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*J Immunol* published online 13 July 2012
http://www.jimmunol.org/content/early/2012/07/13/jimmunol.1200584

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NK Cell Response to Vaccinia Virus Is Regulated by Myeloid-Derived Suppressor Cells

Carl Fortin,* Xiaopei Huang,* and Yiping Yang*†

NK cells are critical for the innate immune control of poxviral infections. Previous studies have shown that NK cells are efficiently activated in response to infection with vaccinia virus (VV), the most studied member of the poxvirus family. However, it remains unknown whether the activation of NK cells in response to VV infection is tightly regulated. In this study, we showed that myeloid-derived suppressor cells (MDSCs) rapidly accumulated at the site of VV infection. In vivo depletion of MDSCs led to enhanced NK cell proliferation, activation, and function in response to VV infection. This was accompanied by an increase in mortality and systemic IFN-γ production. We further demonstrated that the granulocytic-MDSC (G-MDSC) subset was responsible for the suppression on NK cells and that this suppression was mediated by reactive oxygen species. These results indicate that G-MDSCs can negatively regulate NK cell activation and function in response to VV infection and suggest that manipulation of G-MDSCs could represent an attractive strategy for regulating NK cell activities for potential therapeutic benefits.

The Journal of Immunology, 2012, 189: 000–000.

Natural killer cells are crucial in innate immune control of various viral infections (1, 2). Clinically, individuals who are defective for NK cell function usually suffer from severe and recurrent viral infections (3). NK cells also play a critical role in the control of poxviruses. In response to poxviral infection, NK cells are activated and migrate to the site of infection, leading to effective viral control (4–8). In a model of infection with vaccinia virus (VV), the most studied member of the poxvirus family, recent studies have shown that multiple pathways are required for the effective activation of NK cells and the subsequent control of VV infection in vivo. These include both TLR2-dependent and -independent pathways (7, 9), as well as the NKG2D pathway (10). However, it remains unknown whether and how the activation of NK cells is regulated in response to VV infection. Tight control of NK cell activation is desired, as it may avoid the potential collateral damage elicited by the unopposed infection of NK cells.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that play an important role in the regulation of the immune system (11). They consist of myeloid progenitor cells, immature macrophages, immature dendritic cells (DCs), and immature granulocytes. In mice, MDSCs are characterized by the expression of CD11b and Gr-1. They can be further divided into two subsets: granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs), defined by CD11b⁺Ly6G⁻Ly6Clow and CD11b⁺Ly6G⁻Ly6Chigh, respectively (12). It is generally considered that both subsets have distinct immunosuppressive properties (13).

The importance of MDSCs in regulating immune responses was first discovered in cancer patients and was that the accumulation of MDSCs at tumor sites suppresses tumor immunity and promotes tumor growth (14, 15). Since then, extensive studies have established a prominent role for MDSCs in the regulation of T cell responses in mice during tumor progression (11, 16). Recent studies have also demonstrated the ability of MDSCs to modulate NK cell function in tumor models (17–19). In addition to tumor models, MDSCs have been shown to expand in other experimental models, including transplantation (20–22) and autoimmune diseases (23, 24).

MDSC expansion has also been observed in response to various infections including polymicrobial sepsis (25, 26) and parasitic (27), bacterial (28), and viral infections (29, 30). However, it remains largely undefined with regard to how MDSCs modulate the immune response during an infection. In this study, we evaluated whether MDSCs could influence the host’s immune response, specifically NK cell response, to VV infection in vivo. Our results showed that both G-MDSCs and M-MDSCs rapidly accumulated at the site of infection with VV. In vivo depletion of MDSCs promoted NK cell proliferation, activation, and function in response to VV infection, leading to increased mortality and IFN-γ production. We further demonstrated that G-MDSCs were responsible for the suppression of NK cells upon VV infection and that this suppression was mediated by reactive oxygen species (ROS) production.

Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Mice were used between 8 and 10 wk of age. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Duke University (Durham, NC).

VV

The Western Reserve strain of VV was purchased from American Type Culture Collection (Manassas, VA). VV was grown in TK-143B cells.
(American Type Culture Collection) and purified by a 35% sucrose cushion as described (10). The titer was determined by plaque assay on TK-143B cells, and VV was stored at −80°C until use. For in vivo studies, 2 × 10^6 PFU, or as indicated, of live VV in 0.05 ml 1 mM Tris (pH 9) was injected into mice i.p.

**Abs and flow cytometry**

Allophycocyanin-conjugated anti–IFN-γ, PE-conjugated anti-CD49b/Pan-NK Cells (clone DX5), PE-Cy5-conjugated anti-CD3e (clone 145-2C11), FITC-conjugated anti-CD49b/Pan-NK cells (clone DX5), FITC-conjugated anti-granzyme B (clone NGZB), PE-conjugated anti-CD27 (clone LG3A10), allophycocyanin-conjugated killer cell lectin-like receptor G1 (KLRL1; clone 2F1), PE-Cy5-conjugated anti–Gr-1 (clone RB6-8C5), allophycocyanin-conjugated CD11b (clone M1/70), PE-conjugated Ly6G (clone 1A8), and FITC-conjugated Ly6C (clone AL-21) were purchased from BD Biosciences (San Diego, CA). To assess the production of IFN-γ and granzyme B intracellularly, splenocytes were incubated with 5 ng/ml PMA, 10 ng/ml ionomycin, and 5 μg/ml brefeldin A for 4 h at 37°C. Intracellular staining was performed as previously described (31). FACSCanto (BD Biosciences, San Diego, CA) was used for flow cytometry event collection, which was analyzed using FACSDiva software (BD Biosciences).

NK cell proliferation was assessed with the FITC BrdU Flow Kit according to the manufacturer’s instruction (BD Biosciences). Briefly, mice were injected with 2 mg BrdU i.p. 1 h before the sacrifice. BrdU^+ cells were identified after staining with anti–BrdU-FITC and subject to FACS analysis.

**Ovarian VV titer assay**

Viral load in the ovaries was measured by plaque-forming assay as described (7). In brief, female mice were sacrificed 36 h postinfection, and ovaries were harvested and stored at −80°C. Ovaries from individual mice were homogenized and freeze-thawed three times. Serial dilutions were injected with 2 mg BrdU i.p. 1 h before the sacrifice. BrdU^+ cells were marked as described (31). FACSCanto (BD Biosciences, San Diego, CA) was used for flow cytometry event collection, which was analyzed using FACSDiva software (BD Biosciences). The Gr-1 hybridoma (clone RB6-8C5) was a generous gift from Dr. G. Kelsoe (Duke University, Durham, NC). The Gr-1 hybridoma was grown in serum-free medium in a CELLine CL 1000 bioreactor (Wilson Wolf, New Brighton, MN) for 5 d. GM-CSF and IL-4 were replenished on days 2 and 4. On day 5, CD11c^+ DCs were harvested for NK cell stimulation.

**NK–DC coculture system**

DCs were generated from the bone marrow cells in the presence of GM-CSF and IL-4 as described (7). In brief, bone marrow cells were harvested from femurs and tibiae of mice and cultured in the presence of mouse GM-CSF (1000 U/ml) and IL-4 (500 U/ml; R&D Systems, Minneapolis, MN) for 5 d. GM-CSF and IL-4 were replenished on days 2 and 4. On day 5, CD11c^+ DCs were harvested for NK cell stimulation.

NK–DC coculture was performed as described (32) with some modifications. In brief, purified DX5^+CD3^- NK cells (2 × 10^6) were cocultured with CD11c^+ DCs (1 × 10^5) at an NK/DC ratio of 2:1. The coculture was subsequently infected with VV with a multiplicity of infection of 1, relative to DCs, for 20 h at 37°C. To assess the production of IFN-γ intracellularly, the coculture was incubated with 50 ng/ml PMA, 50 ng/ml ionomycin, and 5 μg/ml brefeldin A (BD Biosciences) for 4 h at 37°C. For intracellular production of granzyme B, the coculture was incubated with 5 μg/ml brefeldin A only for 6 h at 37°C.

For NK suppression assays, MDSCs were enriched from spleens of mice that were infected with VV for 24 h with anti-CD11b microbeads (Miltenyi Biotec, Auburn, CA). Enriched CD11b^+ splenocytes were then stained with FITC-conjugated anti–Ly6C and PE-conjugated anti–Ly6G, sorted by flow cytometry, and then added to the coculture at the indicated ratio.

In some experiments, 1000 U/ml catalase (Sigma-Aldrich, St. Louis, MO), 0.5 mM N^ε-monomethyl-l-arginine (L-NMMA; Calbiochem, San Diego, CA), or 0.5 mM N(O)-hydroxy-nor-l-arginine (nor-NOHA; Calbiochem, San Diego, CA) were added to the coculture to inhibit ROS and NO production or arginase activity, respectively, by MDSCs, as described (12).

**Statistical analysis**

A two-sided, unpaired Student t test with 95% confidence bound and Welch’s correction was used for all statistical analysis except for Kaplan-Meier plot, for which log-rank (Mantel-Cox) test analysis was used. Data are presented as mean ± SD. All statistical analyses were performed using GraphPad Prism Version 5.0d for Mac OS X (GraphPad, San Diego, CA).

**Results**

**Rapid accumulation of MDSCs at the site of VV infection**

To study a potential role of MDSCs in the immune response to VV infection in vivo, we first examined whether MDSCs accumulated at the site of VV infection. Mice were infected with VV i.p., and the presence of MDSCs in the peritoneal cavity was evaluated at different days postinfection. Twenty-four hours postinfection, both the percentage and absolute numbers of G-MDSCs (CD11b^+ Ly6G^+Ly6C^low) and M-MDSCs (CD11b^+ Ly6G^-Ly6C^hi/hi) were markedly increased in the peritoneal exudates of the infected mice compared with the naive controls (Fig. 1A). Although the number of M-MDSCs remained relatively unchanged thereafter, that of G-MDSCs declined somewhat after the peak at 24 h postinfection (Fig. 1B). In contrast, the percentage and absolute numbers of G-MDSCs and M-MDSCs in the spleen and bone marrow were markedly increased in the peritoneal exudates of the infected mice compared with the naive controls (Fig. 1A). Although the number of M-MDSCs remained relatively unchanged thereafter, that of G-MDSCs declined somewhat after the peak at 24 h postinfection (Fig. 1B). In contrast, the percentage and absolute numbers of G-MDSCs and M-MDSCs in the spleen and bone marrow were markedly increased in the peritoneal exudates of the infected mice compared with the naive controls (Fig. 1A). Although the number of M-MDSCs remained relatively unchanged thereafter, that of G-MDSCs declined somewhat after the peak at 24 h postinfection (Fig. 1B).
marrow remained relatively steady during the course of infection. These results showed that both subsets of MDSCs accumulated rapidly at the site, which is the peritoneal cavity, of VV infection in vivo.

Depletion of MDSCs leads to increased mortality and IFN-γ production

To evaluate the biological significance of MDSC accumulation at the site of VV infection in vivo, we examined the effect of MDSC depletion with the anti–Gr-1 Ab. The anti–Gr-1 Ab used in this study (clone RB6-8C5) binds to two epitopes, LY6G and LY6C (13), and therefore can induce the depletion of both subsets of MDSCs in vivo (17). Indeed, we found that a single injection of 0.15 mg anti–Gr-1 Ab resulted in an almost complete depletion of both M-MDSCs and G-MDSCs (Fig. 2A). A similar degree of depletion was also observed in the bone marrow (data not shown). We first examined the effect of MDSC depletion on the mortality of mice infection with VV. The depletion of MDSCs significantly \( p < 0.01 \) reduced the survival of infected mice (Fig. 2B). We next determined the cause of death in MDSC-depleted mice. Our results ruled out overwhelming viral infection as a cause of death because the viral titer was lower in MDSC-depleted mice (Fig. 2C). An alternative explanation would be that mice died of an uncontrolled immune and/or inflammatory response. As IFN-γ is linked with such an outcome (33), we then measured the amount of IFN-γ in serum and peritoneal cavity of infected mice. The quantity of IFN-γ was significantly \( p < 0.01 \) higher in serum and peritoneal cavity of depleted mice 24 h postinfection (Fig. 2D). Together, these results show that MDSC-depleted mice have an increased mortality due, at least in part, to increased amounts of IFN-γ, but not an overwhelming viral infection.

Depletion of MDSCs enhances NK cell activation and function

NK cells are pivotal for the control of VV infection as well as serve as an early source of IFN-γ (4–8). Because we observed that MDSC-depleted mice had a reduced viral load and increased IFN-γ production, we hypothesized that MDSCs may control NK cell activation and function in response to VV infection. We first determined the effect of MDSC depletion on NK cell proliferation by pulse BrdU labeling. NK cells were characterized as DX5+CD3− cells as described previously (7, 10). Indeed, the majority of these cells expressed the NK cell-specific marker NKp46 (Fig. 3A). We found that the percentage of BrdU-positive NK cells was significantly \( p < 0.05 \) increased in MDSC-depleted mice both in the spleen and peritoneal cavity (Fig. 3B), leading to a significant \( p < 0.01 \) increase in total NK cell numbers in peritoneal cavity,
but not in spleen, upon MDSCs depletion (Fig. 3B). These results suggest that MDSCs negatively regulate NK cell proliferation in vivo.

It has been shown that the CD27high NK cell subset represents more potent, fully activated cells (34), and KLRG1 expression correlates the terminal activation of NK cells (35). Thus, we next examined how MDSC depletion affected the expression of CD27 and KLRG1 on splenic and peritoneal cavity NK cells 36 h postinfection. We found that mice devoid of MDSCs had an increase in CD27 and KLRG1 double-positive NK cells in both spleen and peritoneal cavity (Fig. 3B), suggesting NK cell activation is enhanced in the absence of MDSCs. We further observed that the production of IFN-γ and granzyme B by splenic (Fig. 4A, 4B) and peritoneal (Fig. 4C) NK cells was significantly higher in MDSC-depleted mice.

Collectively, these results suggest that MDSCs negatively regulate the proliferation, activation, and function of NK cells in response to VV infection in vivo.

To further support this observation, we examined the effect of MDSC depletion in the absence of NK cells on viral titers in vivo. Consistent with our previous observation (7), depletion of NK cells led to a marked increase in viral titers (Fig. 4D). We showed in this study that the effect of MDSC depletion on viral titers was abrogated in the absence of NK cells (Fig. 4D). These results further confirm that the effect of MDSCs on VV infection in vivo is mediated through suppression of NK cells.

G-MDSCs suppress NK cell activation and function

Which MDSC subset was responsible for the observed suppression of NK cell activation and function? To address this question, we used a previously described in vitro NK–DC coculture system (7, 10). G-MDSCs and M-MDSCs were sorted from spleens of infected mice and added to the NK–DC coculture at different MDSC/NK ratios, followed by infection with VV. The addition of G-MDSCs to the coculture resulted in a significant (p, 0.05) reduction in IFN-γ and granzyme B production by NK cells in a dose-dependent manner (Fig. 5). In contrast, M-MDSCs were not suppressive for IFN-γ and granzyme B production by NK cells (Fig. 5B). These results demonstrate that G-MDSCs suppress NK cell activation in vitro.

Having established in vitro that G-MDSCs were the suppressive subset, we next investigated their suppressive capacities for NK cell activation in vivo. Purified G-MDSCs were adoptively transferred into mice, followed by infection with VV. Mice...
transferred with G-MDSCs had a significant reduction in both total NK cell numbers (p < 0.05) and BrdU-positive NK cells (p < 0.01) in the peritoneal cavity compared with the control mice that did not receive G-MDSC transfer (Fig. 6A), indicating that G-MDSCs suppress NK cell proliferation in vivo. To investigate whether this G-MDSC-mediated reduction in NK cell numbers was functionally relevant, we examined the impact of G-MDSC transfer on viral load. We observed that the viral titer was significantly (p < 0.01) increased in mice that received G-MDSCs (Fig. 6B). These results demonstrate that G-MDSCs are responsible for the observed NK cell suppression during VV infection in vivo.

**G-MDSC–mediated suppression of NK cell activation is mediated by ROS**

Having established that G-MDSCs are responsible for suppressing NK cell activation and function, we next sought to determine the mechanism(s) by which G-MDSCs mediate suppression on NK cells. It has been shown that MDSCs can suppress T cell responses by a variety of mechanisms, including the production of NO and ROS, the induction of arginase-1 activity, and others (36). To address whether these mechanisms also apply to the suppression of NK cells in response to VV infection, we added inhibitors of these factors to the G-MDSC/NK coculture system. The results showed that only catalase, the inhibitor of ROS production, could reverse G-MDSC-mediated suppression on NK cells, whereas L-NMMA (inhibitor of NO production) or nor-NOHA (inhibitor of arginase-1) had no effects on G-MDSC-mediated suppression (Fig. 7). When added to the coculture in the absence of G-MDSCs, none of these inhibitors, including catalase, impaired NK cell activation (data not shown). These results strongly suggest that G-MDSC–mediated suppression on NK cell activation during VV infection is mediated by ROS.

**CD244 characterizes G-MDSCs that are responsible for NK cell suppression**

Although G-MDSCs are functionally distinct from neutrophils, it has been difficult to separate G-MDSCs from neutrophils phenotypically. A recent study has shown that tumor-derived G-MDSCs can be characterized by the expression of CD244 in that G-MDSCs are CD244+CD11b+Ly6G+, whereas neutrophils are CD244−CD11b−Ly6G+ (37). Thus, we wanted to know whether CD244 also identified G-MDSCs in response to VV infection. Twenty-four hours postinfection with VV i.p., similar numbers of CD244+ and CD244−CD11b+Ly6G+ cells were found in the spleen. However, the majority of CD11b+Ly6G+ cells in the peritoneal cavity were CD244+ (Fig. 8A). We further showed that the addition of sorted CD244+, but not CD244−, CD11b+Ly6G+ cells to the NK cell coculture resulted in a significant (p < 0.05) reduction in IFN-γ and granzyme B production by NK cells (Fig. 8B). These results suggest CD244 expression identifies peritoneal G-MDSCs that are responsible for the suppression of NK cells in response to VV infection.

**Discussion**

These data suggest that the substantial increase in total peritoneal cavity NK cells is a result of NK cell proliferation as well as NK cells migrated from other places such as spleen.

**FIGURE 7.** G-MDSC–mediated suppression of NK cells is mediated by ROS. DX5+CD3− NK cells were cocultured with CD11c+ DCs and infected with VV or left uninfected (Control). In some wells, G-MDSCs purified from spleens of infected mice were added at a 2:1 G-MDSC/NK ratio (VV+G-MDSC). In addition, where indicated, 1000 U/ml catalase, 0.5 mM L-NMMA, or 0.5 mM nor-NOHA was added to inhibit ROS, NO production, and arginase, respectively. Twenty-four hours postinfection, NK cells were assayed for intracellular IFN-γ and granzyme B (GRB). (A) FACS plots of intracellular IFN-γ and GRB production by NK cells are shown with the percentage of IFN-γ− or GRB-positive cells among DX5+CD3− NK cells indicated. (B) The mean percentage ± SEM of IFN-γ− or GRB-positive cells among DX5+CD3− NK cells is provided. Data shown are representative of three independent experiments.
CD244 and infected with VV or left uninfected (Control). In some wells, sorted dependent experiments. (A) Mice were infected with 2 × 10^6 PFU of VV i.p. Twenty-four hours postinfection, cells from spleen and peritoneal exudates (PE) were stained with anti-CD244, anti-CD11b, and anti-Ly6G. The events were gated according to CD244 expression, and the percentage of CD244^- and CD244^+CD11b^-Ly6G^- cells among total cells is indicated. (B) DX5^+CD3^- NK cells were cocultured with CD11c^- DCs and infected with VV or left uninfected (Control). In some wells, sorted CD244^- (CD244^-) or CD244^+ (CD244^+) CD11b^-Ly6G^- cells were added to the coculture at the CD11b^-Ly6G^- NK cell ratio of 1:2. NK cells were assayed for intracellular IFN-γ and granzyme B (GRB) production 24 h later. FACS plots show the percentage of IFN-γ- or GRB-positive cells among DX5^-CD3^- NK cells. Data are representative of two independent experiments.

In this study, we showed that MDSCs rapidly accumulated at the site of infection with VV. Removal of MDSCs in vivo led to enhanced NK cell proliferation, activation, and function in response to VV infection as well as an increase in mortality and IFN-γ production. We further demonstrated that CD244 expression characterized the G-MDSC subset responsible for the suppression on NK cells and that this suppression was mediated by ROS.

NK cells are critical for the control of viral infections. Thus, in the setting of VV infection, multiple pathways have evolved to ensure effective activation of NK cells and the subsequent control of VV infection in vivo. Previous studies have shown that efficient NK cell activation depends on both TLR2-dependent and -independent pathways (7, 9), as well as the NKG2D pathway (10). This study demonstrates for the first time, to our knowledge, that NK cell response to VV infection is also negatively regulated by G-MDSCs. This tight control of NK cell activation could potentially avoid collateral damage such as mortality and systemic inflammation elicited by the exuberant NK cell activation.

The accumulation of MDSCs in mice in response to viral infection has been shown in different viral infections (29, 30). However, the role of MDSCs in regulating NK cell responses during the early phase of infection has not been addressed. Thus, this is the first study, to our knowledge, showing a critical role of G-MDSCs in regulating the NK cell response to a viral infection. Although MDSCs have been shown to modulate NK cell function in tumor models, it still remains unknown which MDSC subset is responsible for modulation of NK cell function in tumor-bearing hosts (17–19). Our results also provided evidence that both M-MDSCs and G-MDSCs accumulate in the peritoneal cavity in response to VV infection. Although M-MDSCs appear not involved in the suppression of NK cells, we cannot rule out the possibility of their role in regulating T cell response late in VV infection.

The observation that mice depleted of MDSCs in vivo were associated with an increase in mortality and systemic IFN-γ production, but a reduction in viral load, suggests that the increased mortality is likely due to an uncontrolled inflammatory response rather than an infection. Although it is correlated with an increase in systemic IFN-γ production, other cytokines and factors may also contribute to the increased mortality. In line with this notion, a recent study indicates that hepatic acute-phase proteins can control inflammatory responses during infection by promoting MDSC function (26). Further studies are needed to dissect whether other elements are also deregulated, contributing to the increased mortality upon MDSC depletion in vivo.

Several mechanisms have been proposed for MDSC-mediated suppression of immune responses, ranging from receptor–ligand interactions to soluble mediators (11, 13). Although most of the studies involving mechanism(s) of suppression were performed in tumor models, there are a few utilizing infectious models. In a model of *Mycobacterium bovis* bacillus Calmette-Guérin infection, M-MDSCs were found to impair T cell priming in draining lymph nodes by NO production (28). In polymicrobial sepsis, MDSC-mediated suppression was found to be associated with the secretion of IL-10 (25). In models of parasitic infection, IFN-γ-induced NO production was responsible for MDSC-mediated suppression of T cell responses (38, 39).

In contrast to MDSC-mediated suppression on T cells, in this study, we found that ROS production was required for NK cell suppression by G-MDSCs. This may explain why M-MDSCs do not suppress NK cells, as M-MDSCs produced little ROS (11). Although this result suggests that ROS produced by G-MDSCs may be delivered to NK cells to mediate the suppression, we cannot exclude the possibility that receptor–ligand interactions may also be required for the suppression of NK cell activation. Indeed, membrane-bound TGF-β on MDSCs has been shown to be responsible for the induction of NK cell anergy in tumor-bearing mice (17), and the blockade of Nkp30 on NK cells in hepatocellular carcinoma patients reversed the suppression by MDSCs (18). These observations suggest that in some tumor models, the suppression of NK cells by MDSCs involves receptor–ligand interactions. It is not clear whether receptor–ligand interactions are also involved in G-MDSC–mediated NK cell suppression in the setting of VV infection. Future studies are required to investigate this possibility. A recent report showed that lack of Ly49H^- NK cells may afford additional protection against ectromelia viral infection (40). Thus, it is possible that G-MDSCs may interact with Ly49H^- NK cells for the suppressive effect.

In summary, we have provided evidence that MDSCs accumulate rapidly at site of viral infection and suppress NK cell activation and function during the early phase of the immune response against VV infection. We have further shown that it is the G-MDSC subset that mediates ROS-dependent suppression on NK cells. Our study may suggest a novel strategy using G-MDSCs to modulate NK cell activity for potential therapeutic benefits in viral infections.
Acknowledgments
We thank Nancy Martin and Lynn Martinke from the Cancer Center Facility, Duke University, for excellent technical assistance in cell sorting.

Disclosures
The authors have no financial conflicts of interest.

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