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NK Cells Regulate CD8+ T Cell Priming and Dendritic Cell Migration during Influenza A Infection by IFN-γ and Perforin-Dependent Mechanisms

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An effective immune response against influenza A infection depends on the generation of virus-specific T cells. NK cells are one of the first-line defenses against influenza A infection. We set out to delineate the role of NK cells in T cell immunity using a murine model of influenza A infection with A/PR/8/34. We show that early T cell recruitment mainly occurs in the posterior mediastinal lymph node (pMLN). Depletion of NK cells significantly impaired both dendritic cell (DC) and T cell recruitment into the pMLN. A similar reduction of T cell recruitment was observed when migration was blocked by pertussis toxin, suggesting that migration of pulmonary NK cells and DCs regulates cell recruitment to the pMLN. T cell recruitment was dependent on IFN-γ, and transfer of IFN-γ−competent naive NK cells into IFN-γ−/− mice restored T cell recruitment, whereas IFN-γ−deficient NK cells failed to do so. In addition, NK cell depletion reduced the uptake and transport of influenza A virus by DCs, and significantly impaired the virus-specific T cell response. Both IFN-γ−/− and perforin−/− mice showed reduced viral Ag transport by DCs, suggesting that the ability of NK cells to influence virus transport depends on IFN-γ and perforin. In summary, our data suggest that NK cells play a critical role in the initiation and shaping of the T cell response after influenza A infection. The Journal of Immunology, 2012, 189: 2099–2109.

Infection with influenza A virus has caused four pandemics in the last century, with a cumulative death toll >50 million (1, 2), and still leads to 250,000–500,000 annual deaths today (3). In 2009, a novel strain of swine-origin pandemic influenza A/H1N1 emerged and spread to >214 countries (4). NK cells are an early line of defense against influenza A infection and are important for initiating the CTL response against influenza A in vitro (5) and in vivo (6). Depletion of NK cells renders mice more susceptible to influenza A infection (7). The protective role of NK cells in influenza A infection is not well understood, but it has been shown that NK cells recognize and lyse influenza A-infected cells in vitro (8) and in vivo (9). This effector function is largely mediated through perforin because perforin−/− mice reportedly displayed delayed viral clearance and loss of NK-mediated cytotoxicity (10). Influenza A virus infection also results in greatly increased NK cell-derived pulmonary IFN-γ production in mice (11).

Dendritic cells (DCs) potentiate the effector functions of NK cells through secretion of IL-12 (11) and IL-15 (12) post-influenza A infection. Pulmonary DCs migrate to lung draining lymph nodes (DLNs), where they prime both CD8+ and CD4+ T cell responses (13, 14). The CD8+ T cell response is impaired if DC migration is defective as in CCR7−/− mice (15). CD8+ T cells play an important role in controlling viral spread and limiting disease progression by recognizing and lysing infected cells (16). Adoptive transfer of cytotoxic T cell clones recognizing the nucleoprotein of influenza A virus has been shown to protect naive mice from a lethal dose of influenza A virus (17).

Using a murine model of influenza A/PR/8/34 infection, we showed that NK cell depletion impaired DC and T cell recruitment to the posterior mediastinal lymph node (pMLN). IFN-γ produced by NK cells was crucial for DC and T cell recruitment to the pMLN. Furthermore, virus uptake by DCs depended on perforin-mediated lysis of influenza A-infected cells by NK cells. Hence our data show that NK cells regulate the CD8+ T cell response against influenza A by controlling immune cell migration and virus transport.

Materials and Methods

Mice

C57BL/6 and OT-I transgenic mice were purchased from Charles River Laboratories (Wilmington, MA). IFN-γ−/− and perforin−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Seven- to 8-wk-old wild type (WT) and 7- to 10-wk-old genetically modified mice were used in all experiments. All mice were maintained under specific pathogen-free conditions within National University of Singapore’s animal holding unit and used according to institutional guidelines. All procedures were under the Institutional Animal Care and Use Committee protocol numbers 137/08 and 087/10.

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; DC, dendritic cell; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine; DLN, draining lymph node; HA, hemagglutinin; IP-10, IFN-γ inducible protein 10; i.t., intratracehally; MLN, mediastinal lymph node; p.i., postinfection; pMLN, posterior MLN; PT, pertussis toxin; WT, wild type.

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**Virus and infection**

Influenza A/P/8/34 was obtained from American Type Culture Collection (Manassas, VA) and recombinant influenza A/P/8/34 containing chicken OVA epitope SIINFEKL (OT-1 PR8) was a kind gift of Dr. P. Thomas and Prof. Peter Doherty (St. Jude Children’s Hospital, TN). Virus was propagated in the allantoic fluid of 10-12-day-old embryonated chicken eggs at 37°C as previously described (18). Virus infectivity was determined using a plaque assay with Madin-Darby canine kidney cells as described previously (19). Mice were anesthetized by i.p. injection of a mixture containing ketamine (100 mg/kg; Sigma-Aldrich, St. Louis, MO) and medetomidine (15 mg/kg; Orion Pharma, Espoo, Finland). A total volume of 20 μL sterile saline containing 5 PFU influenza A virus was administered intranasally (i.n.). Atipamezole (5 mg/kg; Orion Pharma) was administered i.p. as re- versal to the anesthesia postinfection (p.i.).

**Generation of 1,1'-dioctadecyl-3,3,3',3'-tetramethylinodiocarbocyanine-labeled virus**

Influenza A virus harvested from allantoic fluid was labeled with the lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylinodocarbocyanine (DiD; Molecular Probes, Invitrogen, Singapore) as previously described (20) with modifications. In brief, virus-containing allantoic fluid was concentrated in a Vivascin 20 centrifugal concentrator with a membrane pore size of 100,000 m.w. (Sartorius, Goettingen, Germany) and passed through a 0.2-μm filter. Twenty microliters of the DiD stock solution (20 mg/ml dissolved in DMSO) was added to 1 ml of the allantoic fluid and incubated for 1 h at room temperature with frequent mixing. Nonincorporated DiD was removed by concentrating at 13,000 × g for 5 min. The supernatant containing the virus was further purified by ultracentrifugation over a single 20–50% sucrose step gradient at 112,000 × g for 90 min. The layer containing the virus was harvested and run over NAP-5 columns (GE Healthcare, Singapore) or Zeber desalting columns (7000 m.w. cutoff; Thermo Fisher Scientific, Singapore) according to the manufacturer’s protocol to remove the sucrose.

**Single-cell suspension**

Cells were euthanized and the lungs were removed, cut into small pieces, and digested with Liberase Blendzyme (Roche Diagnostics, Singapore) for 45 min. A single-cell suspension was obtained after passing through a 61-μm cell strainer (BD Pharmingen, Singapore). The mediastinal lymph nodes (MLNs) were removed and passed through a 61-μm cell strainer (BD Pharmingen). For analysis of DCs, MLNs were first digested with Liberase Blendzyme (Roche Diagnostics) for 30 min before passing through a 61-μm cell strainer (BD Pharmingen). Bronchoalveolar lavage fluid (BALF) was collected through aspiration of the lungs with 1 ml PBS three times through an incision at the trachea.

**NK cell depletion**

The hybridoma cell line (HB 191) against NK 1.1 (clone PK136) was purchased from American Type Culture Collection. Cells were cultured in Hybridoma SFM (Life Technologies, Singapore), and the supernatant was harvested. Ab was purified using fast protein liquid chromatography (GE Healthcare, Singapore) and quantified using Bradford assay (Thermo Healthcare, Singapore) or Zeber desalting columns (7000 m.w. cutoff; Thermo Fisher Scientific, Singapore) according to the manufacturer’s protocol to remove the sucrose.

**NK cell purification**

Splenic NK cells were isolated using EasySep Mouse NK Cell Enrichment Kit (STEMCELL, Singapore) according to the manufacturer’s protocol. Purity of isolated NK cells was >70% as determined by flow cytometry. Cells were resuspended in PBS and injected i.v. into mice (1–1.5 × 10⁶ cells/mouse).

**OT-I T cell isolation and CFSE labeling**

OT-I T cells were purified from spleen and lymph nodes of OT-I mice using Ficoll (GE Healthcare) and anti-CD8 Ab-coated MACS beads (Miltenyi Biotec, Singapore). CD8⁺ T cells were >95% pure as determined by flow cytometry. Purity of isolated CD8⁺ T cells was 5 μM violet CFSE (Molecular Probes, Invitrogen) for 15 min at 37°C. The reaction was quenched with culture medium. Cells were washed twice and resuspended in PBS. Mice were injected i.v. with 1 × 10⁶ T cells.

**Pulmonary cell migration assay**

Mice were anesthetized by i.p. injection of a mixture containing ketamine (100 mg/kg; Sigma-Aldrich, Singapore) and medetomidine (15 mg/kg; Orion Pharma, Newbury, U.K.). Fifty microliters CFSE (8 μM; Invitrogen) was administered intratracheally (i.t.). Atipamezole (5 mg/kg; Orion Pharma) was administered i.p. as anesthetic reversal. Mice were then injected with 5 PFU of influenza A virus (H1N1) 6 h after CFSE inoculation. pMLNs were collected for analysis at day 3 p.i.

**Real-time PCR**

pMLNs were harvested from infected mice at days 1 and 2 p.i. mRNA extraction was carried out using RNAeasy mini kit (Qiagen, Singapore), and cDNA was transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed using GoTaq Real-Time PCR Systems with BRYT Green Dye (Promega) on 7500 real-time PCR system (Applied Biosystems, Singapore). IFN-γ inducible protein 10 (IP-10) and GAPDH were amplified with following primers, respectively: 5'-GGAGCGTGCCGTGCAA-3' (forward) and 5'-GCTCCTCCTATGGCCCTCATT-3' (reverse); 5'-AGGGCGGCTGCTGAGATG-3' (forward) and 5'-GCAGAAGGGGGCGGAGATGAT-3' (reverse).

**Apoptotic cell delivery**

Secondary fibroblasts (passage 48) were infected at 2 PFU/cell overnight and induced apoptosis with UV irradiation (60 mJ/cm²) using a UV cross-linker (Spectronil, Westbury, NY). These virus-infected apoptotic cells were stained with 5 μM violet CFSE (Molecular Probes, Invitrogen) for 15 min at 37°C. The reaction was quenched with culture medium. Cells were washed twice, resuspended in PBS, and transferred i.t. to WT, NK cell-depleted, or perforin−/− mice (≈2 × 10⁶ cells/mouse). Lungs were harvested 2 h after cell transfer, and viral uptake was determined by tracking the uptake of CFSE⁺ cells.

**Immunohistochemistry**

Lungs were removed, embedded into OCT compound (Tissue-Tek; Miles, Elhart, IN), and immediately frozen on dry ice. Frozen lung sections of 4 μm were mounted onto Superfrost Plus Slides (Fisher Scientific) and dried overnight before staining. TUNEL staining of the lung sections was carried out using in situ Cell Death Detection Kit, TMR red (Roche Diagnostics, Asia Pacific), according to manufacturer’s protocol. Human anti-hemagglutinin (anti-HA; H1N1) was a kind gift from Dr. Brendon Hanson (Defence Science Organization, Singapore). Alexa Fluor 647 goat anti-human IgG (Invitrogen, Singapore) was used as the secondary Ab for HA staining. pMLNs were harvested and fixed overnight with 2% paraformaldehyde in 20% sucrose. After fixation, pMLNs were washed in 20% sucrose for at least 1 h, embedded into OCT compound (Tissue-Tek, Miles), and immediately frozen on dry ice. Frozen lymph node sections of 4 μm were mounted onto Superfrost Plus Slides (Fisher Scientific) and dried overnight before staining. Goat anti-mouse CD11b (BD Pharmingen) and rat anti-mouse peripheral lymph node addressin (BD Pharmingen) were incubated with sections overnight. Alexa Fluor 488 goat anti-goat IgG (Invitrogen), DyLight 546 goat-anti rat IgG/IgM (Jackson Immunoresearch, West Grove, PA), and DAPI were used as the secondary Ab for B-cells, and Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) was used as the secondary Ab for HA staining. pMLNs were harvested and fixed overnight with 2% paraformaldehyde in 20% sucrose. After fixation, pMLNs were washed in 20% sucrose for at least 1 h, embedded into OCT compound (Tissue-Tek, Miles), and immediately frozen on dry ice. Frozen lymph node sections of 4 μm were mounted onto Shandon Polysine Slides (Fisher Scientific) and dried overnight before staining. Goat anti-mouse CCL21 (R&D Systems) and rat anti-mouse peripheral lymph node addressin (BD Pharmingen) were incubated with sections overnight. Alexa Fluor 647 cyanine anti-goat IgG (Invitrogen), DyLight 546 goat-anti rat IgG/IgM (Jackson Immunoresearch, West Grove, PA), and DAPI were then stained for 1 h. Acquisition of images was performed on a fluorescent microscope (Carl Zeiss, Singapore).

**Flow cytometry**

Single-cell suspensions of lung samples were stained with FITC anti-B220, Pe-Cy7 blue anti-CD3, PerCP blue anti-CD4, PE anti-CD8α, PerCP 5.5 anti-MHC class II, PE anti-CD103, allophycocyanin anti-CD11b, Pacific blue anti-CD11c, and PE anti-NK 1.1—specific Abs (eBioscience, Immucolonec, Singapore; BD Pharmingen, NJ). Fc-blocking Ab (Clone 2.4G2; BioLegend, Genomax, Singapore) was used before staining with specific Ab at 4°C. For IFN-γ and CD107-α staining, cells were treated with monensin (5 μM/ml; Invitrogen) and brefeldin A (BD Pharmingen) in the presence of FITC anti-CD107-α (eBioscience) for 4 h. Cells were then stained for surface markers, fixed, and permeabilized using fixation/ permeabilization buffer (eBioscience). Intracellular staining with allophycocyanin anti–IFN-γ was then performed. For HA staining, cells were stained for surface markers and then permeabilized using fixation/ permeabilization buffer (eBioscience). Human anti-HA (H1N1) was used as primary Ab and then followed with staining of Alexa Fluor 480 goat anti-human IgG (secondary Ab) (Invitrogen). Influenza A virus and infection. CD8⁺ T cells with H-2Dβ/ASNNENMETL (influenza A [PR8] NP) Pro5 MHC Pentamer (ProImmune, Oxford, U.K.) was performed according to the manufacturer’s protocol. Acquisition of samples was performed on
Cyan ADP (Beckman Coulter, CA) or Fortessa (BD). Data were analyzed using FlowJo v9.3 software (Tree Star, Ashland, OR).

Statistics analysis
One-way ANOVA, Mann–Whitney, and Student t tests were used for all analysis using Prism v5 (GraphPad Software, La Jolla, CA).

Results
NK cell depletion attenuated T cell response to influenza A infection
A/H1N1 is a common strain of influenza A that caused two major epidemics in the past (1918 and 2009). In this study, we used a low dose of influenza A to infect mice, closely resembling the physiological conditions under which humans are infected (21). We infected C57BL/6 mice with 5 PFU A/PR/8/34 (H1N1) and analyzed the kinetics of NK cell recruitment and activation. NK cells numbers started increasing within 24 h in the lung and almost tripled over the next 3 d (Fig. 1A). In the BALF, the number of NK cells had increased 17-fold at day 2 p.i. and remained elevated during the first 4 d p.i. (Fig. 1B). In the lung DNL, the number of NK cells had increased 13-fold 3 d after the infection (Fig. 1C). These data confirm that NK cells are rapidly recruited to the site of infection and secondary lymphoid tissue in response to influenza A infection. Next, we assessed the effector function of NK cells in the lung p.i. by staining for intracellular IFN-γ and surface CD107-α expression, a marker that has been shown to correlate with NK cytotoxicity (22). Intracellular IFN-γ levels in NK cells increased 5-fold at day 3 p.i. (Fig. 1D). Concurrently, surface expression of CD107-α (Fig. 1E) was augmented 4-fold, indicating that pulmonary NK cells increased their effector functions p.i.

To understand the importance of pulmonary NK cells in T cell priming in the lung DLNs, we depleted NK cells 24 h before influenza A infection (Supplementary Fig. 1). In NK cell-depleted mice, total cell (Fig. 2A) and T cell infiltration (Fig. 2B) to the pMLN was reduced 2-fold compared with WT at day 3 p.i., whereas no difference was observed in the anterior MLNs (Fig. 2A). These data demonstrate that initiation of inflammation is exclusive to the pMLN, and that cell infiltration to the pMLN was partially dependent on NK cell-mediated mechanisms. We then determined the virus-specific CD8 T cell response in NK cell-depleted and WT mice. A significant reduction in D8–NP366–374+ CD8+ T cells was observed at days 7 and 9 p.i. in the pMLN (Fig. 2C), and a 2-fold reduction in the D8–NP366–374+ CD8+ T cell number was found in the lung on day 11 p.i. in NK cell-depleted mice (Fig. 2D). We also determined the viral titer in WT and NK cell-depleted mice using a plaque-forming assay. Viral replication peaked at day 4 p.i. (Fig. 2E). A significant increase in viral burden was evident at day 2 p.i. in NK cell-depleted mice, whereas no difference was observed at the peak of viral load (Fig. 2E), suggesting that NK cell depletion did not greatly enhance viral replication in a sublethal infection.
NK cell-enhanced T cell recruitment to the pMLN in response to influenza A infection depends on IFN-γ

Because NK cells mainly exert their antiviral effect through direct cytotoxicity and secreted IFN-γ, we infected IFN-γ−/− and perforin−/− mice to determine whether NK cell-mediated T cell recruitment to the pMLN post influenza A infection depends on these effector functions. Infection of both CD3+CD4+ and CD3+CD8+ T cells was measured (Fig. 3). Anti-NK 1.1 Abs were injected i.p. on days −1 and 4 p.i. At various time points p.i., cells from the pMLN (A) and lung (B) were isolated and stained for Dp·NP366–374 pentamer binding to CD3+CD8+ T cells. Data shown are mean ± SEM representing at least nine mice from three independent experiments. Pulmonary viral load was determined at various days p.i. using a plaque-forming assay (C). Data shown are mean ± SEM representing at least three mice from two independent experiments.*p < 0.05, **p < 0.01, ***p < 0.001, using one-way ANOVA and Student t test.

FIGURE 2. NK cell depletion attenuates T cell response during influenza A infection. Mice were treated with anti-Asialo GM-1 Ab (i.p.) 24 h before infection with influenza A/PR/8/34 (5 PFU) or PBS. At day 3 p.i., both the pMLN and anterior MLNs were harvested and total cell numbers enumerated (A). Recruitment of both CD3+CD4+ and CD3+CD8+ T cells was measured (B). Anti-NK 1.1 Abs were injected i.p. on days −1 and 4 p.i. At various time points p.i., cells from the pMLN (C) and lung (D) were isolated and stained for Dp·NP366–374 pentamer binding to CD3+CD8+ T cells. Data shown are mean ± SEM representing at least three mice from two independent experiments. Pulmonary viral load was determined at various days p.i. using a plaque-forming assay (E). Data shown are mean ± SEM representing at least three mice from two independent experiments.*p < 0.05, **p < 0.01, ***p < 0.001, using one-way ANOVA and Student t test.
Impaired migration of pulmonary cells reduces T cell recruitment to the pMLN in response to influenza A infection

To further understand whether migration of pulmonary cells or lymph node resident cells contributed to T cell recruitment to the pMLN, we administered pertussis toxin (PT) concurrently with virus. PT inhibits chemokine functions including CCR7-dependent efflux from the lung (23, 24), whereas T cell mobility is not directly affected (23). PT administration abrogated CD8+ and CD4+ T cell recruitment to the pMLN significantly at day 3 p.i. (Fig. 4D). Because migration of pulmonary DCs and NK cells is likely to be blocked by PT, we used CFSE to trace pulmonary cell migration during infection. We detected both CFSE+DCs and CFSE+ NK cells in the pMLNs, whereas neither CFSE+DCs nor CFSE+ NK cells were detectable in PT-treated mice, indicating that both pulmonary DCs and NK cells do migrate to the pMLN in response to influenza A infection (Fig. 4E). Furthermore, B cells (NK1.1+ CD3−), which do not migrate to the pMLN during the early stage of influenza A infection, were CFSE−, suggesting that CFSE is unlikely to leak into the pMLN (Fig. 4E).

IP-10 and CCL-21 play a role in NK cell-mediated T cell recruitment

To gain better insight into the molecular pathways underlying the NK cell-mediated T cell recruitment to the pMLN, we investigated the expression of chemokines, especially IP-10 and CCL-21, in the pMLN. IP-10 has been shown to promote NK cells, T cell, and possibly DC recruitment (25). CCL-21, constitutively expressed in lymphoid tissue, is a ligand for the CXCR7 receptor that is expressed on both T cells and DCs (26). We harvested pMLNs at days 1 and 2 p.i., and assayed for the IP-10 mRNA expression level in WT, NK cell-depleted, and IFN-γ−/− mice. The level of
IP-10 mRNA increased 6-fold at day 1 p.i. in WT mice, whereas NK cell-depleted and IFN-γ−/− mice showed little increment (Fig. 5A). However, at day 2 p.i., IP-10 expression returned to the baseline and no difference was observed among the groups (Fig. 5A). To address the role of CCL-21, we stained pMLN sections for CCL-21 and high endothelial venule marker, peripheral lymph node addressin (clone MECA-79) at day 3 p.i. in WT, NK cell-depleted, and IFN-γ−/− mice. A marked reduction in CCL-21 expression was evident in NK cell-depleted and IFN-γ−/− mice compared with WT mice (Fig. 5B).

Perforin-dependent generation of apoptotic bodies is required for efficient influenza A virus uptake by DCs

Ag uptake and presentation by DCs are critical for priming T cell responses (13). NK cells have been reported to interact with DCs and affect their maturation and activation (27–29). We therefore determined whether NK cells regulated virus-specific T cell responses by controlling DC function during infection using an in vivo T cell proliferation assay. CFSE-labeled OT-I CD8+ T cells were adoptively transferred to mice that were infected with OT-I PR8 (50 PFU) the following day. Four days p.i., the number of proliferating OT-I-specific CD8+ T cells was significantly reduced in NK cell-depleted compared with WT mice, suggesting NK cell depletion restricted the pool of Ag-presenting DCs (Fig. 6A). To further test whether NK cells were involved in efficient uptake and transport of influenza A Ag by DCs, we compared the number of HA bearing DCs between WT and NK cell-depleted mice. A significant reduction in number of HA+DCs in the pMLN was found in NK cell-depleted mice at day 3 p.i. (Fig. 6B), which suggested a deficiency in virus-carrying DC migration to the pMLN in NK cell-depleted mice. However, no difference in HA levels in pulmonary DCs between two groups was observed (Supplemental Fig. 3). Next, we labeled influenza A with the lipophilic dye, DiD, which is attached to the lipid layer of the virus and has previously been used to follow influenza A virus infections in vivo (20, 30). DiD is not incorporated into progenitor virus released from infected cells, and DiD therefore allowed us to track Ag uptake by DCs from primary infected cells. p.i. with DiD-
labeled influenza A virus (200 PFU), we analyzed DiD expression by pulmonary DCs (Supplemental Fig. 4) and observed a significant increase of DiD+CD103+ DCs in the pMLN 2 d p.i. (Fig. 6C). In contrast, a significant decrease in DiD+DCs was observed in NK cell-depleted mice compared with WT mice (Fig. 6C). To better understand the requirement of NK cell effector functions on Ag uptake, we infected IFN-γ−/− and perforin−/− mice with DiD-labeled influenza A. The number of DiD+ DCs in the pMLN of IFN-γ−/− and perforin−/− mice was similar to those in NK cell-depleted mice, suggesting that IFN-γ and perforin contribute to the virus transport by DCs to the pMLN (Fig. 6D, 6E). Because IFN-γ affects the migration of DCs, it was possible that virus uptake by lung DCs was not deficient in IFN-γ−/− mice. Consistent with this possibility, we observed that IFN-γ−/− mice had similar numbers of DiD+ DCs in the lung 2 d p.i. compared with WT mice (Fig. 6H). In contrast, the number of pulmonary DiD+ DCs was significantly reduced in both NK cell-depleted and perforin−/− mice (Fig. 6F, 6G). These data suggested that perforin-dependent cytotoxicity was required for efficient virus uptake by pulmonary DCs, whereas IFN-γ mediated the Ag transport to the pMLN. We performed a pulse-chase experiment to study the possible role of NK cells in Ag uptake of pulmonary DCs in an ongoing infection. WT, NK cell-depleted, and perforin−/− mice were infected with 5 PFU influenza A and inoculated with DiD-labeled influenza A virus (3.5 × 10^4 PFU) at d 3 p.i. Pulmonary DCs were harvested for analysis after 4 h. Significant viral replication is unlikely to occur in the 4 h of infection, and hence allows us to study the virus uptake in pulmonary DCs in an ongoing infection. A significant reduction in DiD+DCs was observed in NK cell-depleted and perforin−/− mice in the lung (Fig. 6I), suggesting that efficient Ag uptake of pulmonary DCs is partially dependent on NK cells through perforin-mediated pathways in an ongoing infection. Taken together, our data strongly suggest that NK cells are important for virus uptake by pulmonary DCs during the early phase of influenza A infection.

Next, we set out to understand how perforin promoted viral uptake in the lung. It is generally believed that viral Ags are acquired through infection of DCs and by endocytosis of infected cells by DCs (31–35). One possibility is that the absence of perforin might lead to defective endocytosis by DCs. To address this, we used the uptake of FITC-dextran as an indication of endocytosis. However, no difference was found in the uptake of FITC-dextran between WT and perforin−/− mice in vitro (data not shown). Recently, Desch and colleagues showed that CD11b−CD103+DCs preferentially take up Ags from apoptotic bodies (36). We then hypothesized that NK cell-mediated killing of infected cells through perforin-dependent pathway would lead to generation of apoptotic bodies containing viral Ag, which would...
FIGURE 6. NK cells limit the availability of virus-loaded DCs. OT-I CD8 T cells were isolated from spleens and lymph nodes from OT-I transgenic mice. These cells were then labeled with CFSE and adoptively transferred into NK cell-depleted and WT mice (1 x 10^6 cells/mice) 24 h before infection with OT-I PR8 virus (50 PFU). Day 4 p.i., the pMLNs were harvested and the percentage of CFSE^{low}SIINFEKL^{+}CD3^{+}CD8^{+} T cells measured (A). HA containing DCs in the pMLN from WT and NK cell-depleted mice were determined by intracellular staining (B). WT, NK cell-depleted, IFN-γ^{−/−}, and perforin^{−/−} mice were infected with DiD-labeled influenza A (200 PFU). Day 2 p.i., DCs from the pMLN were harvested and measured (C). DiD^{+} DCs in the pMLN were also enumerated in IFN-γ^{−/−} (D) and perforin^{−/−} mice (E). Similarly, DiD^{+} DCs were measured in the lung of NK cell-depleted and WT mice at day 2 p.i. (F). Numbers of pulmonary DiD^{+} DCs were also measured in perforin^{−/−} mice at day 2 p.i. (H). Mice were infected with 5 PFU influenza A virus for 3 d and then infected again with DiD-labeled virus (3.5 x 10^3 PFU). Lungs were harvested and analyzed for DiD uptake by DCs at 4 h after DiD virus inoculation. Reduced numbers of DiD^{+} DCs were observed in NK cell-depleted and perforin^{−/−} mice (I). Data shown are mean ± SEM representing at least eight mice from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, using one-way ANOVA and Mann–Whitney U test.
then facilitate the viral Ag uptake. To address this, we determined the level of apoptosis in the lung from WT, NK cell-depleted, and perforin \(^{-/-}\) mice at day 2 p.i. using in situ TUNEL staining. In agreement with our hypothesis, WT mice had a higher intensity of TUNEL\(^+\) cells in their lung sections, whereas NK cell-depleted and perforin \(^{-/-}\) mice displayed a reduced level of TUNEL staining intensity (Fig. 7A). We then went on to determine whether perforin could also affect the phagocytosis of apoptotic cells by DCs. We transferred CFSE-labeled virus-infected apoptotic mouse fibroblasts to WT, NK cell-depleted, and perforin \(^{-/-}\) mice (\(~2 \times 10^7\) cells/mouse), and harvested lungs 2 h later to determine the uptake of CFSE\(^+\) cells. No difference in uptake of CFSE\(^+\) cells was observed in CD11b\(^+\)CD103\(^+\) DCs or CD11b\(^+\) CD103\(^-\) DCs among three groups, indicating perforin did not affect phagocytosis of apoptotic cells (Fig. 7B). Hence perforin is likely to affect the apoptosis of infected cells, which then facilitate the viral uptake by pulmonary DCs. Taken together, our data demonstrated that NK cells regulate the T cell response to influenza A by promoting uptake and transport of influenza A virus by DCs to the pMLN, which is required for efficient priming of T cell in the DLN.

**Discussion**

In this study, we demonstrate that NK cells are rapidly recruited and activated by a sublethal dose of influenza A/PR/8/34 virus despite the previous observation that immune responses were attenuated with a low dose of infection (37). This dose of virus mimics human infection as limited virus is shed through aerosol droplets (21) and infected mice recover over 14 d, which enabled us to track the immune response at later time points. We describe a novel function of NK cells in shaping the T cell response through modulation of the recruitment of DCs and T cells to the pMLN during the early phase of infection. IFN-\(\gamma\) produced by NK cells upon infection effectively enhanced DC migration and T cell recruitment.
to the pMLN, mediated partially through chemokines such as IP-10 and CCL-21. Besides limiting the number of T cells, NK cell depletion also greatly impaired Ag uptake by DCs because of the lack of perforin-mediated killing of infected lung cells. By both means, NK cells were able to influence the available pool of Ag-presenting DCs in the pMLN, and thus affected the overall magnitude of virus-specific CD8+ T cell response. Our data provide the first evidence that NK cells regulate homing of DCs and T cells to the pMLN after influenza A infection, and that perforin-dependent killing was required for Ag uptake and subsequent transport to the pMLN by DCs.

In the pMLN, successful generation of a CTL response depends on Ag presentation by mature DC and recruitment of naive T cells to the DLNs (15, 38). This process is tightly regulated in a spatiotemporal manner. Although naive T cells are constantly trafficking through the MLNs, only those that have arrived within a limited time window were able to differentiate into effector cells (39). Activated NK cells were required to promote optimal recruitment of both DC and T cells into the pMLN. Inhibition of pulmonary NK and DC migration to the pMLN significantly reduced T cell recruitment, implying that recruitment of immune cells to the pMLN is critically dependent on the signals that are received from the primary infection site in the lung. Once recruited, T cells are activated and differentiated into effector cells. This differentiation process is no longer NK cell dependent, as the expression of activation markers such as CD25, CD69, and CD62L was the same in NK cell-depleted and WT mice (data not shown).

One of the key factors that contributed to NK cell-mediated T cell infiltration is IFN-γ. This cytokine has been shown to influence the induction and expansion of the CTL response in vitro and in vivo (40, 41). In influenza A infection, IFN-γ-deficient mice did not show higher mortality (42), but reduced migration of CD8+ T cell into the lung (43). Our data provide clear evidence that IFN-γ-deficient mice have impaired T cell recruitment to the pMLN, and that naive NK cell deletion restored T cell recruitment. IFN-γ was also found to be important in mediating DC migration from lung to the pMLN. These data accord with the previous study of IFN-γ-deficient mice using an allograft model (44). Expression of chemokines such as IP-10 and CCL-21 in the pMLN was markedly reduced by NK cell depletion and IFN-γ deficiency, suggesting these chemokines may be involved in cell recruitment to the pMLN. IP-10 is an IFN-γ-regulated chemokine, whereas CCL-21 was not previously reported to be directly regulated by IFN-γ.

Induction of the influenza A-specific T cell response depends on the maturation of DCs and their ability to present Ag efficiently in the lung DLNs (45). In NK-depleted mice, virus-specific T cell generation was attenuated, suggesting a possible defect in DC function. We enumerated both CD11b+ and CD103+ DC subsets in NK cell-depleted and WT mice, and measured the activation markers CD80, CD86, MHC class II, and CD70 on DCs. There was no difference in the gross number or in the expression of activation markers between NK cell-depleted and WT mice (data not shown). Hence we analyzed the migration of virus-carrying DCs using intracellular HA staining, DiD-labeled virus in a pulse-chase experiment. There were less HA+DCs and DiD+CD103+ DCs using intracellular HA staining, DiD-labeled virus in a pulse-chase experiment. This strongly supports the view proposed in earlier studies that perforin did not only have an effector function but also a regulatory role in immunity (46, 47). Perforin was found to be important in regulating CTL response to LCMV (48), Thielers’s virus (49), and HSV infection (50). In a murine cytomegalovirus infection model, a perforin-dependent mechanism was found to limit Ag presentation that led to a contraction of the CTL response (47, 51). Other reports have demonstrated the opposite effect, namely, that NK cell-mediated killing of target cells enhances cross-presentation and the attendant T cell response (52). Another study demonstrated enhanced endocytosis by CD8+ DCs after effective killing of allogeneic cells by NK cells (53). Our data support the latter view by showing that NK cell-mediated killing of infected cells enhanced uptake of influenza A viral Ags by pulmonary DCs, especially the CD103+ DC subset, which was shown to be important in priming the CTL response during influenza A infection (30). This effect of perforin-mediated cytotoxicity probably enhanced the pool of apoptotic cell-associated Ags, which are preferentially taken up by pulmonary CD103+ DCs for CD8+ T cell priming (36). In summary, NK cells are required for optimal Ag uptake by DCs during influenza A infection.

To our knowledge, this is the first evidence that NK cells are critical in promoting antiviral adaptive immunity through T cell recruitment, DC migration, and attendant Ag presentation capacity. These findings suggest that activation of NK cells by vaccine adjuvant would enhance the CD8+ T cell response against influenza A infection, which would be another therapeutic avenue for protection from influenza A infection and other pulmonary diseases.

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Disclosures

The authors have no financial conflicts of interest.

References


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**Supplementary Figure 1.** NK cell depletion efficiency. NK cell depleting antibodies were injected i.p.. Pulmonary NK cells were stained with CD3 and NK 1.1 at D2 post treatment. Plots shown are representative data from 2 independent experiments.

**Supplementary Figure 2.** Viral titer in knockout and WT mice. Mice were infected with 5pfu of influenza A infection. Lungs were collected and homogenized. Viral titer was determined using plaque forming assay. Data shown are mean ± SEM representing experiments with at least 6 mice.

**Supplementary Figure 3.** HA expression level of pulmonary DCs from WT and NK cell-depleted mice. Mice were infected with 5pfu of influenza A virus. At D3 p.i, the pulmonary DCs were collected and analyzed for HA expression using intracellular staining. Data shown are mean ± SEM representing experiments with at least 9 mice.

**Supplementary Figure 4.** DC gating strategy for DiD analysis. Live cells were gated for FSC and SSC and single cells were further gated out using pulse width. B220^−^CD4^−^CD8^−^ cells were then gated out. DCs were then gated as I-A/E^+^CD11c^+^. CD11b^+^CD103^-^ and CD11b^-^CD103^+^ subsets were then gated out and DiD expression of each subset was gated according to the control mice.