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CD69 Deficiency Enhances the Host Response to Vaccinia Virus Infection through Altered NK Cell Homeostasis

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ABSTRACT

During the host response to viral infection, the transmembrane CD69 protein is highly upregulated in all immune cells. We have studied the role of CD69 in the murine immune response to vaccinia virus (VACV) infection, and we report that the absence of CD69 enhances protection against VACV at both short and long times postinfection in immunocompetent and immunodeficient mice. Natural killer (NK) cells were implicated in the increased infection control, since the differences were greatly diminished when NK cells were depleted. This role of NK cells was not based on an altered NK cell reactivity, since CD69 did not affect the NK cell activation threshold in response to major histocompatibility complex class I NK cell targets or protein kinase C activation. Instead, NK cell numbers were increased in the spleen and peritoneum of CD69-deficient infected mice. That was not just secondary to better infection control in CD69-deficient mice, since NK cell numbers in the spleens and the blood of uninfected CD69$^{-/-}$ mice were already augmented. CD69-deficient NK cells from infected mice did not have an altered proliferation capacity. However, a lower spontaneous cell death rate was observed for CD69$^{-/-}$ lymphocytes. Thus, our results suggest that CD69 limits the innate immune response to VACV infection at least in part through cell homeostatic survival.

IMPORTANCE

We show that increased natural killer (NK) cell numbers augment the host response and survival after infection with vaccinia virus. This phenotype is found in the absence of CD69 in immunocompetent and immunodeficient hosts. As part of the innate immune system, NK lymphocytes are activated and participate in the defense against infection. Several studies have focused on the contribution of NK cells to protection against infection with vaccinia virus. In this study, it was demonstrated that the augmented early NK cell response in the absence of CD69 is responsible for the increased protection seen during infection with vaccinia virus even at late times of infection. This work indicates that the CD69 molecule may be a target of therapy to augment the response to poxvirus infection.

Vaccinia virus (VACV) is a member of the *Poxviridae* family and was used as a vaccine to eradicate the variola virus, which is from the same family. Since then, it has commonly been used in research as a vaccine vector model. It is a large DNA virus with a linear double-stranded DNA genome that encodes <200 proteins (1). It has a broad cellular tropism and infects almost any cell line in culture. Members of this virus family do not usually establish persistent or latent infections and have a low mutation rate (2). VACV infection is initially controlled by the innate immune response, but it can be eradicated only by adaptive immunity, and *Rag*$^{-/-}$ mice finally succumb to the infection (3). Natural killer (NK) cells are crucial players in the first line of defense against viral infections. Through their expression of a range of germ line-encoded receptors, they are able to recognize virus-infected cells either by direct recognition of viral proteins or by sensing of infection-induced reductions in major histocompatibility complex class I (MHC-I) levels and expression of stress molecules. Upon this recognition, NK cells become activated, proliferate, and use a range of effector mechanisms to control the infection. These include the production of cytokines, such as gamma interferon (IFN-γ), which represents a key player in antiviral defense; the secretion of granzyme B (GrzB) and perforin-containing cytotoxic granules in the immune synapse with the infected cells; and the expression of death-inducing ligands of the tumor necrosis factor (TNF) superfamily of receptors. VACV-infected cells have increased sensitivity to NK cell lysis. However, they can also modulate their function by mechanisms that allow them to evade the immune system (1).

CD69 is a transmembrane C-type lectin protein that is highly expressed in leukocytes upon infection, and the gene for CD69 is located in the NK cell complex in both humans and mice. CD69 is constitutively expressed on thymocytes at certain stages of negative and positive selection (4), bone marrow B cell precursors (5), NK cells, invariant NK T cells (iNKT cells), memory-phenotype T cells (6), and tissue-resident memory T cells (7), among other cell types. It is not detected on naive conventional T and B cells, but it is upregulated on these cells when they encounter a cognate antigen and when the cells are stimulated by cytokines (8). CD69 acts in T and B lymphocyte migration, interacting in *cis* with the receptor sphingosine-1-phosphate receptor 1 (S1P1), inducing its internalization (9). However, the control of NK cell migration depends on $S1P_5$, which has not shown to interact with CD69 (10).

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CD69 deficiency leads to exacerbated disease in different T cell-dependent autoimmunity and allergy experimental models (11–13), and this was related to decreased transforming growth factor β production and increased Th17 responses. In NK cell-sensitive tumor models, CD69 deficiency leads to an increased antitumor response mediated by NK cells at the tumor site (14). Interestingly, in tumor and some autoimmunity models, treatment with an anti-CD69 monoclonal antibody (MAb) reproduced the $CD69^{-/-}$ phenotype (12, 15). In the case of bacterial infection with *Listeria monocytogenes*, CD69 deficiency resulted in poor control of the bacterial burden, accompanied by increased type I and II interferon (IFN) production and increased levels of leukocyte apoptosis (16).

Though CD69 is rapidly expressed in viral infections (17–19), its role in the immune response to these infections has never been studied to date. We aimed at analyzing the effect of CD69 deficiency on the course of VACV infection, and we found that CD69 deficiency results in increased early NK cell-dependent control of the infection. This is not related to altered NK cell reactivity but rather to increased NK cell numbers, a finding which is consistent with the decreased cell death rate. These results show how CD69 can affect immune responses to infection through its role in NK cell survival control.

MATERIALS AND METHODS

Mice. C57BL/6, BALB/c, and $Rag2^{-/-}$ BALB/c mice, all of which were $CD69^{+/+}$ or $CD69^{-/-}$, were bred and housed under specific-pathogen-free conditions in the animal facilities of the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. All mice used in this study were between 6 and 10 weeks of age. $CD69^{-/-}$ mice had been backcrossed on the C57BL/6 and the BALB/c backgrounds at least nine times. All procedures involving animals and their care were approved by the ISCIII Ethics Committees and were conducted according to institutional guidelines.

Cell isolation. Bone marrow was collected from two femurs of each mouse. Blood was collected in 10 ml of 2 mM EDTA–phosphate-buffered saline (PBS), and the numbers provided below are white blood cell counts per 1 ml of blood. Thymus and spleen tissues were disaggregated, and cells were washed in PBS. The erythrocytes in all samples were lysed with ammonium chloride potassium (ACK) solution, and leukocytes were labeled and analyzed by flow cytometry.

Antibodies and immunological reagents. *In vitro* cultures were performed in complete Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, and 2 mM L-glutamine at 37°C. NK cell proliferation was assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation. Briefly, 1×10^6 PFU of VACV was injected intraperitoneally (i.p.) into $Rag2^{-/-}$ mice 24 h before sacrifice. Splenocytes were incubated with 10 μM BrdU and 1×10^6 PFU of VACV for 1 h to restimulate the cells. In *in vivo* studies, mice were injected intraperitoneally with 1×10^6 PFU of VACV, and at 2 days after infection, the mice were treated with 1 mg of BrdU for 3 h before they were sacrificed. The incorporated BrdU was stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Ab) according to the manufacturer's instructions (FITC BrdU flow kit; BD Biosciences), and the cells were analyzed by flow cytometry.

NK cells were ablated by a single intravenous (i.v.) injection of 100 μg of anti-asialo GM1 (eBioscience, San Diego, CA) or 50 μg of anti-asialo GM1 (Wako Chemicals USA, Richmond, VA) in 200 μl PBS 1 day before infection. Control mice received the same dose of rabbit IgG (Sigma-Aldrich) by the same schedule. At 2 days after infection, the mice were sacrificed and analyzed. The completeness of NK cell depletion was determined by the absence of NKp46-positive ($NKp46^+$) cells in the spleen and blood.

Abs and flow cytometry. Cells were incubated with anti-CD16/32 (Fc-block 2.4G2; BD Biosciences, Franklin Lakes, NJ, USA). The following antibodies against mouse intracellular and surface antigens were purchased from eBioscience (San Diego, CA): anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7 or clone Ly-2), anti-CD11b (clone M1/70), anti-CD11c (clone N418 or clone HL3), anti-CD19 (clone eBio1D3), anti-CD25 (clone 3C7), CD49b (clone DX5), anti-CD69 (clone H1.2F3), anti-CD107a (clone eBio4A3), anti-CD122 (clone TM-b1), anti-F4/80 (clone BM8), anti-GR1 (clone RB6-8C5), anti-IFN (clone XMG 1.2), anti-NKp46 (clone 29A1.4), and anti-TNF (clone MP6-XT22). For intranuclear staining with anti-mouse T-bet (clone eBio4B10), cells were fixed and permeabilized with a FoxP3/transcription factor buffer set (BD). Cells were analyzed with a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), using BD FACSDiva software (Becton Dickinson), and the data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

Immunohistochemistry. Immunohistochemistry was carried out on 10-μm-thick serial spleen sections from uninfected $Rag2^{-/-}$ $CD69^{-/-}$ and wild-type (WT) mice or VACV-infected mice at 2 days after infection with 10^5 PFU of VACV. Sections were first stained with anti-CD45 or anti-Ki67 and were subsequently stained with hematoxylin.

Vaccinia virus. The Western Reserve (WR) strain of VACV (kindly provided by Daniel Lopez) was grown in CV1 cells cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μM β-mercaptoethanol. The titer was determined by plaque assay on CV1 cells, and the viral stock was stored at −80°C in PBS until use. A total of 1×10^5 or 1×10^6 PFU was injected into $Rag2^{-/-}$ mice and 1×10^7 PFU was injected into BALB/c mice intraperitoneally in 0.2 ml PBS. For survival studies, BALB/c mice were inoculated with 1×10^5 PFU intranasally and were weighed over time.

Ovarian and spleen VACV titer assay. The viral load in the ovaries was measured by plaque-forming assay. In brief, female mice were sacrificed at the times indicated below, and the ovaries and spleen were harvested and stored at −80°C in 0.5 ml of PBS until use. The ovaries and spleen from individual mice were first homogenized and freeze-thawed three times. Serial dilutions were plated on confluent CV1 cells. After 1 day of culture at 37°C, the plates were stained with crystal violet and the plaques were counted.

***Ex vivo* intracytoplasmic staining (ICS).** To assess the intracellular production of IFN-γ and TNF-α, 2×10^6 spleen cells were incubated with RMA-S cells in the presence of brefeldin A (BFA; 5 μg/ml) for 4 h at 37°C and washed. Alternatively, cells were restimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin or RPMI only in the presence of BFA (5 μg/ml) for 4 h at 37°C. Following incubation, the cells were stained for surface molecules, fixed with 4% paraformaldehyde, washed, and incubated with anti-IFN-γ MAb, anti-TNF MAb, and granzyme B MAb in the presence of 0.75% saponin for 20 min at 4°C. Events were acquired and analyzed by flow cytometry as described above. The values presented here were derived by subtracting the values for unstimulated wells (medium alone) from the values for restimulated ones.

Bone marrow chimeras. C57BL/6 recipient mice (CD45.1) were lethally irradiated with a dose of 1,050 rads. $CD69^{+/+}$ (CD45.1/0.2) and $CD69^{-/-}$ (CD45.2) donor mice were sacrificed, and bone marrow was collected from the femurs, tibiae, and humeri. Cells were passed through a 70-mm-mesh-size Nitex mesh. A mixture of 5×10^6 cells of each donor bone marrow sample was injected intravenously through the tail vein into irradiated recipient mice. After 6 to 8 weeks, bone marrow (tibiae and femurs) and spleen cells were harvested and NK cell numbers ($NKp46^+$) and macrophages (F4/80 positive [$F4/80^+$]) were analyzed.

Cell death assay. Splenocytes from noninfected mice were cultured in 24-well plates (1×10^6 cells/ml), and cell death was assayed at different times after culture by staining with propidium iodide (PI), followed by flow cytometric analysis.

Statistical analysis. All data were plotted and statistically analyzed using GraphPad Prism software. Graphs show the means and standard errors of the means (SEMs). Statistical significance was determined using a two-way analysis of variance or an unpaired two-tailed *t* test. A *P* value of <0.05 was considered significant.

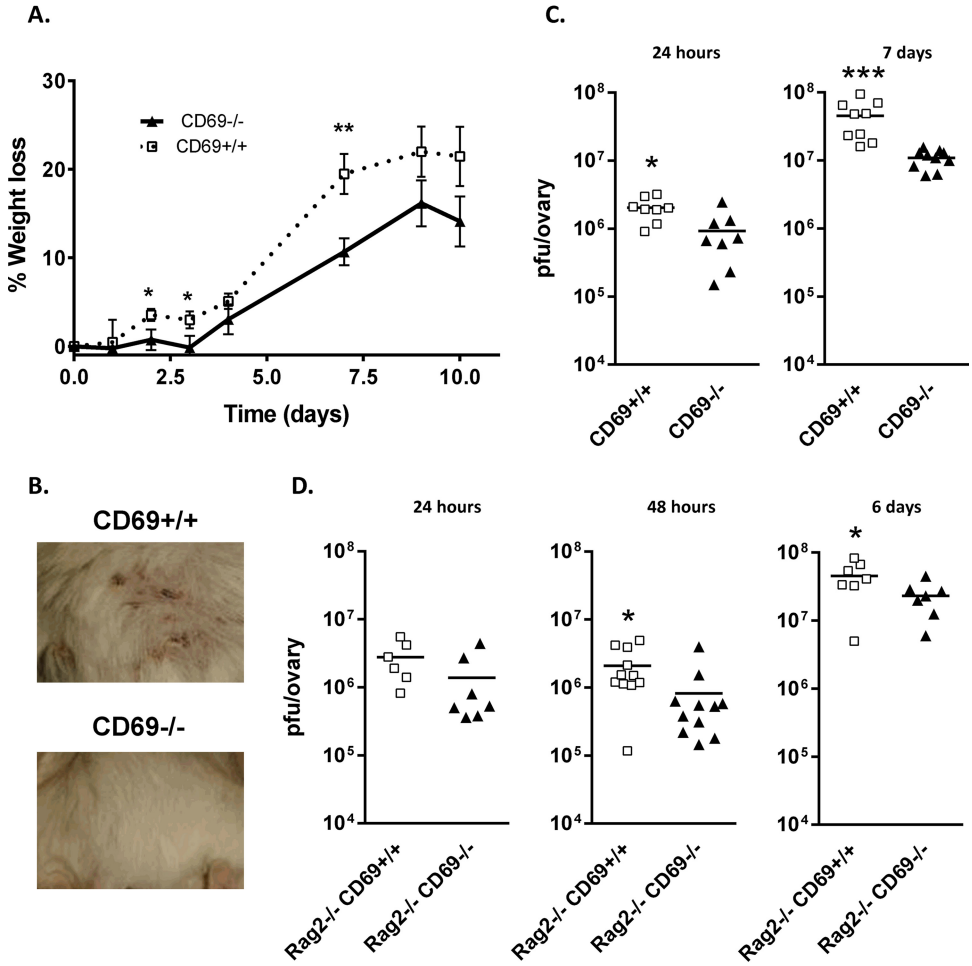

FIG 1 CD69$^{-/-}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice are more resistant to VACV infection than their CD69$^{+/+}$ counterparts. (A) CD69$^{+/+}$ or CD69$^{-/-}$ immunocompetent mice were infected intranasally with 10^5 PFU of VACV WR. Weight loss was evaluated over 10 days. (B) At 10 days after intranasal infection, CD69 mice had developed pustular lesions in the lumbar region, whereas CD69$^{-/-}$ mice were free of them. (C) CD69$^{+/+}$ and CD69$^{-/-}$ mice were infected with 10^7 PFU of VACV WR intraperitoneally. Their ovaries were collected and analyzed for the viral load at 24 h and 7 days after infection. (D) Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice were infected with 10^5 PFU of VACV WR, and their ovaries were collected at 24 h, 48 h, and 6 days after infection. (E) CD69$^{+/+}$ and CD69$^{-/-}$ mice (left) and Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$CD69$^{-/-}$ mice (right) were infected with 10^6 PFU of VACV WR intraperitoneally, and splenic viral titers were measured at 1 day postinfection. The data shown are representative of those from one experiment (A and B) or were pooled from two independent experiments (C to E). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

RESULTS

Enhanced *in vivo* anti-VACV activity in CD69$^{-/-}$ mice mediated by NK cells. During viral infection, expression of CD69 is induced in all leukocytes. To evaluate the role of CD69 in the host response to vaccinia virus, CD69$^{+/+}$ and CD69$^{-/-}$ mice were intranasally infected with 10^5 PFU of VACV, and their weight and appearance were monitored over time. CD69$^{-/-}$ mice showed reduced weight loss relative to their WT counterparts (Fig. 1A). Moreover, we observed that at 10 days postinfection (dpi), all CD69$^{+/+}$mice developed lumbar pustules, whereas CD69$^{-/-}$ mice did not (Fig. 1B). CD69 WT and CD69$^{-/-}$ mice were also infected intraperitoneally, and viral titers in the ovary were analyzed. At 7 dpi, CD69$^{-/-}$ mice had significantly lower titers than WT mice (Fig. 1C). The difference was already significant 1 day after infection (Fig. 1C), which suggested that CD69 is involved in the early antiviral immune response. Indeed, CD69$^{-/-}$ Rag2$^{-/-}$ mice also had lower viral titers than CD69 WT Rag2$^{-/-}$ mice at 2 and 6 dpi (Fig. 1D), and this trend was already observable at 1 dpi (Fig. 1D). No significant differences in the viral load in the spleen at 1 dpi were observed when either the load in CD69$^{+/+}$ mice was compared with that in CD69$^{-/-}$ mice or the load in Rag2$^{-/-}$ CD69$^{+/+}$ mice was compared with that in Rag2$^{-/-}$ CD69$^{-/-}$ mice (Fig. 1E). Therefore, increased control of viral infection was also observed in CD69$^{-/-}$ mice in the absence of adaptive immunity.

Previous studies showed that NK cells are crucial for VACV clearance *in vivo* in WT mice (20). To investigate a possible contribution of NK cells to the enhanced antiviral response observed in CD69$^{-/-}$ mice, CD69$^{-/-}$ and WT mice were treated with an NK cell-depleting anti-asialo GM1 MAb 1 day before being infected with VACV, and virus titers were analyzed at 1 dpi. The anti-asialo treatment eliminated the significance of the differences in the viral loads between CD69$^{-/-}$ and WT mice (Fig. 2A). In the Rag2$^{-/-}$ mouse background, the depletion of NK cells not only eliminated the advantage that CD69$^{-/-}$ mice had but also ren-

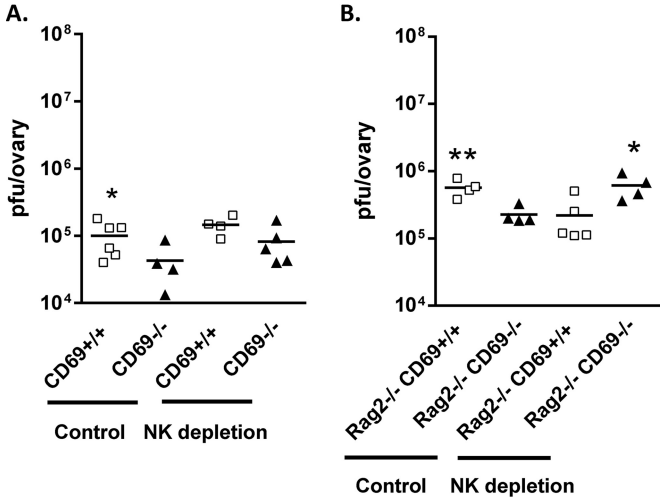

FIG 2 NK cells mediate the increased resistance to VACV WR in CD69$^{-/-}$ mice and Rag2$^{-/-}$ CD69$^{-/-}$ mice. CD69$^{+/+}$ and CD69$^{-/-}$ mice (A) and Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$CD69$^{-/-}$ mice (B) were treated with 50 μg of NK cell-depleting anti-asialo GM1 or rabbit serum control IgG i.v. 1 day before infection. Mice were then infected with 1×10^6 PFU of VACV WR i.p. Their ovaries were harvested and assayed for the viral load at 1 day postinfection. The data shown were pooled from two independent experiments and are representative of those from four independent experiments (A) or are from one experiment and are representative of those from three independent experiments (B). *, $P < 0.05$; **, $P < 0.01$.

dered them more susceptible to infection than CD69$^{+/+}$ mice (Fig. 2B), pointing to a differential effect of CD69 deficiency on NK cells and other leukocytes present in Rag2$^{-/-}$ mice. Altogether these results show that the increased early control of viral infection observed in CD69$^{-/-}$ mice is majorly mediated by NK cells.

Similar NK cell reactivities in both VACV-infected CD69$^{-/-}$ and CD69$^{+/+}$ mice but increased NK cell numbers in VACV-infected CD69$^{-/-}$ mice. We proceeded to analyze the NK cell function in CD69$^{-/-}$ mice. For this, we analyzed cytokine and GrzB production and the degranulation of NK cells (NKp46$^+$ cells) from infected mice. At early times of infection and in uninfected mice, the percentages of IFN-γ-producing (IFN-γ$^+$) and TNF-α-producing (TNF-α$^+$) cells within NK cells were equivalent in CD69$^{-/-}$ and WT splenocytes after PMA restimulation (Fig. 3A) as well as in Rag2$^{-/-}$ CD69$^{-/-}$ and Rag2$^{-/-}$ CD69$^{+/+}$ splenocytes after restimulation with PMA or RMA-S cells (Fig. 3B and data not shown), pointing to the fact that CD69 does not affect the cytokine production capacity or the activation threshold of NK cells. Thus, the NK cell-mediated increased viral resistance observed in the CD69$^{-/-}$ mice does not appear to be due to increased NK cell reactivity. In contrast, differences in the numbers of reactive, cytokine-producing NK cells were observed. The spleens of infected CD69$^{-/-}$ mice had significantly augmented numbers of IFN-γ- and TNF-α-producing NK cells (Fig. 3C) and a tendency toward increased NK cell numbers in general (Fig. 3D). The percentages of IFN-γ$^+$ and TNF-α$^+$ cells among CD4$^+$ and CD8$^+$ T cells were also unaffected by the CD69 deficiency (Fig. 4A), but increased numbers of IFN-γ$^+$ and TNF-α$^+$ CD4$^+$ and CD8$^+$ T cells were present in the spleens of CD69$^{-/-}$ mice at 24 h postinfection (Fig. 4B). Thus, like for NK cells, the reactivity of noncognate, innately responding T cells is unaltered in CD69$^{-/-}$ mice, but the T cells are increased in number.

The increased NK cell numbers were not due to an augmented proportion of NK cells in the spleens of CD69$^{-/-}$ mice but were due to increased total cell counts (Fig. 3D and data not shown). At 24 h postinfection, CD69-deficient mice had higher numbers of total spleen, blood, and peritoneum cells (Fig. 3D and 4C). All the splenic and peritoneal main leukocyte subtypes, CD4$^+$ and CD8$^+$ T cells, B cells (CD19$^+$), dendritic cells (DCs; CD11c^{hi}), macrophages (CD11b^{hi}, F4/80$^+$, CD11c$^-$), monocytes (CD11b^{hi}, F4/80$^-$, CD11c$^-$, Gr1^{int}, SSC^{low}), neutrophils (CD11b^{hi}, F4/80$^-$, CD11c$^-$, Gr1^{hi}, SSC^{int}), and eosinophils (CD11b^{hi}, F4/80$^-$, CD11c$^-$, Gr1^{low}, SSC^{hi}), were increased in number (Fig. 4D and E), but no major differences in their percentages were found (data not shown). At 7 days after infection, CD69$^{-/-}$ mice had approximately 40% more splenic leukocytes than WT mice (Fig. 3D), and numbers of all the main lymphocyte subsets were also increased to the same extent (Fig. 4F).

Likewise, at 2 and 6 dpi, Rag2$^{-/-}$ CD69$^{-/-}$ mice also had increased spleen leukocyte numbers relative to Rag2$^{-/-}$ CD69$^{+/+}$ mice (Fig. 5A), which translated into an increased splenic leukocyte density, as seen by immunohistochemical staining with an anti-CD45 MAb (Fig. 5B). The numbers of NK cells were also significantly higher in CD69$^{-/-}$ Rag2$^{-/-}$ mice (Fig. 5A), but their percentage was unaltered (data not shown). Upon *ex vivo* restimulation, the number of IFN-γ$^+$, CD107$^+$, and GrzB-producing (GrzB$^+$) NK cells among CD69$^{-/-}$ Rag2$^{-/-}$ spleen cells was also higher (Fig. 5C and D). We also detected a tendency toward increased numbers of NK cells producing TNF-α, even though this was not statistically significant (Fig. 5C). The numbers of other main leukocyte subtypes found in Rag2$^{-/-}$ mice were also increased in the spleens of CD69$^{-/-}$ Rag2$^{-/-}$ mice (Fig. 5E). These data reveal a positive correlation between viral infection control and effector NK cell numbers in CD69$^{-/-}$ mice.

Similar NK cell proliferation rates in CD69$^{-/-}$ Rag2$^{-/-}$ and CD69 WT Rag2$^{-/-}$ mice. The observation that Rag2$^{-/-}$ CD69$^{-/-}$ mice present an increased accumulation of NK cells in the spleen led us to assess whether CD69 is associated with the control of NK cell proliferation in antiviral immunity. The proliferation activity of splenic NK cells of uninfected and VACV-infected Rag2$^{-/-}$ CD69$^{-/-}$ and Rag2$^{-/-}$ CD69$^{+/+}$ mice was determined on the basis of their capacity to incorporate BrdU after 2 days of infection. Similar percentages of BrdU-positive (BrdU$^+$) were found in splenic NK cells from the Rag2$^{-/-}$ CD69$^{-/-}$ and Rag2$^{-/-}$ CD69$^{+/+}$ mice (Fig. 6A), although the number of BrdU$^+$ NK cells in the spleens of Rag2$^{-/-}$ CD69$^{-/-}$ mice was increased (Fig. 6B). Equivalent results were obtained when the proliferation of splenic NK cells from infected mice was analyzed with an *ex vivo* pulse of BrdU (data not shown). Furthermore, histological sections of tissue from spleens obtained 2 days after infection were stained by immunohistochemistry for Ki67, a nuclear protein associated with rRNA transcription, and a slight increase in Ki67 staining in CD69$^{-/-}$ mice compared to that in CD69 WT mice was observed, consistent with increased numbers of cycling cells (Fig. 6C). NK cells were analyzed for the expression of CD122 (interleukin-2 receptor β [IL-2Rβ]) and CD25 (IL-2Rα) in the spleen (Fig. 6D) and in bone marrow (Fig. 6E), but no differences in the percentages of NK cells expressing these components of the IL-2 receptor were found. These results show that CD69$^{-/-}$ NK cells proliferate at a rate similar to that of CD69$^{+/+}$ NK cells but that increased numbers of proliferating NK cells are found in CD69$^{-/-}$ mice.

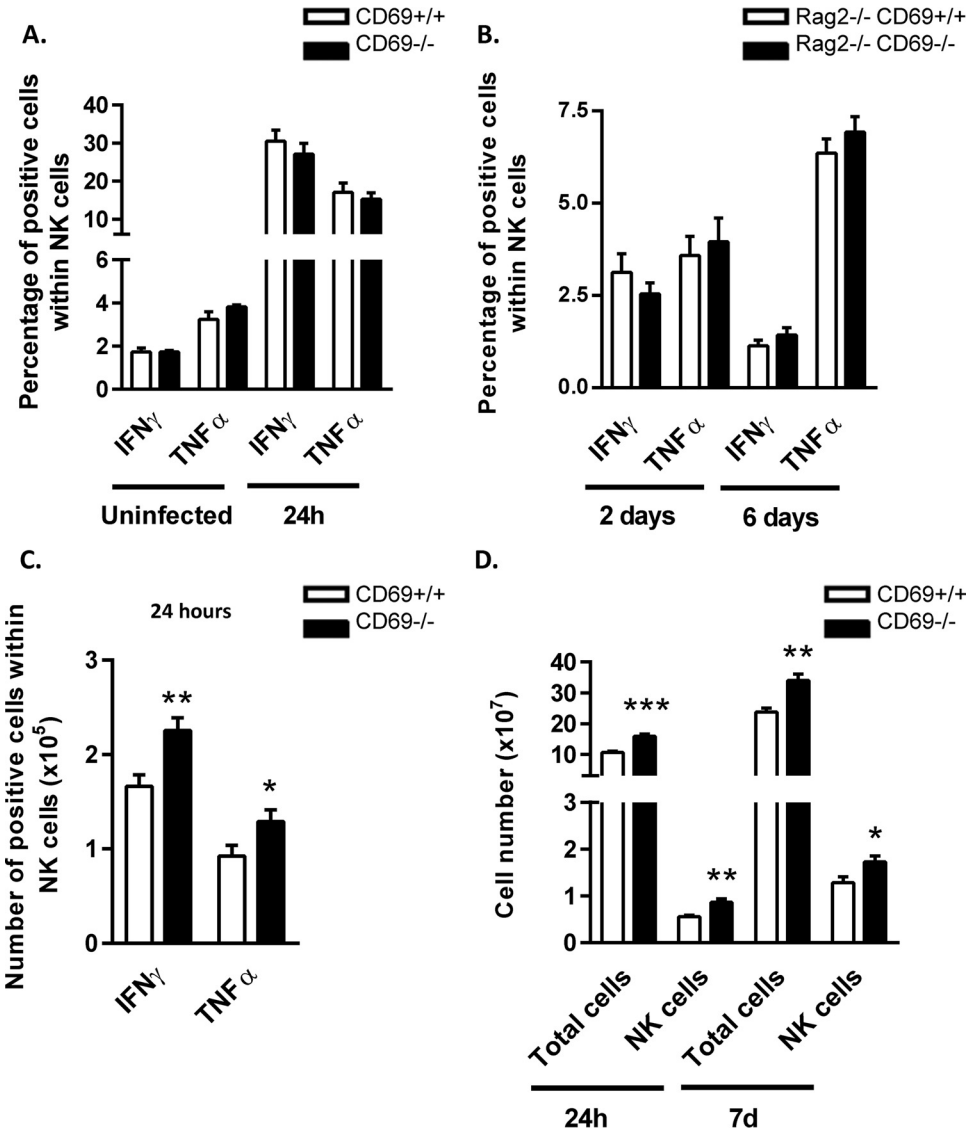

FIG 3 Unaltered NK cell activation in CD69$^{-/-}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice infected with VACV. (A) CD69$^{-/-}$ and WT mice were infected i.p. with 1×10^7 PFU of VACV WR or left uninfected and were sacrificed at 24 h after infection. Spleen cells were cultured with PMA/ionomycin and BFA for 4 h and stained for cell surface markers and intracellular cytokines. The percentages of NK cells producing IFN-γ or TNF-α are shown. (B) Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice were infected with 1×10^5 PFU i.p. of VACV WR. Spleen cells were collected at 2 and 6 days after infection and restimulated with RMA-S cells over 4 h; intracellular IFN-γ and TNF-α levels in NK cells were measured, and the percentages of cytokine-producing cells are plotted. (C) The numbers of IFN-γ- and TNF-α-producing NK cells in CD69$^{-/-}$ and CD69$^{+/+}$ mice were measured at 24 h after infection as described in the legend to panel A. (D) Total cell and NK cell numbers in spleens of CD69$^{+/+}$ and CD69$^{-/-}$ mice at 24 h and 7 days (7d) after infection. The data were pooled from two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Increased NK cell numbers in uninfected CD69$^{-/-}$ mice. The differences in cellularity between CD69$^{-/-}$ and CD69 WT mice at such early time points of infection may indicate that the cellularity is already altered before the infection. Therefore, we analyzed uninfected mice, and we observed that total cell and NK cell numbers were already increased in the spleens of both CD69$^{-/-}$ and CD69$^{-/-}$ Rag2$^{-/-}$ mice (Fig. 7A). Moreover, the spleens of CD69 WT and CD69$^{-/-}$ mice with mixed bone marrow chimeras had higher proportions of CD69$^{-/-}$ NK cells than CD69$^{+/+}$ NK cells among total donor-derived NK cells at steady state, indicating that increased NK cell numbers in CD69$^{-/-}$ mice are due to an NK cell intrinsic effect. An equivalent observation was not made for other cell types, since, for example, the proportions of CD69$^{-/-}$ F4/80^{+} cells were even lower than those of CD69$^{+/+}$ F4/80^{+} cells among total donor-derived F4/80^{+} cells (Fig. 7B). An NK cell intrinsic effect of CD69 deficiency is substantiated by the fact that CD69 was expressed on all NK cells of all the organs analyzed, even in the earliest maturation stages (Fig. 7C and D).

Steady-state NK cell numbers can be affected by NK cell recirculation. S1P5 is a member of the sphingosine phosphate receptor family, and T-bet-dependent S1P5 expression has been reported to control NK cell recirculation through the mediation of egress from lymphoid organs, finally affecting splenic NK cell counts (10). We reasoned that CD69 could have an effect on NK cell recirculation

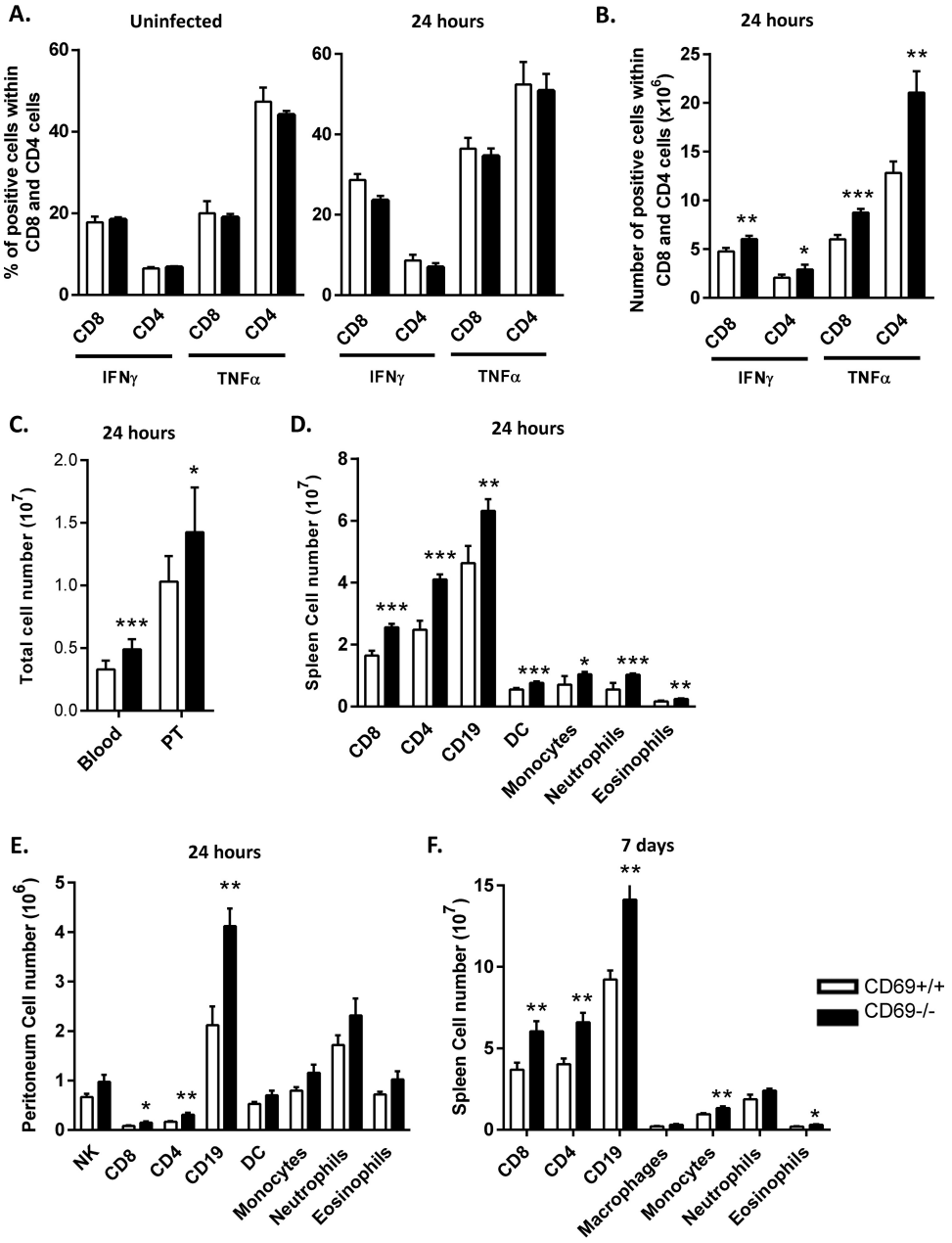

FIG 4 CD69$^{-/-}$ mice show increased numbers of activated T cells and total leukocytes in the early response to VACV WR. WT and CD69$^{-/-}$ mice were not infected or were infected with 10^7 PFU of VACV WR i.p. and were sacrificed at 24 h or 7 days postinfection, as indicated. (A and B) Spleen cells were stimulated with PMA/ionomycin and BFA over 4 h. The percentages (A) and numbers (B) of IFN-γ- and TNF-α-producing CD8$^+$ and CD4$^+$ lymphocytes were measured in uninfected mice (A, left) and in infected mice at 24 h after infection (A, right, and B). (C) Total cell numbers in peritoneum (PT) and blood at 24 h after infection. (D and E) Numbers of the indicated splenic (D, F) and peritoneal (E) lymphoid and myeloid subpopulations at 24 h (D, E) and 7 days (F) after infection. The data were pooled from two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

through altered T-bet expression, and we characterized T-bet expression by bone marrow and spleen NK cells. However, we did not observe differences in the expression of T-bet by NK cells at either site at 1 day after infection (Fig. 7E). Thus, CD69 deficiency does not alter the NK cell number through altered T-bet expression.

The spontaneous cell death rate is reduced in CD69$^{-/-}$ NK lymphocytes. We also tested whether increased immune cell survival contributed to the observed greater number of NK cells in CD69$^{-/-}$ mice. Cell death is difficult to test for *in vivo* because dead cells are rapidly eliminated. Thus, we cultured Rag2$^{-/-}$ CD69$^{-/-}$ and Rag2$^{-/-}$ CD69 WT splenocytes *in vitro* and analyzed NK cells for spontaneous cell death by PI staining at different times of culture. No significant differences were found at early time points. However, we observed significantly reduced percentages of dead cells in Rag2$^{-/-}$ CD69$^{-/-}$ mice after 60 h of culture (Fig. 8). These data suggest that the lower spontaneous cell death rate in CD69$^{-/-}$ mice contributes to the lymphocyte accumulation observed in the spleen and that CD69 has a role in regulating NK cell survival.

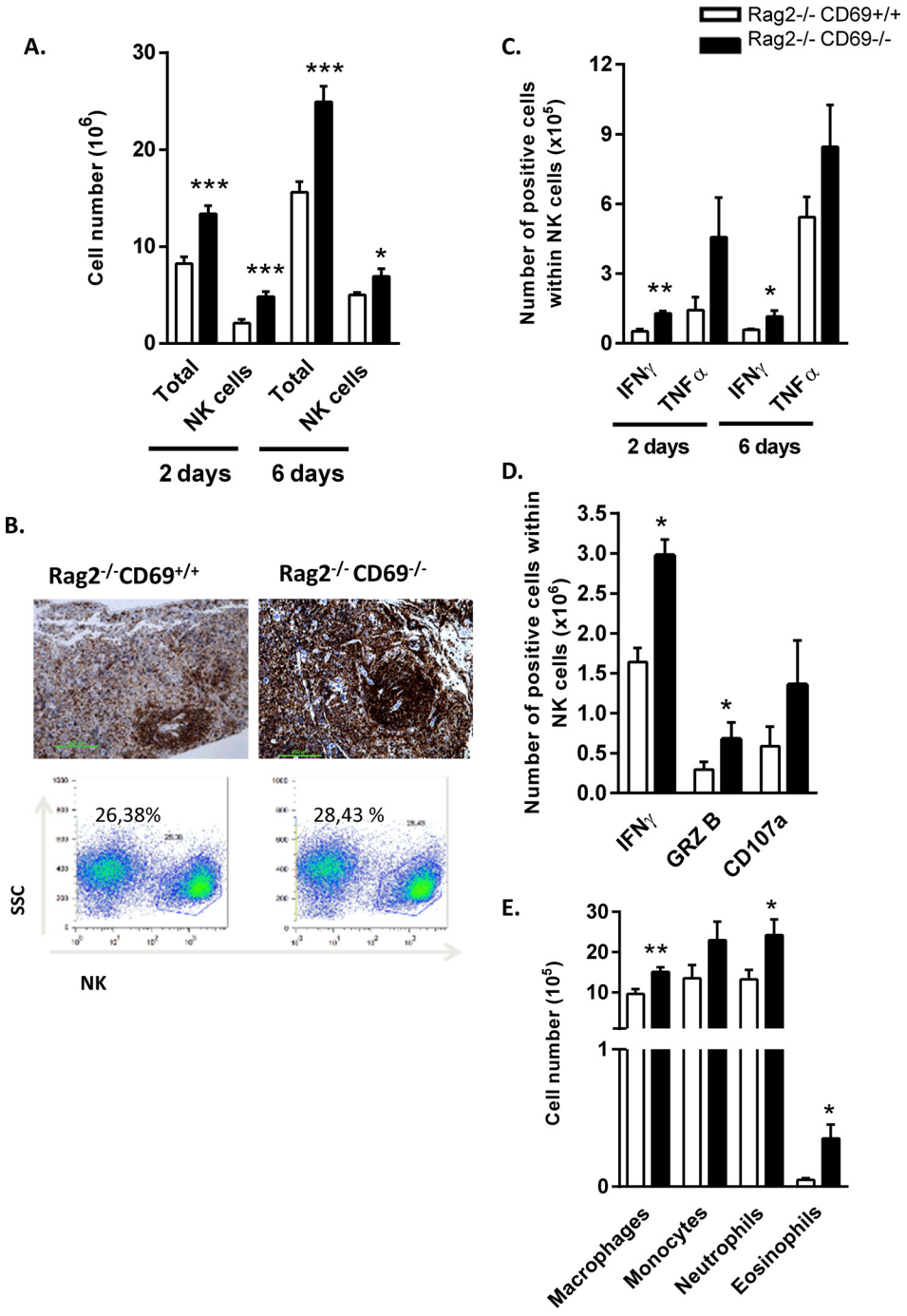

FIG 5 VACV-infected Rag2$^{-/-}$ CD69$^{-/-}$ mice show increased accumulation of splenocytes and reactive NK cell numbers. (A to D) Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice were infected with 1×10^5 PFU of VACV i.p. and were sacrificed on the indicated day postinfection. (A) Splenic total cell and NK cell (DX5 positive) numbers at 2 and 6 days postinfection. (B) (Top) Immunohistochemical analysis of spleen by staining with anti-CD45 MAb (the images are from one mouse and are representative of those from seven mice); (bottom) representative plots of NK cell staining (DX5 positive) at 1 day after infection. SSC, side scatter. (C) Spleen cells were collected at 2 and 6 days after infection and restimulated with RMA-S cells over 4 h; the numbers of IFN-γ- and TNF-α-positive NK cells are shown. (D) Spleen cells were collected at 24 h postinfection and cultured with PMA/ionomycin and BFA over 4 h. The numbers of IFN-γ-, GrzB-, and CD107a-positive NK cells are shown. (E) The numbers of different splenic myeloid subpopulations. (A and C to E) The data presented were pooled from two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

DISCUSSION

In this work, we show an early NK cell-mediated increase in VACV infection control in CD69$^{-/-}$ mice consistent with increased NK cell numbers at peripheral sites in these mice. Two observations point to the suggestion that the increased activated NK cell numbers in the CD69$^{-/-}$ mouse spleens are not secondary to a differential evolution of infection. On the one hand, increased numbers of splenic NK cells were already present in uninfected

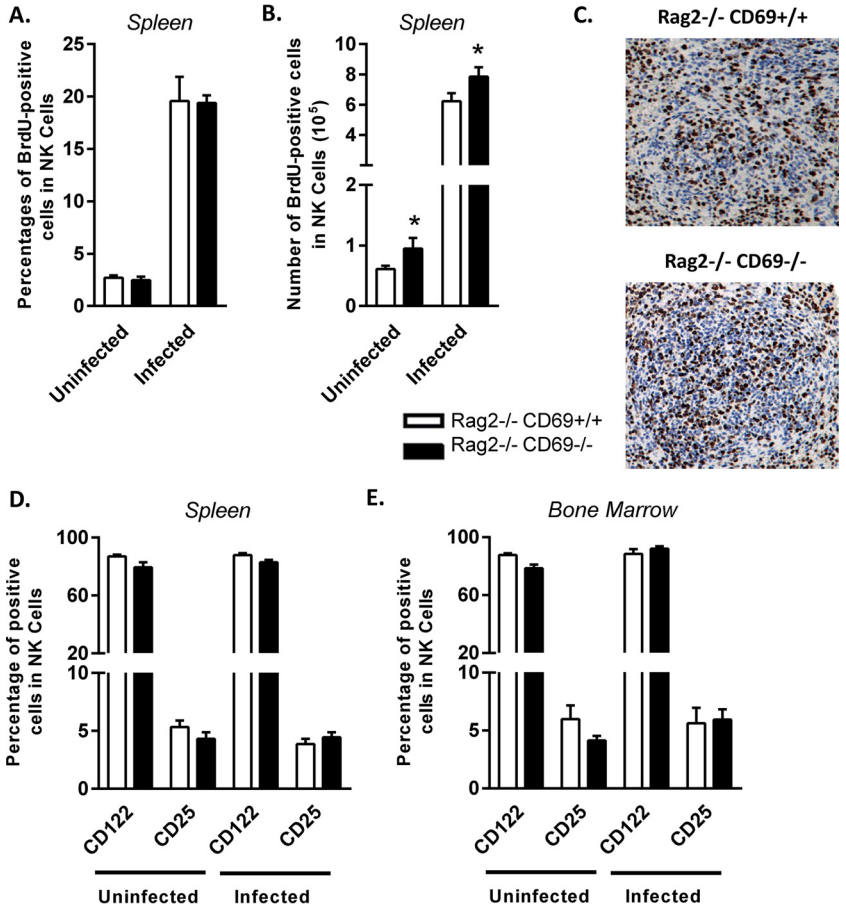

FIG 6 Unaltered NK cell proliferation rate but increased numbers of proliferating NK cells in VACV-infected Rag2$^{-/-}$ CD69$^{-/-}$ mice. (A and B) Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice were infected with 1×10^6 PFU of VACV WR i.p. or not infected. At 2 days after infection, mice were injected i.p. with 1 mg of BrdU for 3 h, and then they were sacrificed. Spleens were collected and stained. The percentages (A) and numbers (B) of BrdU-positive cells within NK cells (NKp46$^+$) in the spleen are shown. The data for uninfected mice represent data pooled from two independent experiments, and the data for infected mice represent data from one experiment. *, $P < 0.05$. (C) After 2 days of infection, histologic sections of spleen were stained for anti-mouse Ki67 by immunohistochemistry. The images are from one mouse and are representative of those from seven mice. (D and E) The percentages of splenic (D) and bone marrow (E) NK cells positive for the alpha and beta chains of the IL-2 receptor were determined in infected and noninfected mice. The data were pooled from two independent experiments.

mice, and this was translated to higher numbers of cytokine-producing cells upon *in vitro* restimulation. On the other hand, spleen viremia was not significantly different between CD69$^{+/+}$ and CD69$^{-/-}$ mice. However, it has been suggested that lymphoid organs are not sites of VACV replication but, rather, are sites of viral particle drainage. Unlike the spleen, ovaries are the organs that sustain the highest virus replication rate in systemic VACV infection (21). The infection induces the recruitment of NK cells, among other leukocyte types, into the ovary (21).

The higher NK cell counts in CD69$^{-/-}$ mice do not seem to be due to an increased NK cell proliferation capacity but, rather, are due, at least in part, to a lower rate of spontaneous cell death.

CD69 has a negative regulatory role in a number of autoimmune diseases and in the antitumor immune response. In the first report of its role in the immune response to infection, we showed that CD69-mediated immune regulation was beneficial in the course of *Listeria monocytogenes* bacterial infection, that CD69 deficiency led to impaired control of *L. monocytogenes* infection starting at early time points, and that this was related to the enhanced production of type I and II IFNs and increased leukocyte apoptosis (16). In contrast, we show here that negative immune modulation by CD69 is detrimental in the case of the infection with VACV in different mouse strains. This differential effect may be due to the different interaction of the pathogen with the augmented immune response seen in the CD69$^{-/-}$ mouse. On the one hand, *L. monocytogenes* exploits the immune activation to induce massive lymphocyte apoptosis. Through the secretion of listeriolysin toxin, it induces the apoptosis of lymphocytes sensitized by type I IFN. Upon phagocytosis of large amounts of apoptotic lymphocytes, the macrophages secrete IL-10, inducing immune suppression and favoring bacterial outgrowth. In contrast to *L. monocytogenes*, VACV employs a large part of its genome to the avoidance of immune recognition and activation (1). Thus, the enhanced immune response of the CD69$^{-/-}$ mice may be detrimental in one case and beneficial in the other.

We have previously shown that CD69$^{-/-}$ mice had an NK cell-mediated augmented antitumor immune response (14). Similar to the present results, NK cell reactivity was also unaltered in the absence of CD69, but the enhanced antitumor response was mediated by increased NK cell accumulation. Higher numbers not only of NK cells but also of total leukocytes were already reported at steady state. Thus, it is likely that CD69 is also involved in the

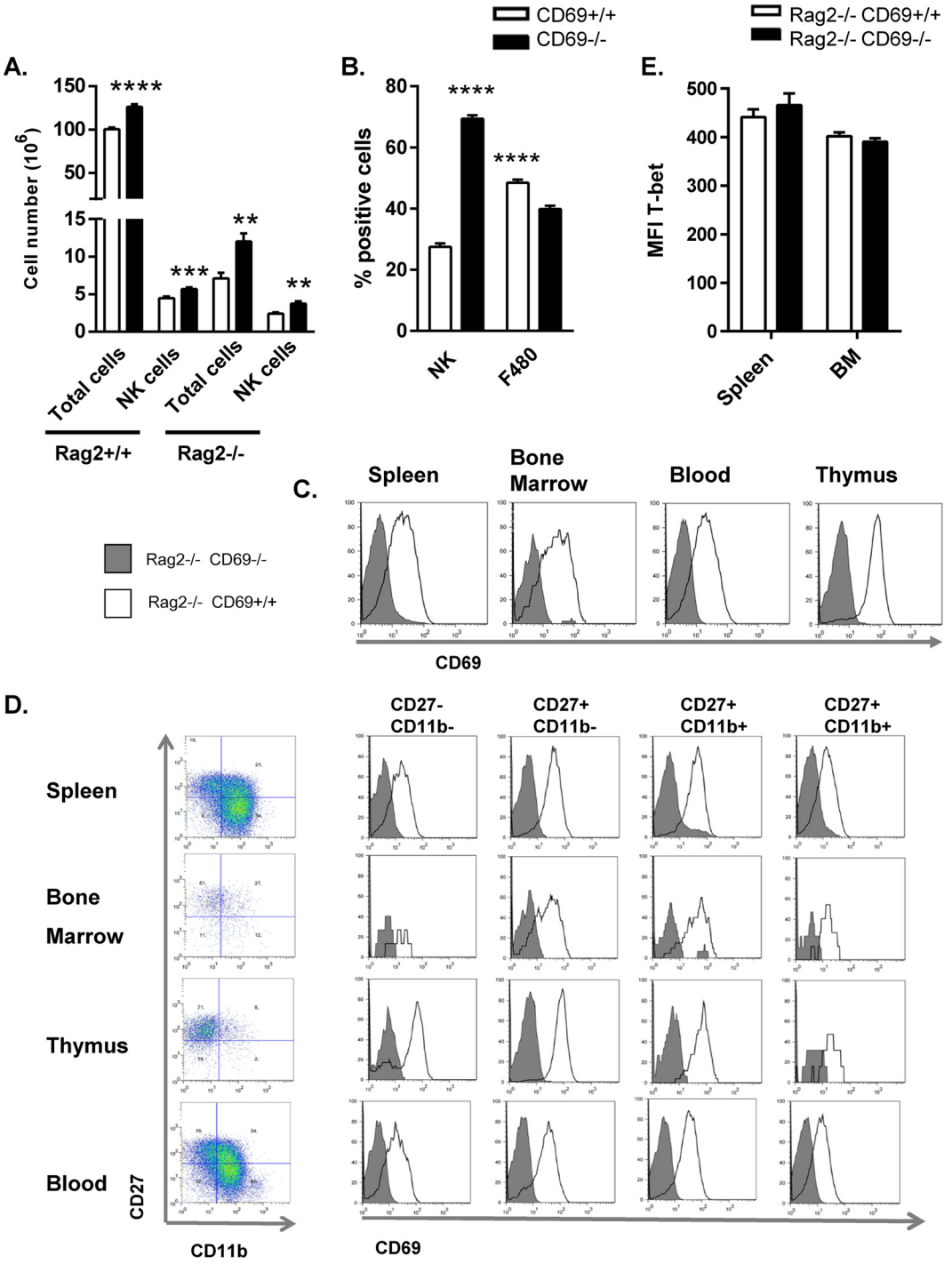

FIG 7 NK cell distribution and CD69 expression in NK cells. (A) Total cell and NK cell numbers in spleens of unmanipulated Rag2$^{+/+}$ and Rag2$^{-/-}$ mice. (B) C57BL/6 recipient mice (CD45.1) were reconstituted with a mixture of wild-type (CD45.1/0.2) and CD69$^{-/-}$ (CD45.2) bone marrow cells. The values represent the percentage of CD69$^{+/+}$ and CD69$^{-/-}$ mouse NK cells or macrophages among total NK cells or total macrophages, respectively. (C and D) Spleen, bone marrow, thymus, and blood cells were collected from unmanipulated Rag2$^{-/-}$ CD69$^{+/+}$ and CD69$^{-/-}$ mice and stained for NKp46, CD27, CD11b, and CD69. Overlays of CD69 profiles gated on total NK cells (C) or NK cells in the different maturation stages (D) from Rag2$^{-/-}$ CD69$^{+/+}$ (solid line) and Rag2$^{-/-}$ CD69$^{-/-}$ (gray-filled area, background control) mice are shown. (E) Rag2$^{+/+}$ and Rag2$^{-/-}$ mice were infected with 1×10^6 PFU of VACV WR i.p. At 1 day after infection, spleen and bone marrow (BM) cells were analyzed for T-bet expression on NK cells (NKp46^{+}). The graphs show the mean fluorescence intensity (MFI) of intranuclear T-bet staining in spleen and bone marrow NK cells. (A, B, and E) The data were pooled from two experiments. (C and D) Results from one representative of two experiments are shown. **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.001$.

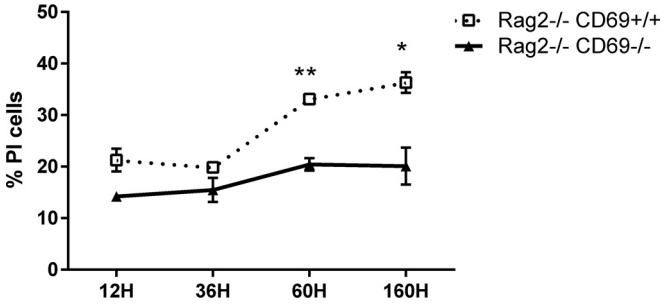

FIG 8 Attenuation of spontaneous cell death in Rag2$^{-/-}$ CD69$^{-/-}$ mice. Cell survival was assessed by PI staining of unfractionated splenic cells of uninfected Rag2$^{-/-}$CD69$^{-/-}$ and Rag2$^{-/-}$CD69$^{+/+}$ mice at different times (in hours [H]) of culture. Results from one representative of two experiments are shown. *, $P < 0.05$; **, $P < 0.01$.

homeostatic regulation of NK cells as well as of other leukocyte subsets. The fact that the early effect of CD69 deficiency on viral control depends on NK cells is likely due to the fact that these cells are of chief importance in the early control of this type of infection (20). The possibility of an effect of CD69 deficiency on the role of other cell types, especially at later time points of infection, is not excluded.

The results of previous studies performed by our group showed that CD69 deficiency did not affect the percentage of VACV-specific CD8$^+$ T cells at 7 days postinfection, implicating that CD69 does not affect either the priming or the rate of expansion of virus-specific cells (6). However, total cell numbers were not recorded then, and considering that steady-state T cell numbers are increased in the CD69$^{-/-}$ mouse, it is expected that this is translated to increased VACV-specific CD8$^+$ T cell numbers during the primary adaptive immune response, which likely contribute to the lower viral titers observed at day 7.

We did not observe any differences in the percentages of IFN-γ- and TNF-α-producing NK cells upon *ex vivo* restimulation with PMA or cells of the MHC-I-deficient cell line RMA-S between CD69 WT and CD69$^{-/-}$ mice. The strong activation with PMA overrides the NK cell activation thresholds set by the process of NK cell tuning (22), and thus, the results with PMA may reflect just an equivalent capacity of CD69 WT and CD69$^{-/-}$ mouse NK cells to produce cytokines. Instead, stimulation with MHC-I-deficient targets does not override those thresholds (22). Therefore, the equivalent cytokine production upon restimulation with RMA-S cells reflects the fact that even if CD69 is expressed constitutively on NK cells at different maturation stages as well as during infection, it does not participate in NK cell tuning (23).

Altogether, this work points to a role for CD69 in the resolution of viral infection through a role in the homeostatic control of NK cells.

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