIG. 1. DC depletion abolishes type I IFN production during primary HSV-1 infection. CD11c/DTR-Tg mice were i.p. injected with PBS (0.5 ml/mouse) or DT (100 ng/mouse) 24 h prior to hind-FP infection with HSV-1 at 5×10^6 PFU per FP. Thirty hours p.i. with HSV-1, spleens were harvested for FACS analysis of DC frequencies. (A) Representative FACS contour plots depicting the percentages of total CD11c⁺ MHC-II⁺ DCs. (B) Thirty hours p.i. with HSV-1, serum samples were harvested via cardiac blood withdrawal. Type I IFN levels were determined by virus neutralization assay. Values represent the means $\pm$ standard deviations of pooled data from two independent experiments with two to three mice per group. DT + HSV-1, DT-treated HSV-1-infected mice ($n = 5$); PBS + HSV-1, PBS-treated HSV-1-infected mice ($n = 6$); naïve mice ($n = 4$). Statistical analyses using the Mann-Whitney test revealed P values of <0.05 (*) between the PBS-treated HSV-1-infected group and naïve control group and between the PBS-treated HSV-1-infected group and the DT-treated HSV-1-infected group.

on mouse L929 cells. Briefly, serial twofold dilutions of 50 μ l of IFN-containing serum were made in IMDM without serum in a 96-well plate, and 0.1 ml of L929 cells (5×10^4 cells) was added per well. Following overnight incubation, cells were challenged with 50 μ l of EMC virus at a multiplicity of infection of 1. After incubation for 24 h, the degree of cytopathy was quantified. Individual wells were ranked on a scale of 1 to 4, with 1 being all alive and 4 being all dead. One unit of IFN activity is defined as the inverse of the dilution that gave 50% protection against the cytopathic effect of EMC virus in L929 cells.

Quantitation of HSV-1 in FP and SC tissues. The level of infectious HSV-1 in the FP and spinal cord (SC) was determined as described previously (31). Briefly, individual FPs and the SC were removed and stored in complete medium at -80°C . Tissues were disrupted by homogenization in 1-ml glass grinders (Wheaton, Millville, NJ) and centrifuged at high speed ($400 \times g$). The resultant cell-free homogenate was assayed at various dilutions on Vero cell monolayers in 12-well tissue culture plates overlaid with 0.5% methylcellulose. Plaques were visualized following fixation of the monolayers with 10% buffered formalin and staining with 0.5% crystal violet.

Statistical analyses. When the experimental design involved comparison of two groups, the Mann-Whitney t test was used. When experimental designs involved multiple treatments, analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used. Statistical analyses were performed using GraphPad 4.0 software (GraphPad, Inc., San Diego, CA). The P value of significant differences is reported. Unless otherwise stated, plotted data represent the means $\pm$ standard errors of the means (SEMs) of the results.

RESULTS

DC depletion abolishes type I IFN production during primary HSV-1 infection. The CD11c-DTR-Tg mouse model utilizes a DT-based system that allows for the inducible, transient ablation of CD11c⁺ DCs following treatment with DT (50). This model has successfully been used to study the role of DCs in microbial immunity (30, 31, 36, 43) and in the development of airway hypersensitivity (60). We therefore took advantage of the CD11c-DTR-Tg model to examine whether DCs are

important for triggering optimal NK cell activation and cytotoxicity following infection with HSV-1. The efficiency of DC depletion was verified in mice 30 h p.i. Non-DC-depleted HSV-1-infected mice had a higher frequency of DCs than DT-treated HSV-1-infected mice and naïve control mice (Fig. 1A).

We next examined the functional implications of DC ablation, specifically with regard to cytokine production, following HSV-1 infection. DC-derived cytokines, including IFN- α/β , IL-12, IL-15, and IL-18, have been implicated in NK cell activation in vitro (62). Of these, IFN- α/β seem to be especially important for NK cell cytotoxicity (62). We therefore examined serum type I IFN levels in DC-ablated and non-DC-ablated mice 30 h after HSV-1 infection. DC ablation led to a significant decrease in the production of functional type I IFN ($P < 0.05$) (Fig. 1B); no significant differences were found between DC-ablated HSV-1-infected and non-HSV-1-infected control mice (Fig. 1B). The depletion of macrophages did not affect HSV-1 pathogenesis (31), nor did it affect type I IFN production (data not shown). These data confirm that, as observed for other viral infections (12), DCs are the principal source of type I IFN during the early immune response to HSV-1. We next examined the implications of reduced type I IFN production for NK cell activation.

DC depletion impairs NK cell activation. Several cell surface molecules, including B220, Thy-1, CD2, CD16, LFA-1, CD69, CD45RA, and CD45RO, are known to be upregulated on activated murine and human NK cells (63). Of these, B220 and CD16 are thought to be particularly important determinants of NK cytotoxicity, with B220 being a marker of non-

FIG. 2. DC depletion impairs optimal NK cell activation. (A) Spleens were harvested from DT-treated (DT + HSV-1), PBS-treated (PBS + HSV-1), and naïve DTR-Tg mice 30 h p.i. with HSV-1 and stained for NK1.1 and CD3. (B) B220 or CD16 expression by NK1.1⁺ CD3⁻ cells from naïve, DT-treated HSV-1-infected mice (DT + HSV-1), and PBS-treated HSV-1-infected mice (PBS + HSV-1) was analyzed. Representative histograms for one mouse are displayed. These data are representative of the results of two separate experiments using a minimum of four mice per group per experiment.

MHC-restricted killers (8, 31) and CD16 being a marker of antibody-dependent cellular cytotoxicity but not other modes of NK cell-mediated cytotoxicity (34).

To initially determine the cytotoxic potential of NK cells in the absence of DCs, the frequency of B220⁺ NK and CD16⁺ NK cells was examined 30 h after subcutaneous FP infection with HSV-1 in DT-treated and PBS-treated CD11c-DTR-Tg mice. Because secondary lymphoid organs are important sites of DC/NK interaction (7, 16), a specific emphasis was placed on the levels of activated NK cells in the spleens of mice. As previously reported (39, 48), DT treatment does not appear to impair the total frequency of splenic NK1.1⁺ CD3⁻ cells (Fig. 2A). DC-intact mice were found to have a higher frequency of NK1.1⁺ CD3⁻ B220^{hi} (Fig. 2B, left panel) and NK1.1⁺ CD3⁻ CD16^{hi} cells (Fig. 2B, right panel) than DC-ablated and naïve mice. DC-ablated and naïve mice had comparable levels of activated NK cells (Fig. 2A and B). These data extend previously published results (31) and suggest that the absence of DCs impedes the optimal activation, and perhaps the proliferation or recruitment, of NK cells to secondary lymphoid organs following infection with HSV-1. We next examined the effect of DC depletion on NK cell function.

DCs are not required for granule formation by NK cells. Like CD8⁺ cytotoxic T lymphocytes, NK cells utilize the mechanism of granule exocytosis in cell death. Lytic granules, which contain a mixture of potent proteins, release their content into the intercellular space between the NK cell and target cell, whereby the action of these enzymes results in target cell death

(35, 56). Major granule proteins include perforin (Prf), a cell membrane disruptor, and granzymes (Grz), a family of structurally related serine proteases with various substrate specificities (53). To date, 11 granzymes have been identified (54). GrzB, found in both humans and mice, appears to play a particularly important role in triggering target cell death (53). Prf and GrzB function synergistically by forming a single macromolecular complex at the target cell surface, which triggers nuclear damage and results in cell death (41, 50). The signals that regulate granule formation and polarization upon activation are not completely understood. Recent studies, however, suggest that DCs are important for the optimal expression of GrzB and Prf by activated CD8⁺ cytotoxic T lymphocytes and NK cells (15, 39). Whether DCs play a similar role in NK cell granule formation following HSV-1 infection is not known. Ex vivo intracellular staining and flow cytometric analysis was therefore undertaken to analyze the effect of DC ablation on GrzB and Prf expression by activated NK cells 24 to 30 h p.i. with HSV-1. Using this approach, comparable levels of Prf (Fig. 3A and C) and GrzB (Fig. 3B and D) were found for DC-depleted and non-DC-depleted mice. Infected mice, regardless of the presence of DCs, expressed higher levels of Prf (Fig. 3A and C) and GrzB (Fig. 3B and D) after infection than naïve mice. B220⁺ NK1.1⁺ CD3⁻ cells expressed significantly higher levels of Prf (Fig. 3A and C) ($P < 0.05$) and GrzB (Fig. 3C and D) ($P < 0.001$) than did B220⁻ NK1.1⁺ CD3⁻ cells in both DC-ablated and non-DC-ablated mice; no significant differences were observed between these NK cell subsets in naïve

FIG. 3. Intracellular expression of granzyme B and perforin is not affected by the presence of DCs. (A and B) Thirty hours following HSV-1 infection, single-cell splenocyte suspensions were analyzed by flow cytometry. Expression of perforin (A) and granzyme B (B) on NK1.1⁺ CD3⁻ B220⁻ cells (solid black lines), NK1.1⁺ CD3⁻ B220⁺ cells (broken lines), and isotype controls (solid gray lines) was analyzed. (C and D) Average perforin (C) and granzyme B (D) MFIs were calculated. Bars represent means $\pm$ SEMs of the results from two independent experiments using a minimum of three mice per treatment group per experiment. Statistical analyses were performed by using ANOVA followed by Tukey's multiple comparison test, and results are indicated by asterisks. PBS + HSV-1, PBS-treated HSV-1-infected mice; DT + HSV-1, DT-treated HSV-1-infected mice; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mice. These data suggest that the absence of DCs does not lead to the generation of dysfunctional NK cells as measured by lytic-granule protein expression on a per-cell basis. Given these results, we sought to more thoroughly gauge the functionality of NK cells in vivo.

DC depletion impairs optimal NK cell cytotoxicity. An in vivo flow cytometric-based NK cytolytic assay, which takes advantage of the property of NK cells of selectively lysing $\beta 2$ m^{-/-} cells, was used to examine NK cell function in DC-depleted and non-DC-depleted mice during infection with HSV-1. $\beta 2$ m^{-/-} (NK sensitive) and wild-type (NK resistant) murine splenocytes were labeled with the fluorochrome CFSE at the concentrations CFSE^{hi} and CFSE^{lo}, respectively, combined at a 1:1 ratio, and used as target cells for the in vivo NK cytotoxicity assay. Twenty-four to 30 h p.i. with HSV-1, 8 to 10 million target cells were injected i.v. into DC-ablated and nonablated CD11c-DTR-Tg mice. Five hours postinjection of target cells, mice were sacrificed and their spleens and ILN were harvested. The extent of NK cell-mediated cytolytic activity in each of these organs was then assayed through measuring the loss of the CFSE^{hi} cell population.

Pretreatment of C57BL/6 mice with the synthetic double-stranded RNA-like polymer poly(I:C), a known inducer of type I IFN and NK cell activation (14), results in a complete loss of the CFSE^{hi} target cell population amounting to 96.9% and 96.6% total NK cell-mediated cytotoxicity in the spleens and ILN (Fig. 4A and B), respectively. Mice depleted of NK cells via treatment with anti-NK1.1 IgG antibody (PK136) 36 h prior to poly(I:C) treatment had nearly equivalent levels of CFSE^{hi}

and CFSE^{lo} target cell populations (Fig. 4A and C), indicating a nearly complete loss of NK cell-mediated cytotoxicity, which confirms the NK1.1⁺ cell-specific nature of the in vivo cytotoxicity assay results. DC-depleted CD11c-DTR-Tg mice infected with HSV-1 displayed 31.8% NK-specific lysis, compared to 80.9% in non-DC-depleted HSV-1-infected CD11c-DTR-Tg mice. Interestingly, the levels of NK cell-mediated cytotoxicity were comparable for naïve, DT-treated HSV-1-infected, and DT-treated-only CD11c-DTR-Tg mice, with 24.9%, 31.8%, and 41.1% total cytotoxicity, respectively (Fig. 4A and C). The same overall patterns of NK cell cytotoxicity were seen in the ILN of mice (Fig. 4B and D). Collectively, these data suggest that cytolytic function is highly dependent upon the presence of functional DCs. Furthermore, these data indicate the potential presence of at least two distinct NK cell subpopulations with regard to the requirement for DC help: (i) a perpetually activated NK cell population in naïve and DT-treated-only mice; and (ii) an NK cell population requiring DC help for optimal activation following infection with HSV-1.

Optimal IFN- γ production by NK cells is dependent on DCs. In vitro evidence has shown NK cell IFN- γ production to be regulated by DCs (18), yet the relevance and magnitude of this phenomenon in vivo has not been examined. Intracellular staining and flow cytometric analysis revealed DC depletion to result in significantly lower frequencies and absolute numbers of IFN- γ -producing NK cells (Fig. 5A and C). The ablation of DCs appears to impact the physiological functionality of NK cells as well, for even stimulation with the DC-independent stimuli PMA and ionomycin resulted in a significant reduction

FIG. 4. DC depletion impairs optimal NK cell cytotoxicity. Twenty-four to 30 h after HSV-1 infection, *in vivo* cytolytic activity was measured as described in Materials and Methods. (A and B) Representative histograms depicting percent CFSE^{lo} (B6 control splenocyte) and CFSE^{hi} ($\beta 2$ m^{-/-} splenocyte) target cell peaks in the spleen (A) and ILN (B). Up to 1×10^4 CFSE-positive cells were collected for analysis. (C and D) To calculate specific lysis, the following formula was used: ratio = (percent CFSE^{lo}/percent CFSE^{hi}). For percentage of specific lysis in the spleen (C) and ILN (D), the following formula was used: $[1 - (\text{ratio CFSE}^{\text{lo}}/\text{ratio CFSE}^{\text{hi}})] \times 100$. Bars represent means $\pm$ SEMs of results from four independent experiments with one to three mice per group per experiment. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test, and results are indicated by asterisks. Poly I:C, poly(I:C)-treated mice; PBS + HSV, PBS-treated HSV-1-infected mice; dep, depleted; HSV, HSV-1 infected; DT, DT treated; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

in IFN- γ -producing NK cells (Fig. 5B and C). Moreover, based on the results of mean fluorescence intensity (MFI) analysis, DC depletion was found to significantly impair the ability of NK cells to produce IFN- γ (Fig. 5D). Interestingly, IFN- γ expression was limited to a specific subset of NK cells that expressed low levels of the B220 activation marker (Fig. 5D). Despite the clear presence of the B220^{lo} NK cell subset (Fig. 2B), no IFN- γ expression could be detected in the spleens of mice (data not shown). Together, these data suggest that DCs

regulate the IFN- γ -producing potential of NK cells and that IFN- γ production is limited to a specific B220^{lo} subset of NK cells found in the draining PLN of mice following subcutaneous HSV-1 infection.

NK cell depletion does not enhance susceptibility to FP infection with HSV-1 KOS. In addition to being a first-line defense against infection and malignancy, NK cells make important contributions as modulators of adaptive immune responses (28, 37, 55). Previous studies demonstrated that tran-

FIG. 4—Continued.

sient DC ablation prevented NK cell activation and caused the early onset of morbidity and mortality prior to the onset of peak HSV-1-specific T-cell activity (31). These observations suggested that a critical time frame exists during the immune response to HSV-1 wherein DCs are absolutely required for resistance to HSV-1 infection. Moreover, the studies suggested that NK cells contribute to the adaptive immune response and play a critical role in innate resistance to HSV-1. Given the importance of DCs in NK cell activation following HSV-1 infection, the contribution of NK cells in HSV-1-specific acquired immunity was next examined.

Five days p.i., the HSV-1-specific CD8⁺ T cells were examined via intracellular FACS analysis. Although there were detectable differences in the overall frequencies of HSV-1-specific CD8⁺ TNF- α ⁺ (Fig. 6A, left panel) and CD8⁺ IFN- γ ⁺ cells (Fig. 6A, right panel) in the draining lymph nodes of mice, no significant differences could be detected with respect to absolute cell numbers (Fig. 6B and C). Moreover,

based on a standard PFU assay, no differences could be detected with respect to viral replication at the site of infection (Fig. 7, left panel). Similarly, the depletion of NK cells does not appear to enhance viral crossover into the central nervous system of HSV-1-infected mice (Fig. 7, right panel). These data demonstrate that NK cells do not significantly contribute to the control of HSV-1 replication and spread and that they do not quantitatively affect the development of adaptive immune responses. Collectively, these studies suggest that NK cells do not play a significant role during the critical time frame thought to exist following HSV-1 infection wherein DCs are absolutely required for innate resistance (31).

DISCUSSION

Regarding in vivo IFN- γ production by NK cells, our studies revealed an essential role for DCs. Specifically, transient ablation of DCs led to a marked decrease in the frequency and

FIG. 5. IFN- γ expression by NK cells requires DCs. (A and B) Intracellular staining was used to gauge the total number of IFN- γ -producing NK cells in the PLN of mice 24 h post-HSV-1 infection as described in Materials and Methods. Representative plots depicting the frequencies of NK1.1⁺ CD3⁻ IFN- γ ⁺ cells in the absence (A) or presence (B) of PMA-ionomycin stimulation are depicted. (C) Absolute numbers of NK1.1⁺ CD3⁻ IFN- γ ⁺ cells per PLN. The bars represent the mean absolute numbers $\pm$ SEMs of NK1.1⁺ IFN- γ ⁺ for at least 7 mice per group. (–) and (+), absence and presence, respectively, of PMA-ionomycin stimulation. (D) The average MFIs of IFN- γ expression by NK1.1⁺ CD3⁻ B220⁻ and NK⁺ CD3⁻ B220⁺ in DT-treated-only, DT-treated HSV-1-infected, and PBS-treated HSV-1-infected mice are depicted. Bars represent means $\pm$ SEMs of the results from two independent experiments using a minimum of three mice per treatment group. Statistical analyses were performed by using ANOVA followed by Tukey's multiple comparison test, and results are indicated by asterisks. DT only, DT-treated-only mice; PBS + HSV, PBS-treated HSV-1-infected mice; DT + HSV, DT-treated HSV-1-infected mice; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cellularity of IFN- γ -producing NK cells early after infection and resulted in a significant impairment in the IFN- γ production capacity of activated NK cells. How NK cell IFN- γ responses are regulated by DCs *in vivo* is not completely understood. Based on *in vitro* studies, however, DC regulation of IFN- γ is likely a function of the infectious model being used

and the NK effector cell population being examined. Following murine cytomegalovirus infection, hepatic NK cell activity is exclusively dependent on IL-12, while splenic NK cell IFN- γ is dependent on both IL-12 and IL-18 (45); a synergistic interaction between IL-18 and IL-12 is required for the effective mobilization and activation of pulmonary NK cell IFN- γ re-

FIG. 6. NK cell depletion does not impair cytokine synthesis by HSV-1-specific CD8⁺ T cells. Intracellular staining was used to gauge the total frequency of HSV-1-specific CD8⁺ T-cell responses in the PLN of NK1.1-depleted and non-NK1.1-depleted mice on day 5 post-HSV-1 infection. (A) Representative histograms of TNF-α or IFN-γ production by CD8⁺ T cells are depicted. (B and C) The bars represent the mean numbers of total CD8⁺ T cells producing IFN-γ (B) or TNF-α (C) per PLN. Each bar represents the mean value obtained from a total of two experiments using four mice per treatment per experiment. The bars represent the standard deviations from the means. Statistical analysis revealed no significant differences between any of the groups examined. NK Dep, NK1.1-depleted mice; Non-Dep, non-NK1.1-depleted mice.

sponses after infection with the fungal pathogen *Cryptococcus neoformans* (46); and IFN-α/β is crucial for IFN-γ expression by NK cells in *Leishmania infantum*-infected mice but not in *Leishmania major*-infected mice (48). With respect to HSV-1, a recent report demonstrates IL-18 derived from plasmacytoid

DCs to be the crucial factor in the induction of IFN-γ expression by splenic NK cells following i.v. infection (9). Our studies, in contrast, did not detect a splenic population of IFN-γ-expressing NK cells (data not shown) but rather found IFN-γ production to be the purview of a B220^{lo} NK cell subset localized in the draining

FIG. 7. NK cell depletion does not increase susceptibility to FP infection with HSV-1 strain KOS. To quantify viral replication, FPs and SCs were harvested from mice. Lysates were then prepared and assayed for HSV-1 PFU on permissive Vero cells. (Left) Each data point represents the mean of both hind FPs from a single mouse. (Right) Each data point represents the mean log PFU per gram of SC from an individual mouse. Statistical analysis revealed no significant differences in HSV-1 PFU levels of NK1.1-depleted and non-NK1.1-depleted (Non-Depleted) mice. D5, day 5 p.i.

PLN of mice. This apparent discrepancy may be due to the route of inoculation employed, as the i.v. model induces a systemic viremia (27) and marked inflammation in the spleen, while the subcutaneous FP model results in a localized infection wherein the draining PLN play a primary role in the generation of immunity (2, 40). The PLN environment is probably more conducive than the splenic environment to the optimal priming of IFN- γ expression by NK cells after subcutaneous FP HSV-1 infection, which may explain why, despite the presence of a B220^{lo} NK cell population in the spleen, no IFN- γ could be detected. Collectively, these observations suggest that the tissue and the cytokine milieu in which the DC/NK interaction takes place are critical arbiters of function (26). In vivo studies need to be more actively pursued to delineate the mechanisms underpinning the observed compartmentalization of IFN- γ expression by NK cells and the role of DCs therein.

Recent studies using the CD11c-DTR-Tg mouse model have found DCs to be absolutely essential for the priming of NK cell cytotoxicity (40, 49). Specifically, it was found that naïve NK cells acquire effector functions only after DC priming and that this process was dependent on IL-15 (39) or IL-12 and TLR-9 (48). Data presented herein, in contrast, suggest that DC help is essential for IFN- γ expression but is not absolutely required for triggering NK cell cytotoxicity. The apparent discrepancy between these data is probably due to the cytolytic assay used to measure NK cell function. Our analysis revealed that although the lack of DCs led to a reduction in the frequency of activated cytolytic NK cell subsets in vivo, it did not affect GrzB and Prf expression by NK cells. An increase in GrzB and Prf expression was detectable after infection irrespective of the presence of DCs, which suggests that lytic granule formation, on a per-cell basis, occurs independently of DCs. Unlike resting CD8⁺ T cells (47), resting NK cells are thought to be preternaturally active in that they express perforin and cytotoxic proteins, which are stored in specialized secretory lysosomes, without prior sensitization (11). The in vivo cytotoxicity assay used in our studies revealed a basal level of NK cell cytotoxicity in naïve mice, DC-depleted mice, and DC-depleted HSV-1-infected mice; this is likely a function of constitutive cytotoxic protein expression. Recent in vitro studies have found DC-independent activation of NK cell cytotoxicity to proceed via direct activation of TLR-3 (49, 51) and of TLR-2, TLR-7, and TLR-9 (21, 25, 51), all of which are expressed by human NK cells. In these studies, NK cell activation was induced via the coculture of sorted NK cells with TLR agonists. Functionally, however, within the context of a viral infection, our results demonstrate that although NK-mediated cytolysis can be triggered in the absence of DCs, optimal NK-mediated cytolysis can only take place in the presence of DCs. A bioassay revealed that the ablation of DCs impeded type I IFN production.

Lucas and associates have elegantly demonstrated that type I IFN-induced IL-15 production by DCs is essential for NK cell priming and survival in vivo (39). The reduced frequency of activated NK cells in DC-ablated and DC-ablated HSV-1-infected mice observed in our studies is likely a reflection of the low levels of type I IFN and subsequent low levels of IL-15 (22). The absence of DCs in our studies, however, did not result in the complete elimination of activated NK cells in DC-ablated and DC-ablated HSV-1-infected mice. Rather, these mice had levels of activated NK cells comparable to those of naïve controls. Together, the data argue for a differ-

ential DC requirement in the activation of NK cell cytotoxicity: an NK cell population dependent on DC priming for optimal activation and an NK cell population that is independent of DC priming. O'Leary et al. have shown that NK cells are able to mediate long-lived, antigen-specific adaptive recall responses independent of B and T lymphocytes (44). It is therefore conceivable that the DC-dependent NK cell population observed in our studies is being activated via a heretofore-unknown "antigen presentation" step mediated by DCs which is dependent on type I IFN and IL-15, while the DC-independent NK cell population is being directly activated via pathogen recognition receptors expressed on the cell surface, although this notion requires much more extensive study. The following questions are among those that need to be resolved. Can "DC-dependent" and "DC-independent" NK cells be phenotypically identified and functionally differentiated? What are the exact in vivo requirements of NK cells for DC help? Are the requirements for DC help by NK cells similar in both lymphoid and nonlymphoid compartments during infection?

For mice, no consensus exists with respect to the role of NK cells in intrinsic resistance to HSV-1 and HSV-2 (1, 6, 10, 20, 24, 52, 57–59, 61). Depending on the method of NK cell depletion, strains of virus and mouse utilized, and the route of inoculation, resistance to HSV infection is reduced (6, 10, 20, 52, 57–59) or not substantially affected (9, 24) in the absence of functional NK cells. Studies using mice that lack components of the NK cell activation machinery suggest NK cells to be important for the control of persistent, but not acute, HSV-1 infection (61). Our data confirm and extend these findings. Specifically, the depletion of NK cells did not significantly affect the innate resistance of B6 mice to HSV-1 infection as measured by viral replication and spread. Moreover, the depletion of NK cells did not affect the downstream HSV-1-specific immune response.

Collectively, the studies described herein argue an important selective role for DCs in NK cell activation following HSV-1 infection. Although DCs are important for optimal NK cell activation, they do not appear to be important for the effective control and clearance of acute subcutaneous FP infection with HSV-1. Moreover, in contrast to results for other pathogens (28, 37, 55), NK cells appear to be dispensable in the development of HSV-1-specific T-cell immunity. These findings may have bearing on the development of effective antiviral and antitumor therapies.

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