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Characteristics of NK Cell Migration Early after Vaccinia Infection[1]

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NK cells are critical components in innate immunity, yet little is known about their migration and proliferation during infection. In this report we study the early NK response toward vaccinia. We observed NK migration into the infected peritoneum as early as 6 h after vaccinia inoculation. Interestingly, although NK trafficking to the infected peritoneum depended on $G\alpha_i$ protein-coupled receptors, trafficking to other tissues (including lung, liver, spleen, and bone marrow) did not. We found that despite a dramatic increase in NK numbers at the primary site of infection, their in situ proliferation was low compared with that at other tissue locations. These features are similar to those found for Ag-experienced T cells, suggesting similar patterns of trafficking and proliferation for these lymphocyte subsets. *The Journal of Immunology,* **2005, 175: 2152–2157.**

To maintain a functional immune system that provides protection against a wide range of antigenic insults, the innate and adaptive arms of the immune system have to cooperate. According to present concepts, after infection, NK cells are among the first leukocytes to become activated and thus fill the lag before T and B cells are fully armed effector cells (1, 2). Although the role of NK cells as key players early in an immune response is generally accepted, there is surprisingly little known about early NK cell trafficking in response to a pathogen (3). Determining the NK response to infection is relevant to determining the innate immune response per se and also for assessing the potential role of NK-dendritic cell (DC)[3] cross-talk in directing the adaptive immune response (3–5).

The homing properties of T cells are fairly well established, in that naive cells are mostly limited to secondary lymphoid organs, whereas effector and memory cells can access various nonlymphoid tissues (6–8). Similar to activated/memory T cells, NK cells are found in nonlymphoid organs and are capable of rapidly displaying effector function upon stimulation (9). However, NK requirements for in vivo homing are still poorly defined. Welsh et al. demonstrated increased numbers of NK cells at the site of primary infection (10, 11) and also showed that NK cells proliferate in response to viral infection and type I IFNs (1, 12–14). In most cases, however, it is not clear whether changes in the NK pool at the site of challenge reflects NK migration, in situ proliferation or both. Studies by Salazar-Mather and Biron (15, 16) showed that murine CMV infection leads to migration of NK cells into the liver in response to CCL3 (MIP-1α), which, in turn, is induced by IFN-$\alpha\beta$. However, these studies focus on NK migration into the liver 48 h after infection. In contrast, little is known about the initial NK migration into virally infected tissues, which can be detected far earlier (10) (see below). Because NK cells have direct antiviral effects and can regulate DC activity, the requirements for rapid entry of NK cells into infected sites is relevant to the developing immune response.

VV infection elicits NK activation, proliferation, and accumulation at the site of infection (10, 11, 17, 18), and NK cells have long been proposed to mediate an important element in the protective response against this poxvirus (17, 19), especially in situations of T cell deficiency (20). In this report we study the requirements and characteristics of NK migration occurring early (by 6 h) after i.p. infection with VV. Our data suggest that NK trafficking to the primary site of infection obeys requirements distinct from those of NK trafficking to other tissues. Although NK numbers continue to increase in the infected PC over successive days, our data suggest that their proliferation occurs chiefly at distinct sites, including the spleen. These findings shed light on requirements for NK trafficking and migration after infection.

Materials and Methods

Mice

B6, CD45.1 congenic B6 (B6.SJL), and RAG-1$^{-/-}$ mice were obtained from The Jackson Laboratory; RAG-2$^{-/-}$ mice were purchased from Taconic Farms. All mice were females, age matched, maintained under specific pathogen-free conditions, and used at 6–12 wk of age.

Adoptive transfer and cell sorting

RAG$^{-/-}$ splenocytes were depleted of RBC and labeled with CFSE (Molecular Probes) as described previously (21). Between 0.7×10^6 and 3×10^6 CFSE-labeled cells were injected (i.v., via the tail vein) per recipient, as indicated. In some experiments, $1–5 \times 10^7$ RBC-depleted RAG$^{-/-}$ splenocytes were incubated in 10 ml of HBSS with 1 μg/ml pertussis toxin (Calbiochem) for 30 min at 37°C before washing in HBSS and i.v. injection.

Viruses and viral infection

The Western Reserve vaccinia strain (VV-WR) was obtained from Dr. M. Bevan (University of Washington, Seattle, WA) and from Dr. L. Selin (University of Massachusetts, Worcester, MA). In some experiments, recombinant VV-GFP-JAW-OVA (VV-OVA) or VV-GFP-JAW-P815p (VV-P815p) strains were used. These strains (provided by Dr. J. Yewdell, National Institutes of Health, Bethesda, MD) include the $OVA_{257–264}$ and P815p K^b-restricted epitopes, respectively, fused C-terminally to GFP and the transmembrane region of JAW-1. Mice were infected i.p. with 2×10^6 PFU of the indicated virus (viral titer was determined by plaque assays performed on 143B cells).

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[3] Abbreviations used in this paper: DC, dendritic cell; PC, peritoneal cavity; PTX, pertussis toxin; VV, vaccinia virus; VV-WR, Western Reserve vaccinia strain.

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Tissue preparation

Recipient mice were killed at the time points indicated, and single-cell suspensions were prepared from the spleen, lung, liver, and bone marrow (8). Before tissue removal, mice were perfused with PBS containing 1% FCS and 0.02% azide. Lung and liver tissue was minced and incubated at 37°C for 45 min to 1 h with collagenase D (Roche) in RPMI 1640/10% FCS. The resulting suspension was mashed through a strainer using the soft end of a syringe plunger. Liver cells were pelleted by centrifugation, resuspended in 40% Percoll (Amersham Biosciences), layered on 70% Percoll, and centrifuged at 900 × *g* for 20 min. Cells at the gradient interface were harvested and washed before use. Bone marrow was harvested from the right femur and fibula. Peritoneal lavage was performed by flushing 10 ml of PBS directly into the PC with a 26G1/2 syringe and removing the fluid with an 18G1 syringe. NK numbers in spleen and PC were calculated from multiplication of total live lymphocyte numbers (determined by trypan blue cell count) multiplied by the percentage of NK cells (identified, by flow cytometry, as $NK1.1^+CD3^-$ cells). For PC, the total recovered cell number was obtained by determining cell counts per milliliter of recovered lavage fluid and multiplying this number by 10 (the volume used for the peritoneal wash). PC samples contaminated by blood were not included in the analysis.

Flow cytometry

Samples were treated with 2.4G2.1 (Fc-Block, BD Pharmingen) before additional staining. Cells were typically stained with anti-NK1.1-allophycocyanin (PK136), anti-CD3-PerCP (145-2C11), and anti-CD69-PE (clone H1.2F3; from eBioscience and Pharmingen). Data are shown gated on the NK cell ($NK1.1^+CD3^-$) population. In adoptive transfer experiments, transferred NK cells were distinguished from congenic host cells using anti-CD45.2-PE (104; eBioscience) Ab. For BrdU incorporation assays, BrdU was injected (1 mg/mouse i.p.) 1 h before harvesting. Cells were stained using anti-BrdU-FITC Abs according to the manufacturer's protocol (BrdU Flow Kit; BD Biosciences). Cells were analyzed using FACSCalibur or LSR II (BD Biosciences) instruments and FlowJo software (TreeStar).

Statistical analysis

Unpaired two-tailed Student's *t* test was used for data analysis, calculated using PRISM software (GraphPad). Graphs show mean values, and error bars represent the SD. The *p* values obtained are shown in the figures.

Results

Activation and accumulation of NK cells after VV infection

To determine initial NK trafficking kinetics, we explored the accumulation and phenotype of NK cells in the PC and spleen of B6 mice infected (i.p.) with VV. In keeping with previous work (10, 11) we found that NK cell numbers increased dramatically in the PC by 24 h of infection and peaked around day 3. However, we also detected a significant increase in NK number in the infected PC as early as 6 h after infection (Fig. 1*A*). In contrast, NK cell numbers in the spleen showed a significant decline on days 1–2 as reported previously (11), followed by a marked increase on days 3 and 5 after infection (Fig. 1*A*). Splenic as well as peritoneal NK cells had already up-regulated CD69 (indicative of activation) by 6 h after infection. NK blasting (as determined by forward scatter) was evident by 24 h after VV infection (Fig. 1*B*), consistent with studies of other viruses (13). The fact that both peritoneal and splenic NK cells were similarly activated at these early time points suggests a systemic activating signal, probably mediated by cytokines such as type I IFNs (1). However, the accumulation of NK cells in the infected PC, rather than the spleen, over the next few days suggested directed trafficking and/or proliferation of NK cells at the site of primary vaccinia infection. Because we observed NK accumulation at the site of primary infection as early as 6 h, additional experiments were designed to determine the migration and proliferation properties of NK cells early during the response to VV infection.

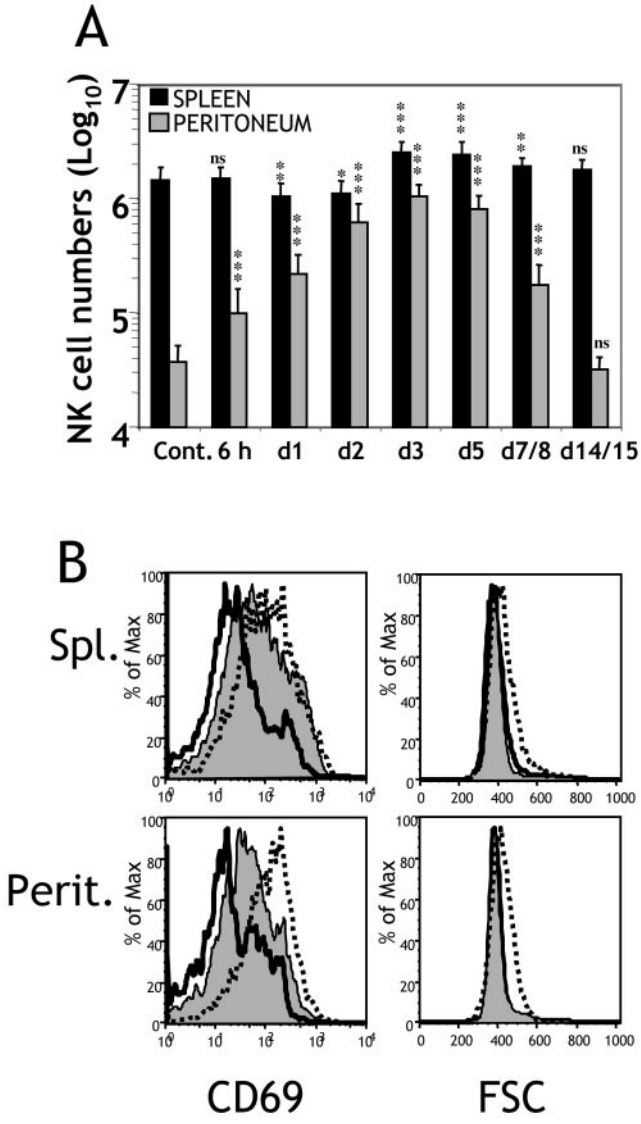

FIGURE 1. NK cell numbers in spleen and PC during the course of infection with VV-OVA B6 mice were infected (i.p.) with 2×10^6 PFU of VV-OVA, and the endogenous NK cell response was analyzed over time. *A*, NK cell numbers were determined in spleen and PC starting at 6 h after infection up to 15 days. The data shown are compiled from at least three experiments for each time point. *B*, CD69 surface expression and forward scatter of NK cells were determined at 6 and 24 h of infection in spleen and PC. Uninfected control is shown in bold, the 6 h point is shown in gray, and the 24 h point is shown as a dotted line. Data are representative of at least three experiments. Statistical analysis compares uninfected (control) to infected samples. In this and following figures: ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$; ns, not significant ($p > 0.05$).

NK cell migration properties

Many chemokine receptors are expressed by NK cells (both resting and activated cells), and there is considerable evidence that chemokines can attract NK cells to sites of inflammation (1, 15, 16, 22). However, the role of classic chemokine signals (mediated through pertussis toxin-sensitive, G protein-coupled receptors (23)) in NK migration to normal and infected sites has not been comprehensively addressed. Furthermore, studies of the requirements for chemotactic factors in NK migration have focused on relatively late time points, days after the primary infection (15, 16). In contrast, little is known about the requirements for the early

trafficking of NK to sites of infection, such as the migration we observed 6 h after VV infection.

To track NK migration, we adoptively transferred CFSE-labeled NK cells into congenic hosts before i.p. infection with VV. Six hours after infection, total NK cells increased in frequency (Fig. 2, *A* and *B*) and number (Fig. 1) in the PC, whereas NK frequencies in other tissues (spleen, liver, lung, and bone marrow) did not increase significantly (Fig. 2*A*). Whether this difference occurred simply because live VV had not spread from the PC at this time point is not yet clear. After perfusion (and Percoll purification for liver samples), the total cell counts typically recovered from the tissues were as follows (numbers $\times 10^6 \pm$ SEM): spleen, 47.8 $\pm$ 4.6; PC, 3.2 $\pm$ 0.4; lung, 4.1 $\pm$ 1.1; bone marrow, 11.6 $\pm$ 1.1; and liver, 3.8 $\pm$ 0.8 ($n = 6$–12 animals for each tissue). These numbers were compiled from both uninfected and 6-h VV-infected animals, and we did not observe an impact of this short term infection on total cell yields (data not shown). Hence, a change in the percentage of NK cells in Fig. 2*A* reflects a change in the absolute NK numbers at the designated sites. Donor NK cells could be detected by flow cytometry in the PC of infected, but not uninfected, animals (Fig. 2*B*), confirming that NK cells migrate to the site of infection within 6 h.

We next sought to determine whether NK migration to infected and uninfected sites was dependent on G_i protein-coupled receptors. Pertussis toxin (PTX) is a potent inhibitor of heterotrimeric G protein-coupled receptors involving $G\alpha_i$ subunits and is well characterized for its ability to block the chemotactic responses of leukocytes, interrupting normal entry into or out of tissues (23–25). Animals were given donor NK cells that were either PTX treated or untreated, and the mice were then left uninfected or were given VV i.p., and tissues were harvested 6 h later.

Analysis of donor NK cells migration showed stringent requirements for trafficking to the PC at 6 h. In the absence of infection, very few donor NK cells could be detected in the PC, whereas donor NK migration was clearly detected in the VV-infected peritoneum (Fig. 2*C*). This migration to the PC was profoundly inhibited by PTX treatment of the donor NK cells (Fig. 2*D*), implying the involvement of a $G\alpha_i$ protein-coupled receptor (8, 22–25). In contrast, migration to other sites (lung liver, bone marrow, and spleen) was not reliably impacted by PTX treatment. We noted a slight inhibition of PTX-treated NK cell migration into the bone marrow, but this difference was not statistically significant in these experiments.

In the previous experiments, PTX- and control-treated donor NK cells were transferred to different hosts, making direct comparison of their migration properties difficult to determine. Hence, we also tested migration of PTX-treated and untreated NK cells in the same environment, allowing for competition between the two donor NK pools. Splenocytes were harvested from $RAG^{-/-}$ mice, and one group of cells was treated with PTX, whereas the second group of cells were not treated with PTX, but instead were labeled with CFSE. The two groups were mixed at a 1:1 ratio and adoptively transferred (i.v.) into CD45 congenic B6.SJL host mice. One hour after adoptive transfer, the host mice were infected i.p. with VV-OVA or VV-WR and were harvested 6 h after infection. Donor NK cell migration to the bone marrow, lung, liver, spleen, and PC was assessed by flow cytometry. In these assays, donor NK cell migration to the uninfected PC was essentially undetectable, whereas migration to the VV-infected PC was observed, but only for the untreated donor NK population (Fig. 3). Both PTX-treated and untreated donor NK cells trafficked similarly in spleen, lung, liver, and bone marrow, although once again, a modest, but reproducible, reduction in PTX-treated NK cell trafficking to the infected bone marrow was observed (Fig. 3). The overall magnitude and requirements for trafficking to those sites were not influenced

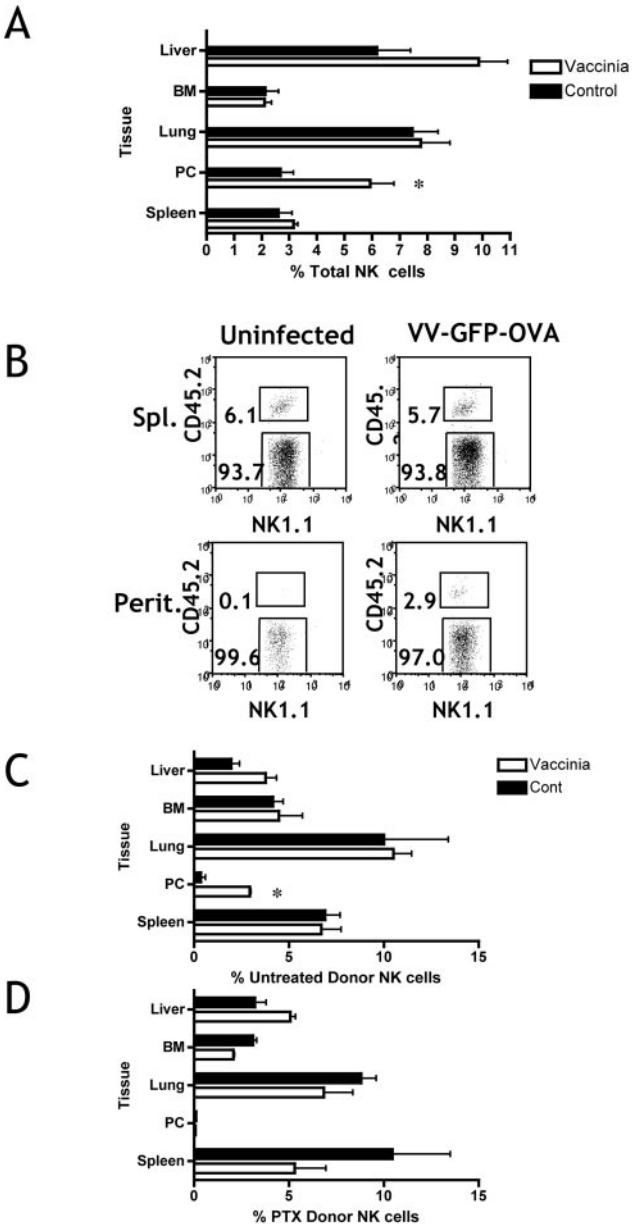

FIGURE 2. Trafficking of adoptively transferred NK cells into spleen and PC of control and infected mice. *A* and *B*, B6. SJL (CD45.1) mice received $CD45.2^+CFSE^+$ NK cells (i.v.) 1 day before infection with 2×10^6 PFU of VV-OVA. Mice were harvested 6 h after infection. *A*, The frequency of total NK cells (identified as $NK1.1^+CD3^-$) was determined in the indicated tissues. Four to six animals were included per group. In statistical comparison between uninfected and infected groups, *, $p = 0.02$ (other differences were not statistically significant). *B*, The percentage of donor ($CD45.2^+$) NK cells among total NK cells was determined by flow cytometry on NK ($NK1.1^+CD3^-$) cells in the spleen and PC. Data shown are representative of two or three animals in each group and are similar to those from two other experiments using VV-OVA or VV-WR. *C* and *D*, CD45 congenic donor NK cells were either mock treated (*C*) or treated with PTX (*D*) before injection. Host animals were then either left uninfected (Cont.) or were infected (i.p.) with VV. Six hours later, the mice were killed, and the frequency of donor-derived NK cells in the indicated tissues was determined. Statistical comparison between uninfected and infected animals was performed: *, $p = 0.001$ (other differences were not statistically significant).

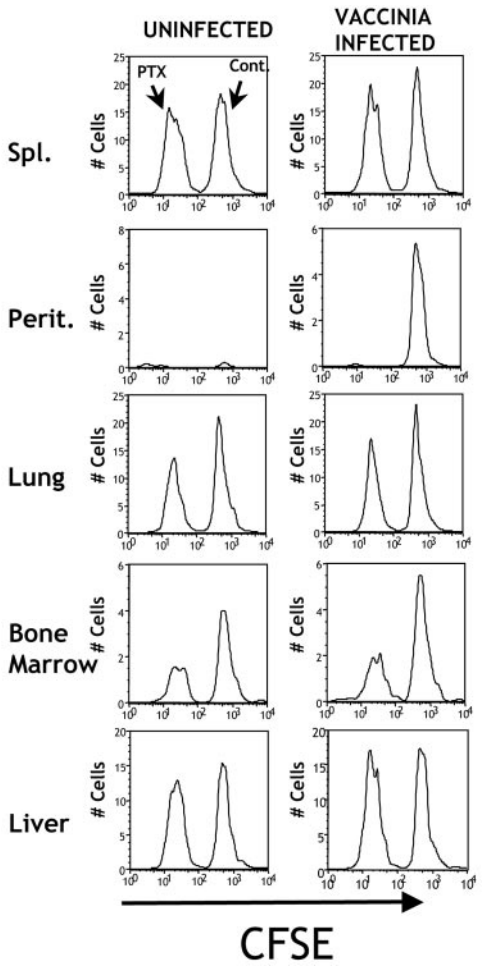

FIGURE 3. Trafficking of adoptively transferred $CFSE^+$ or PTX-treated NK cells in infected and uninfected mice. B6 $RAG^{-/-}$ ($CD45.2^+$) splenocytes were prepared and either labeled with CFSE (control) or treated with PTX. The cells were mixed at a 1:1 ratio, and 2.2×10^6 total cells were transferred into B6.SJL ($CD45.1^+$) mice. Mice were infected (i.p.) with 2×10^6 PFU of VV-OVA 1 h after cell transfer. Mice were harvested 6 h after infection, and the percentage of transferred cells was determined in spleen, PC, lung, bone marrow, and liver. Data are representative of two or three mice per group, and similar data were obtained in an independent experiment.

by VV infection at this time point (Fig. 3 and data not shown) despite the fact that NK cells were activated in at least some of these tissues (Fig. 1*B*).

NK cells proliferate poorly at the site of primary VV infection

Although the data presented above address NK trafficking that occurs early during vaccinia infection, NK numbers at that site continue to rise dramatically over the next 3 days. By 24 h after infection, we observed that NK cells in both the PC and spleen displayed a blasting phenotype (Fig. 1*B*), in line with previous reports suggesting the appearance of NK cell blasts at the site of viral replication (13, 14). Furthermore, NK cell proliferation is known to occur in the spleen and liver after VV infection (18). However, it is currently unclear whether the accumulation of NK cells at the site of infection is achieved by their in situ proliferation, or whether they proliferate in other tissues and then migrate to the vaccination site.

To determine the site of NK proliferation after VV infection, we used a short BrdU pulse approach. Limiting the exposure to a 1-h BrdU pulse is designed to maximize the likelihood that cells incorporated BrdU in the same location from which they were harvested. Thymocytes were used as a positive control to show consistency in BrdU labeling (Fig. 4). On day 1 of VV infection, ~1% of NK cells in the spleen were $BrdU^+$, representing a significant increase in labeling over uninfected controls. In contrast, NK cells in the PC were not significantly labeled with BrdU at this time. By day 2 of infection, proliferation among splenic NK cells increased dramatically, with ~25% NK cells incorporating BrdU, similar to previous reports involving infection with a higher VV dosage (18). In the peritoneal NK pool, there was considerably less BrdU incorporation (average, 10% NK cells incorporating BrdU). This pattern for the site of NK proliferation is surprising because it runs counter to the changes in NK cell numbers at the same time points. On days 1–2 after infection, NK numbers dramatically increased in the PC, but decreased in the spleen. The increase in proliferation on day 2 after infection (Fig. 4) occurred 24 h after blasting cells were found in the spleen and PC (Fig. 1*B*) and 1 day before the peak numbers of NK cells appeared in both locations (Fig. 1*A*). By day 5 after infection, BrdU incorporation in splenic NK cells was still elevated compared with that in uninfected controls, whereas proliferation among NK cells in the PC was not significantly different from that in uninfected controls (Fig. 4). This time point marks the beginning of the decline in NK numbers at both locations (Fig. 1*A*). These data support the model that the accumulation of NK cells in the infected PC is driven primarily by migration of NK cells that migrate from distinct locations (including the spleen).

Discussion

In this report we studied NK trafficking in response to peritoneal infection with VV. Our data indicated that the accumulation of NK cells into the infected PC occurs rapidly (starting by 6 h after infection), and that this trafficking is sensitive to PTX treatment, indicating involvement of a $G\alpha_i$-coupled receptor. In contrast, NK trafficking into spleen, liver, bone marrow, and lung occurred in the absence of infection and was largely insensitive to PTX. Active vaccinia replication in spleen, liver, and lung after i.p. infection has been demonstrated by several groups (17, 26), but these studies have focused on later time points (days after infection) rather than the very early time points studied in this report. Hence, it is possible that the patterns and requirements for NK migration into

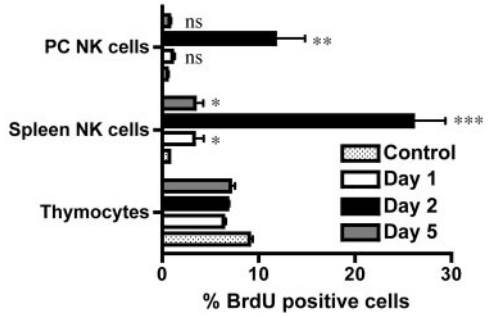

FIGURE 4. Minimal NK proliferation in VV-infected PC. B6 mice were left uninfected (control) or were infected (i.p.) with 2×10^6 PFU of VV-OVA virus for the indicated number of days. One hour before death, the mice received an injection (i.p.) of BrdU (1 mg). Thymus, spleen, and PC cells were analyzed for their BrdU content. Data shown for thymocytes are based on a live gate and serve as a positive control for labeling. Data shown for spleen and PC are for $NK1.1^+CD3^-$ cells only. Data were compiled from four experiments, with four to six animals (from at least two separate experiments) at each time point. Statistical analysis compares values from uninfected vs infected samples.

these tissues may change later in the course of the infection as the viremia becomes more widespread. Together, these data suggest very different trafficking requirements for NK entry into different tissues. Interestingly, these migratory properties of NK cells closely mirror those for memory T cells as determined by Klonowski et al. (8). They found that PTX-treated memory CD8 T cells were unable to traffic to the PC (or lymph nodes), but trafficking to spleen, liver, lung, and bone marrow was insensitive to PTX treatment, and PTX-treated cells were actually increased in number in the peripheral blood (a site not analyzed in our studies) (8). Similar findings were reported by Mazo et al. (27), although these authors noted a partial inhibition of PTX-treated CD8 memory cells to the BM at early time points, similar to our findings with NK cells (Figs. 2 and 3). Similar to our findings with NK cells, these data on CD8 memory cells were unanticipated, because lymphocyte trafficking is so strongly influenced by PTX-sensitive G_i protein-coupled receptors (23–25). However, there were important differences between the studies. Although Klonowski et al. (8) found that memory CD8 T cells migrate to the PC in normal mice, we found NK cell trafficking to this site was minimal in uninfected animals (Fig. 3). This may simply be due to the duration of the migration assay (3 days for the memory CD8 T cell study compared with our 6 h assay) or may reflect actual differences in trafficking cues for normal vs infected PC. Finally, although our data suggest that migration to liver, lung, spleen, and bone marrow is largely unaffected by PTX, this does not exclude a role for G protein-coupled receptors, because some receptors for chemokines and phospholipids can use PTX-insensitive G proteins (22, 28). In any event, our data suggest striking parallels in the trafficking patterns for NK cells and Ag-experienced T cells.

Although NK cells accumulate dramatically in the PC in the first few days after VV infection, NK proliferation was markedly more extensive in the spleen at these times. Together with the fact that the number of splenic NK declines on days 1 and 2 of infection compared with that in uninfected controls, these data fit with a model in which the majority of NK cells proliferate in the spleen (and potentially other tissues) before migration to the site of primary infection (in this case the PC). In this respect, our findings resemble studies of activated CD4 T cells, which accumulate at the Ag-bearing site without proliferating in situ (29). McIntyre et al. (13) used an ex vivo [^{3}H]thymidine incorporation assay to show that NK cells from mouse hepatitis virus-infected PC proliferate and are cytolytic. However, these studies did not determine the fraction of NK cells at the site that proliferate and, hence, their contribution to overall NK numbers (13). In our analysis, using a short term BrdU incorporation assay, we found that proliferation of NK cells in the PC was not statistically significant on days 1 and 5, but proliferation was observed on day 2 of infection. At the same time, NK blasts were evident in the PC (and spleen) by day 1 (Fig. 1*B*). Hence, although local proliferation in the PC may account for some NK accumulation at this site after day 2, it seems likely that the early wave of NK cells appearing in the infected PC on day 1 arose from migration of cells from other tissues. Caution should be taken in extrapolating these results, however, because it is certainly possible that the extent of NK cell proliferation at the site of infection differs for different viruses. Indeed, NK proliferation in the liver after infection with hepatotropic viruses might well have different requirements, because the liver has a large number of resident NK cells (1). In contrast, there are very few NK cells in the uninfected PC (10, 11) (Fig. 1*A*), and we were unable to detect NK trafficking to the normal PC in a short term migration assay (Figs. 2 and 3).

Our data indicate that 6 h is sufficient for the appearance of activated NK cells at the primary site of infection. What signals drive this migration are currently unclear, although our data on the PTX sensitivity of NK trafficking to the infected PC supports the idea that a chemokine or other chemotactic factor may be involved. In other infections, such as with MCMV, NK trafficking to the liver is in response to CCL3, production of which is driven by Type-I IFNs (15, 16). Whether this pathway operates at the very early time points studied in this report is unclear. A distinct question is how these immigrant NK cells might influence the immune response? Aside from direct effector functions of NK cells against the pathogen (of which IFN-γ secretion may be relevant in the response to vaccinia) it has become clear in recent years that interactions between NK cells and DC can have profound effects on the latter population. Because the ratio of NK cells to DC is evidently critical to determining whether the interaction favors maturation or death of immature DC (3, 30, 31), early interactions between these cell populations may have a profound effect on the direction of the immune response. Indeed, in preliminary data we have been able to detect dendritic cells in the vaccinia infected PC (at 6 h post infection) which are presenting virally-encoded peptide Ags in association with class I MHC molecules (data not shown), suggesting that relevant NK-DC cross-talk could begin very soon after pathogen infection. The significance of this potential interaction on the adaptive immune response to vaccinia is currently under investigation. However, it is still unclear whether NK cells play a nonredundant role in protection against vaccinia. Various approaches to NK depletion have suggested that these cells may offer protection against the virus (17, 19, 32), but key T cell populations may also be depleted in these systems. In preliminary data, we have observed much greater sensitivity of $Rag^{-/-}\gamma C^{-/-}$ vs $Rag^{-/-}$ mice to vaccinia infection (data not shown). A prominent difference between these mouse strains is the lack of NK cells in the former but not the latter. However, effects of γC-deficiency on macrophages and DC (33) may complicate this interpretation also. By extending the analysis, pursued in this study, of focusing on time points early after infection, it should be possible to determine the effect which NK cells have on other cell populations (including DC and various T cell populations) as the protective response develops.

In summary, our studies define the sites for NK migration and proliferation at early time points following vaccinia infection and build on our understanding of the initial events in the innate immune response to pathogens.

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Disclosures

The authors have no financial conflict of interest.

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