ORIGINAL INVESTIGATION

Caspase-8 restricts natural killer cell accumulation during MCMV Infection

Yanjun Feng[1] · Lisa P. Daley-Bauer[1] · Linda Roback[1] · Marc Potempa[2] · Lewis L. Lanier[2] · Edward S. Mocarski[1]

Abstract

Natural killer (NK) cells provide important host defense against herpesvirus infections and influence subsequent T cell control of replication and maintenance of latency. NK cells exhibit phases of expansion, contraction and memory formation in response to the natural mouse pathogen murine cytomegalovirus (MCMV). Innate and adaptive immune responses are tightly regulated in mammals to avoid excess tissue damage while preventing acute and chronic viral disease and assuring resistance to reinfection. Caspase (CASP)8 is an autoactivating aspartate-specific cysteine protease that initiates extrinsic apoptosis and prevents receptor interacting protein (RIP) kinase (RIPK)1–RIPK3-driven necroptosis. CASP8 also promotes death-independent signal transduction. All of these activities make contributions to inflammation. Here, we demonstrate that CASP8 restricts NK cell expansion during MCMV infection but does not influence NK memory. $Casp8^{-/-}Ripk3^{-/-}$ mice mount higher NK response levels than $Casp8^{+/-}Ripk3^{-/-}$ littermate controls or WT C57BL/6 J mice, indicating that RIPK3 deficiency alone does not contribute to NK response patterns. MCMV m157-responsive Ly49H$^+$ NK cells support increased expansion of both Ly49H$^-$ NK cells and CD8 T cells in $Casp8^{-/-}Ripk3^{-/-}$ mice. Surprisingly, hyperaccumulation of NK cells depends on the pronecrotic kinase RIPK1. $Ripk1^{-/-}Casp8^{-/-}Ripk3^{-/-}$ mice fail to show the enhanced expansion of lymphocytes observed in $Casp8^{-/-}Ripk3^{-/-}$ mice even though development and homeostasis are preserved in uninfected $Ripk1^{-/-}Casp8^{-/-}Ripk3^{-/-}$ mice. Thus, CASP8 naturally regulates the magnitude of NK cell responses in response to infection where strong activation signals depend on another key regulator of death signaling, RIPK1. In addition, the strong NK cell response promotes survival of effector CD8 T cells during their expansion. Thus, hyperaccumulation of NK cells and crosstalk with T cells becomes amplified in the absence of extrinsic cell death machinery.

Keywords Apoptosis · Necroptosis · Cell death · Proliferation · Herpesvirus · Ripoptosome

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✉ Edward S. Mocarski
  mocarski@emory.edu

1 Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, 1462 Clifton Rd. N.E, Atlanta, GA 30322, USA

2 Department of Microbiology and Immunology and Parker Institute for Cancer Immunotherapy, University of California, San Francisco, CA 94143, USA

Introduction

NK cells are innate lymphocytes that contribute to early control of virus infections. NK-dependent antiviral responses develop rapidly and exhibit robust cytotoxicity [1]. Activation depends on receptors that override inhibitory signals and trigger NK cell expansion, contraction, memory formation [2]. Murine cytomegalovirus (MCMV) has yielded invaluable insights into antiviral NK cell function due to the ability of virus-encoded MHC class I-like glycoprotein m157 to specifically engage host Ly49H, an activating receptor mediating robust proliferation and strong cytotoxic effector responses [3] that dictate early control over MCMV [4, 5]. Following peak expansion, most Ly49H$^+$ NK cells contract to leave a memory-like population that persists [6] in a manner reminiscent of the adaptive T cell response. Human (H)CMV-infected individuals have higher persistent

levels of CD94+NKG2C+ NK cells compared to seronegative individuals [7, 8], suggesting that NK cell memory also follows HCMV infection. Given that comparable patterns of memory have been observed in humans [9] and mice [6], MCMV-infected mice help to provide mechanistic insights into memory NK cell generation.

In addition to the Ly49H receptor, NK cell expansion depends on proinflammatory cytokines and costimulatory receptors. IL-12, IL-18, IL-33 and type I IFNs regulate NK cell proliferation, cytotoxic function and memory formation [10]. IL-12-triggered, STAT4-dependent signal transduction upregulates MyD88 and consequent expression of the IL-18 receptor to promote NK cell proliferation [11]. Type I IFN-induced transcription factors STAT1, STAT2, IRF8 and IRF9 all contribute to NK cell survival during expansion [12–14]. In addition, both IL-12 and type I IFN promote NK cell cytotoxic function and IFNγ production [15, 16]. Signaling through NKG2D augments m157-dependent Ly49H+ NK proliferation, although stimulation via NKG2D alone is not sufficient to trigger NK cell expansion [17]. All of these factors contribute to the levels, differentiation programs and longevity of NK cells, which is mediated by the balance of proliferation and death.

Lymphocyte turnover and cell death occur primarily via cell-intrinsic apoptosis, mediated by Bcl2 family member Bim acting upstream of Bax- and Bak-dependent mitochondrial outer membrane permealization (MOMP) and release of cytochrome *c* [18, 19]. Similar to its role in antiviral T cell contraction, Bim functions during Ly49H+ NK cell contraction and formation of a memory pool [20, 21]. Extrinsic cell death is activated by cell-surface TNF receptor superfamily death receptors (DRs, including Fas, as well as TNFR1 and TRAIL) that drive cytosolic events resulting in death-dependent (apoptotic, necroptotic) and death-independent signaling outcomes [22–25]. Fas death receptor-mediated signaling and Bid amplification of signals [19] contribute to T cell homeostatic turnover [25]. Fas also contributes to contraction following herpesvirus infection [26]. We have recently shown that CASP8-dependent extrinsic apoptosis is dispensable for NK or T cell contraction during MCMV or HSV1 infection of viable and immunocompetent $Casp8^{-/-}Ripk3^{-/-}$ mice [27, 28], a topic that is elaborated in the accompanying review. Our data are consistent with the predominant role of the intrinsic pathway in the shutdown of acute NK cell responses following clearance of viral antigens.

CASP8 was initially believed to support lymphocyte activation due to defective NF-κB signaling via CD244 (or 2B4) and CD16 in mice with T cell-specific deficiency [29, 30], events likely to result from the vital role that CASP8 plays in suppressing RIPK3-mediated necroptosis [31]. Our recent study of $Casp8^{-/-}Ripk3^{-/-}$ mice reveals that the Ly49H+ NK cell expansion peaks higher compared to either $Casp8^{+/-}Ripk3^{-/-}$ or WT C57BL/6 J controls during the acute phase of MCMV infection [27], indicating that CASP8 restricts Ly49H+ NK cell expansion in a necroptosis-deficient background. However, the interpretation of these results is complicated by other environmental considerations, including viral antigen load as well as the abnormal B220+CD3+ T cell accumulation [27, 31] and enhanced DCs numbers that accompany MCMV infection in $Casp8^{-/-}Ripk3^{-/-}$ mice [27].

In this study, we show that CASP8 restricts Ly49H+ NK cell accumulation as well as effector differentiation but is dispensable for memory NK cell development. While our previous study has shown that Ly49H+ NK cell hyperaccumulation in $Casp8^{-/-}Ripk3^{-/-}$ mice requires m157 MCMV antigen, data from the current work show that enhancement requires active virus replication. Further, using a co-transfer system, it appears that CASP8 makes a cell-autonomous contribution to restricting NK cell expansion. Strikingly, RIPK1 elimination in $Casp8^{-/-}Ripk3^{-/-}$ mice completely ablates NK cell responses, although $Ripk1^{-/-}Casp8^{-/-}Ripk3^{-/-}$ mice exhibit normal homeostatic NK cell development [32]. Finally, $Casp8^{-/-}Ripk3^{-/-}$ NK cells support robust memory development that results in normal levels of CD11b^lo CD27+ and higher percentages of Ly6C+ and KLRG1^hi subsets when compared with WT mice.

Materials and methods

Mice, viruses and experimental infection

$Casp8^{-/-}Ripk3^{-/-}$ [31] and $Casp8^{-/-}Ripk3^{K51A/K51A}$ [33] mice were > 97% C57BL/6 J. $Casp8^{-/-}Ripk3^{-/-}$ and $Casp8^{+/-}Ripk3^{-/-}$ littermates were generated by mating $Casp8^{-/-}Ripk3^{-/-}$ and $Casp8^{+/-}Ripk3^{-/-}$ mice. $Ripk1^{-/-}Casp8^{-/-}Ripk3^{-/-}$ mice were generated as described previously [32]. C57BL/6 J mice were obtained from The Jackson Laboratory, used for backcrossing and bred at Emory University Division of Animal Resources. K181-BAC-derived WT virus and K181-BAC-derived Δm157, kindly provided by Wolfram Brune (Heinrich Pette Institute), were propagated in NIH3T3 fibroblasts. Mice were inoculated intraperitoneally with 1×10^6 PFU virus per mouse. The cell culture medium was purified and concentrated for virus stock preparation, which were stored in single-use aliquots at − 80 °C until use.

Plaque assay

Homogenized tissue in DMEM medium was overlaid on NIH3T3 cells plated in 6-well plates. On day 4 post infection, cells were fixed with methanol and stained with Giemsa

for visualizing plaques that were counted with the aid of a dissecting microscope.

Antibodies

For flow cytometry, antibodies to CD16/CD32 (FcγRII/III; Clone 2.4G2), CD45 (Clone 30-F11), CD45.1 (Clone A20), CD45.2 (Clone 104), CD11b (Clone M1/70), NK1.1 (Clone PK136), B220 (Clone RA3-6B2), CD3ε (Clone 17A2), CD3ε (Clone 145-2C11), CD8 (Clone 53–6.7), KLRG1 (Clone 2F1), Ki67 (Clone SolA15) were from BD PharMingen; and CD3ε (Clone 17A2) were from BioLegend; and Ly49H (Clone 3D10) was from eBioscience. For Annexin V analysis, splenocytes harvested from mice were stained with Annexin V (BD Bioscience).

Flow cytometry

Spleens were harvested from euthanized mice, and were pressed through metal sieves for organ disruption. Cells were then incubated with red blood cell lysis buffer and then passed through a 100-mm strainer. In all experiments, 2×10^6 viable cells, as determined by trypan blue exclusion, were stained with antibodies specific for surface and intracellular proteins and were prepared for detection by flow cytometry (BD LSRII cytometer and FACSDiva Software; BD Biosciences). Data were analyzed with FlowJo (TreeStar), and graphed with Prism 7 (GraphPad).

Results

MCMV-infected mice deficient in CASP8 and RIPK3 show enhanced Ly49H$^+$ NK cell accumulation compared to controls [27]. Thus, similar to the cell-autonomous impact on CD8 T cells [27, 28], CASP8 naturally restricts Ly49H$^+$ NK cell expansion, once unleashed necroptosis has been eliminated from the host. To determine whether enhancement was Ly49H$^+$ NK cell specific, the time course of Ly49H$^+$ and Ly49H$^-$ NK cell expansion was determined. Splenocytes from $Casp8^{-/-}Ripk3^{-/-}$, $Casp8^{+/-}Ripk3^{-/-}$ and WT mice were collected at various times following MCMV infection, and NK cell number and phenotype were assessed. Total NK cell numbers were comparable in all groups on days 1.5, 3 and 5 post MCMV infection (Fig. 1a), but expanded to peak levels at 7 dpi that were dramatically higher in $Casp8^{-/-}Ripk3^{-/-}$ mice relative to controls. A similar pattern of contraction occurred in all mice from 9 to 14 dpi. The data suggest that CASP8 restricts NK cell numbers during the expansion phase but is dispensable for cell contraction, a pattern that has been observed with antiviral CD8 T cells in these mice [28]. Numbers of MCMV m157-driven Ly49H$^+$ NK cells [3] increased to significantly higher levels by

7 dpi in $Casp8^{-/-}Ripk3^{-/-}$ mice, as expected [27] (Fig. 1b); whereas, the numbers of Ly49H$^-$ NK cells remained generally comparable independent of genotype over the two-week observation period (Fig. 1c). Consistent with the cell count analysis, percentages of Ly49H$^+$ NK cells were significantly higher in $Casp8^{-/-}Ripk3^{-/-}$ mice at 7 dpi compared to WT and $Casp8^{+/-}Ripk3^{-/-}$ controls, while levels were comparable at 1.5 dpi independent of genotype (Fig. 1d). To further investigate whether Casp8 regulates cells through a cell-autonomous or cell-extrinsic mechanism, equal numbers of CD45.1$^+$ WT and CD45.2$^+$ DKO NK cells were cotransferred into Ly49H-deficient mice. Expansion and contraction of Ly49H$^+$ NK cells were measured in peripheral blood following MCMV infection. DKO cells exhibited increased expansion with a peak that was higher than WT controls (Fig. 2a). Following the peak NK cell response, Ly49H$^+$ NK cells from both genotypes contracted between days 14 and 28 post infection. Consistent with the enhanced accumulation, DKO cells made up the majority of the total Ly49H$^+$ population throughout the infection (Fig. 2b). These data show that Casp8 mediates an NK cell-autonomous restriction of expansion but is dispensable for contraction.

Upon infection, all three genotypes upregulated KLRG1 on Ly49H$^+$ NK cells, suggesting that maturation occurs independently of CASP8 or RIPK3 (Fig. 1e). However, a higher proportion of $Casp8^{-/-}Ripk3^{-/-}$ Ly49H$^+$ NK cells upregulated KLRG1 at 7 dpi, again reminiscent of responding CD8 T cells in these mice [28]. Taken together, CASP8 appears to restrict m157-specific Ly49H$^+$ NK cell accumulation following MCMV infection without playing a role in contraction.

Expansion of NK cells during antiviral prophylaxis

Next, we sought to investigate whether the NK cell response pattern depended on MCMV antigen levels. Differences between Casp8-deficient mice and controls do not result from differential viral replication levels or clearance [27, 28]. Infected mice were treated with cidofovir (CDV) to inhibit virus replication, conditions where T cell expansion patterns were preserved [28]. NK cell responses were investigated at 7 dpi where CDV effectively suppressed viral load to undetectable levels in these mice [28]. Compromised replication, which would be expected to reduce m157 levels markedly, did not impact total or Ly49H$^+$ NK cell responses, but reversed the heightened numbers in $Casp8^{-/-}Ripk3^{-/-}$ mice (Fig. 3a). Interestingly, numbers of Ly49H$^+$ cells in CDV-treated $Casp8^{-/-}Ripk3^{-/-}$ mice actually trended lower than in controls (Fig. 3b) which was reflected in reduced percentages as well (Fig. 3c). CDV-compromised virus levels reduced the levels of NK cell differentiation and percentages of KLRG1^{hi} Ly49H$^+$ NK cells, regardless of genotype (Fig. 3d). Nevertheless, independent
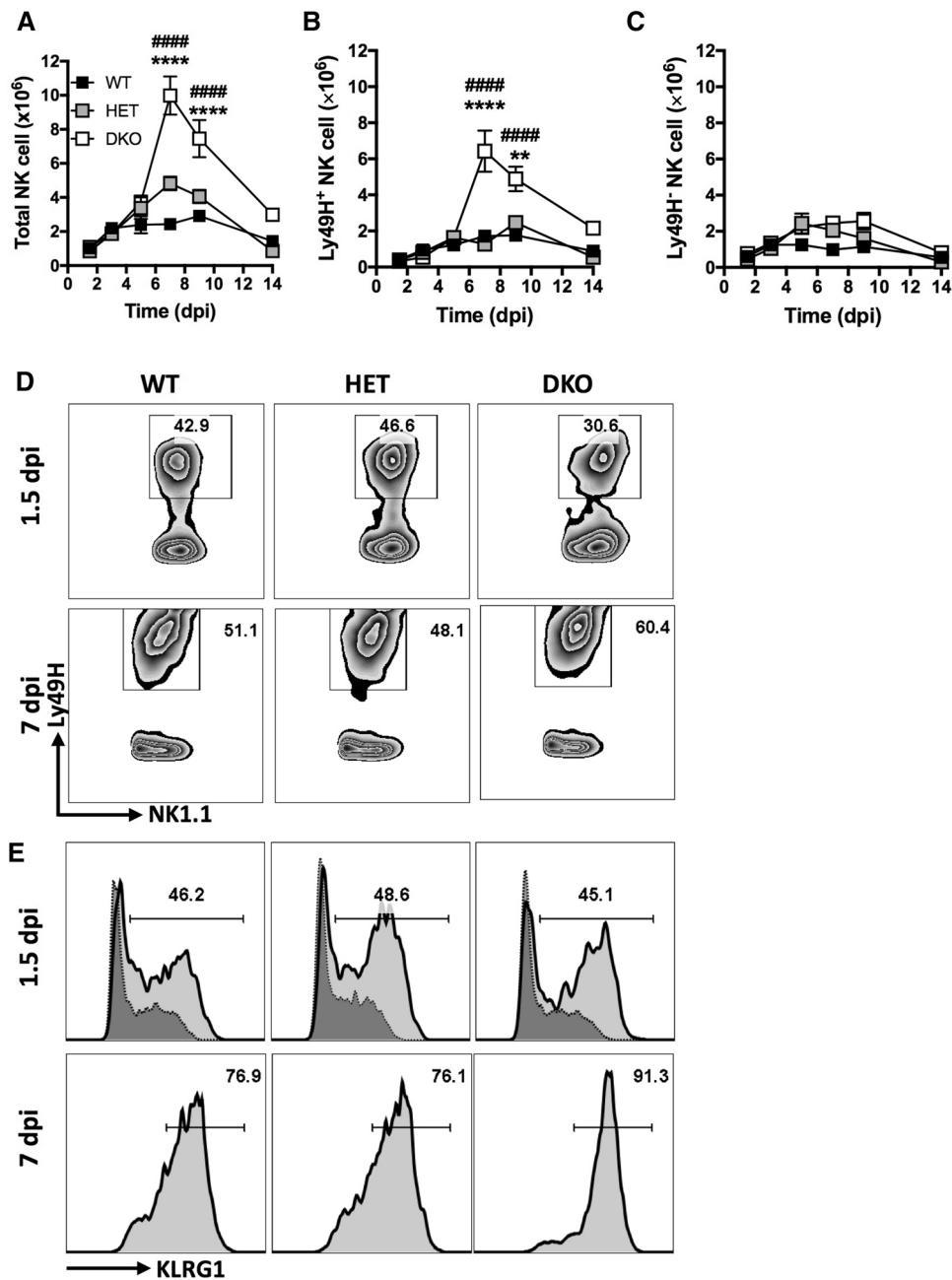
Fig. 1 NK cell expansion, contraction, and differentiation during the acute phase of MCMV infection. 6–8-week-old C57BL/6 (WT), $Casp8^{+/-}Ripk3^{-/-}$ (HET) and $Casp8^{-/-}Ripk3^{-/-}$ (DKO) mice [31] were inoculated intraperitoneally (i.p.) with 1×10^6 PFU MCMV K181-BAC per mouse. Spleens were processed at 1.5, 3, 5, 7, 9 and 14 days post infection (dpi) and stained with antibodies specific for the indicated cell-surface markers. Splenic CD45$^+$B220$^-$CD3$^-$NK1.1$^+$ NK cells were gated to identify Ly49H$^+$ and Ly49H$^-$ NK cells. Graphs comparing numbers of total (**a**), Ly49H$^+$ (**b**), and Ly49H$^-$ (**c**) NK cells. Data are presented as mean numbers ± S.E.M. Representative flow plots comparing the frequencies of Ly49H$^+$ subset out of total NK cells (**d**). Representative his-

tograms showing the frequencies of Ly49H$^+$ NK cells expressing KLRG1 (**e**). C57BL/6 mice were from The Jackson Laboratory and maintained at Emory University Division of Animal Resources, and experimental procedures were conducted in accordance with the National Institutes of Health and Emory University Institutional Animal Care and Use Committee guidelines; K181-BAC virus strain was propagated in NIH3T3 cells, purified and concentrated from clarified cell culture medium; flow cytometry and antibodies (Abs) have been described [28]. Data represent at least two independent experiments using $n = 5$ mice per group. Significant differences between DKO and WT (*) or HET (#) mice are indicated as **$p < 0.01$; *** or ###$p < 0.001$; **** or ####$p < 0.0001$
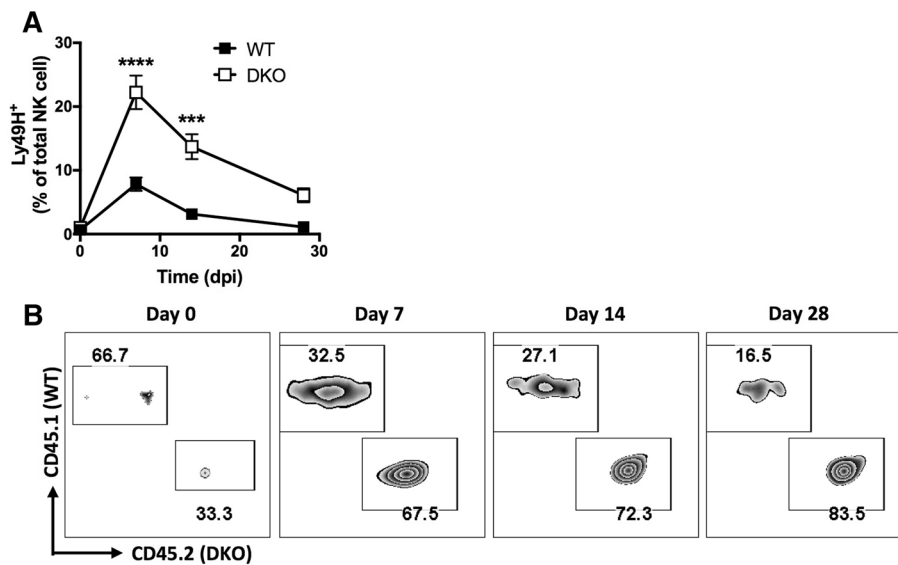
Fig. 2 Cell-autonomous impact of CASP8 on NK cell expansion and contraction during the acute phase of MCMV infection. Equal numbers (10^6) of the purified $TCR\beta^-NK1.1^+$ cells from naïve $CD45.1^+$ WT mice and $CD45.2^+$ DKO mice were mixed (1:1) and co-transferred into Ly49H-deficient recipients, followed with MCMV infection the next day. Graph showing the percentages $\pm$ S.E.M. of $Ly49H^+$ cells in blood on days 0, 7, 14, and 28 post infection (**a**). Representative plots showing the ratios of WT and DKO $Ly49H^+$ NK cells in blood of the recipients on the indicated days post infection (**b**). Data represent one experiment with $n = 3$ mice per group

of CDV treatment, $Casp8^{-/-}Ripk3^{-/-}$ mice exhibited higher percentages of $KLRG1^{hi}$ $Ly49H^+$ NK cells than control mice. These data show that the enhancement of $Ly49H^+$ NK cell number in $Casp8^{-/-}Ripk3^{-/-}$ mice requires active replication, although CASP8 suppresses NK cell differentiation independent of antigen load.

NK hyperaccumulation in different CASP8-deficient settings

To investigate whether $Ly49H^+$ NK cell hyperaccumulation occurs with another necroptosis incompetent, CASP8-deficient strain, NK cells in $Casp8^{-/-}Ripk3^{K51A/K51A}$ mice, where the kinase activity has been specifically disrupted [33], were compared to $Casp8^{-/-}Ripk3^{-/-}$ as well as WT controls. The enhancement of NK cells was observed consistently in either CASP8-deficient setting due to the activation of $Ly49H^+$ cells (Fig. 4). Finally, RIPK1 is a death domain-containing pro-necroptotic kinase involved in regulating apoptosis and pro-survival signaling transduction as a component of the ripoptosome [25]. To understand whether RIPK1 contributed to $Ly49H^+$ NK cell responses, $Ripk1^{-/-}Casp8^{-/-}Ripk3^{-/-}$ mice were infected, and splenocytes were analyzed at 7 dpi. Interestingly, total and $Ly49H^+$ NK cell responses were severely impaired in mice lacking RIPK1 at 7 dpi (Fig. 4), even though uninfected mice exhibit normal NK cell development [32] and homeostasis [27]. This does not appear to be an NK cell-intrinsic dysregulation because similar impairment occurs with CD8 T cells (data

not shown), indicating that RIPK1 contributes to lymphocyte response properties in the absence of CASP8 function. Thus, CASP8 restricts NK cell accumulation during MCMV infection; however, RIPK1 supports hyperaccumulation when CASP8 and RIPK3 are absent.

## Hyperaccumulation of NK cells requires m157–Ly49H interactions

In C57BL/6 mice, only NK cells express the Ly49H receptor and only $Ly49H^+$ NK cells respond directly to the viral m157-encoded glycoprotein [4, 5]. $Ly49H^-$ NK cells are stimulated indirectly, although $Ly49H^+$ NK cells are the drivers [3, 34] such that proliferation and cytotoxicity do not develop to a demonstrable level during infection with Δm157 MCMV [10]. Infection with Δm157 virus failed to drive hyperaccumulation (Fig. 5a) in $Casp8^{-/-}Ripk3^{-/-}$ mice, which showed kinetics distinct from WT MCMV, but comparable to either $Casp8^{+/-}Ripk3^{-/-}$ or WT mice. Thus, the increased accumulation is totally dependent on m157, consistent with data showing $Ly49H^+$ NK cell hyperaccumulation in MCMV-infected $Casp8^{-/-}Ripk3^{-/-}$ mice (See Fig. 1).

NK cell hyperaccumulation dictates survival of antiviral CD8 T cells

Δm157 MCMV has yielded invaluable insights into positive and negative impacts of NK cell function on CD8 T cell responses [35–38]. In addition to the striking impact on NK

Fig. 3 Patterns of NK cell responses during antigen level compromise. Mice were administered 5 mg/kg/day CDV i.p. in PBS, starting from 3 day prior to infection with MCMV as described in Fig. 2 and previously [28]. All groups were euthanized and splenocytes were analyzed at 7 dpi. Bar graphs comparing the numbers of total (**a**) and Ly49H^{+} (**b**) NK cells. Data are presented as mean ± S.E.M. Representative flow plots showing the percentages of Ly49H^{+} cells within the total NK population (**c**). Representative histograms showing the percentage of Ly49H^{+} NK cells expressing KLRG1 (**d**). Data represent two independent experiments with $n = 4$ mice per group
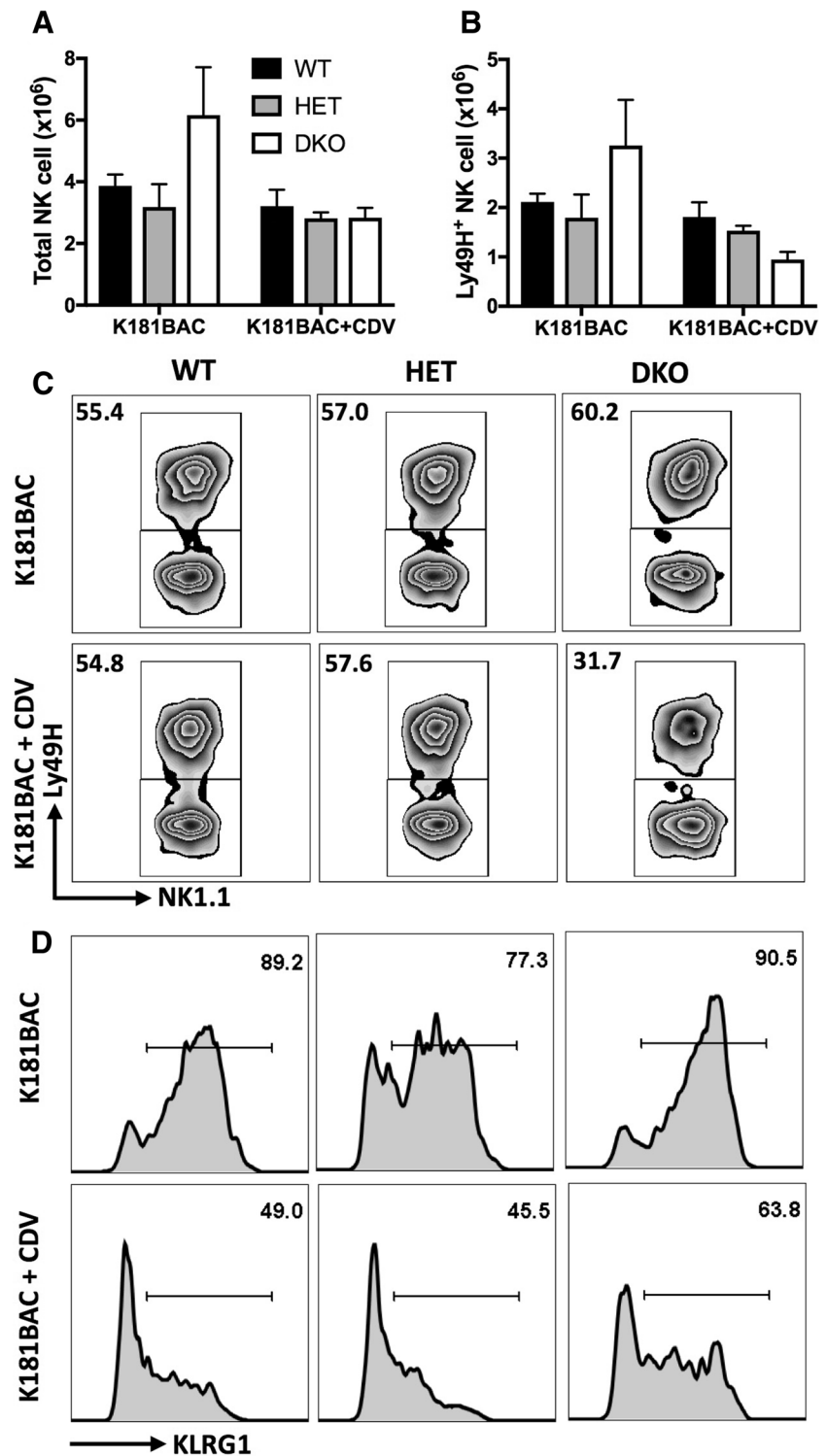
cell hyperaccumulation, both total (Fig. 5b) and M45-specific (Fig. 5c, d) CD8 T cell responses returned to control levels during Δm157 MCMV infection of $Casp8^{-/-}Ripk3^{-/-}$ mice. KLRG1^{hi}CD127^{lo} and KLRG1^{hi}CD127^{hi} effector subsets failed to hyperaccumulate (Fig. 5e). Δm157 virus infection resulted in higher frequencies of fixable viability staining

(FVS^{+}) dead cells in spleens of $Casp8^{-/-}Ripk3^{-/-}$ mice at 7 dpi compared to m157-expressing K181-BAC virus (Fig. 5f), suggesting that the Ly49H^{+} NK cell response supported $Casp8^{-/-}Ripk3^{-/-}$ splenocyte survival. $Casp8^{-/-}Ripk3^{-/-}$ mice exhibited higher frequencies of annexin V[+] $Casp8^{-/-}Ripk3^{-/-}$ CD8 T cell subsets (Fig. 5g),

Fig. 4 Assessment of NK cell responses in another CASP8-deficienct strain and the contribution of RIPK1 to the response. WT, DKO, *Casp8–/–Ripk3*K51A/K51A (*C8–/–R3K51A;* [33]) and *Ripk1–/–Casp8–/–Ripk3–/–* (TKO, [32]) mice were infected with MCMV as described in Fig. 2. Bar graphs comparing the mean numbers ± S.E.M. of the splenic total (**a**), Ly49H+ (**b**), and Ly49H− (**c**) NK cells at 7 dpi. Representative flow plots showing the percentages of CD45+CD3−B220− cells expressing NK1.1 (**d**) or Ly49H (**e**). Combined data of *n* = 7 mice from two independent experiments are shown
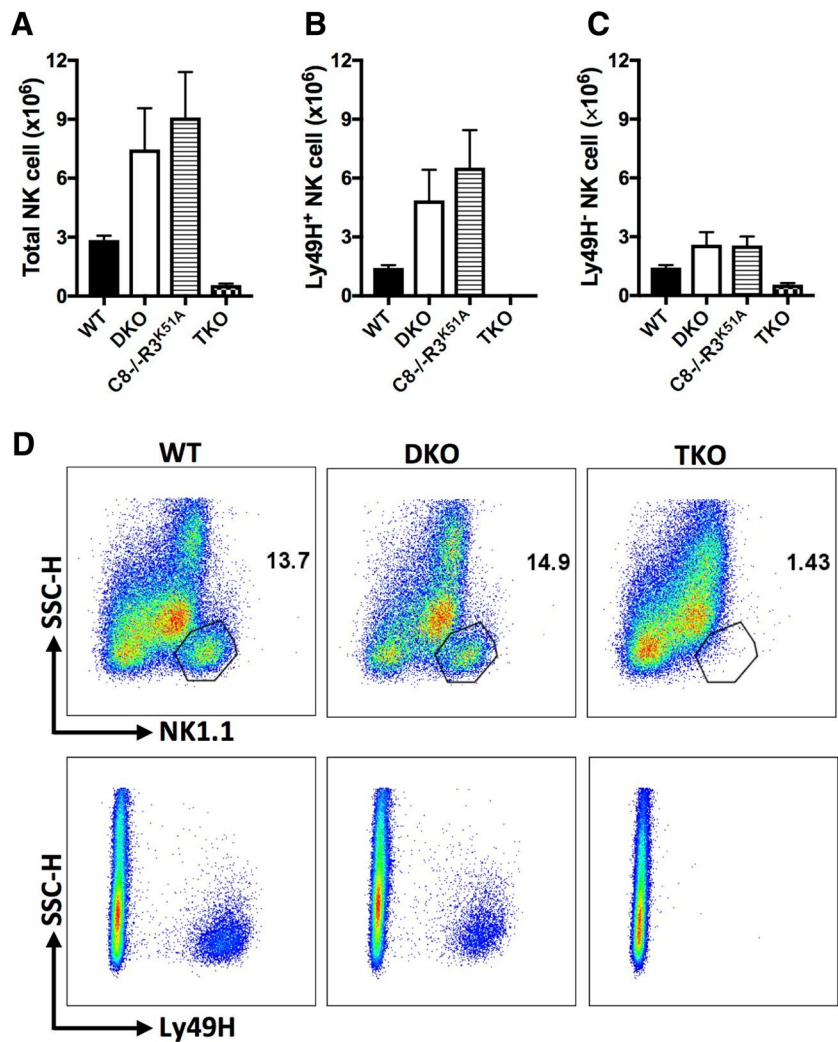

suggesting that greater numbers of T cells underwent apoptosis in *Casp8–/–Ripk3–/–* mice when the Ly49H+ NK cell response was compromised. Overall, these experiments implicate the m157-driven Ly49H+ NK response in supporting *Casp8–/–Ripk3–/–* T cell survival as they hyperaccumulate following MCMV infection.

To determine whether NK cell-autonomous CASP8 and RIPK3 contributed to *Casp8–/–Ripk3–/–* CD8 T cell accumulation, CFSE-labeled total CD45.2+ WT or *Casp8–/–Ripk3–/–* splenocytes that contained 10^6 CD8 T cells were transferred into congenic CD45.1+ recipients, followed by infection with K181-BAC or Δm157 virus 24 h later (Fig. 6a). The numbers of endogenous CD45.1+ Ly49H+ NK cells were comparable in all K181-BAC-infected recipients but were compromised during infection with Δm157 virus (Fig. 6b). The numbers of responding Ly49H+ NK cells appeared to be generated independent of the donor cell genotype. Strikingly, hyperaccumulation of total and M45-specific *Casp8–/–Ripk3–/–* CD8 T cells was compromised in Δm157 virus-infected mice

(Fig. 6c), indicating that WT Ly49H+ NK cells sufficed to enhance *Casp8–/–Ripk3–/–* CD8 T cell accumulation. Cell proliferation assessment by CFSE dilution revealed that the m157-driven Ly49H+ NK cell response did not have an impact on *Casp8–/–Ripk3–/–* CD8 T cell proliferation (Fig. 6d), reinforcing the CD8 T cell-intrinsic underpinnings of hyperaccumulation [28]. Altogether, Ly49H+ NK cells have an impact independent of CASP8 and RIPK3 on *Casp8–/–Ripk3–/–* CD8 T cell proliferative potential following MCMV infection, by supporting the survival of *Casp8–/–Ripk3–/–* CD8 T cells.

Cytokines, together with m157-driven Ly49H receptor signaling, contribute to memory NK cell formation after contraction [10]. Memory NK cells express high levels of markers related to differentiation and maturation [6]. We sought to investigate whether the heightened acute response of CASP8-deficient NK cells altered memory formation. NK cell numbers and memory cell differentiation markers were assessed in WT, *Casp8–/–Ripk3–/–* and *Casp8–/–Ripk3*K51A/K51A mice at 8 wpi. Both CASP8-deficient mutants exhibited
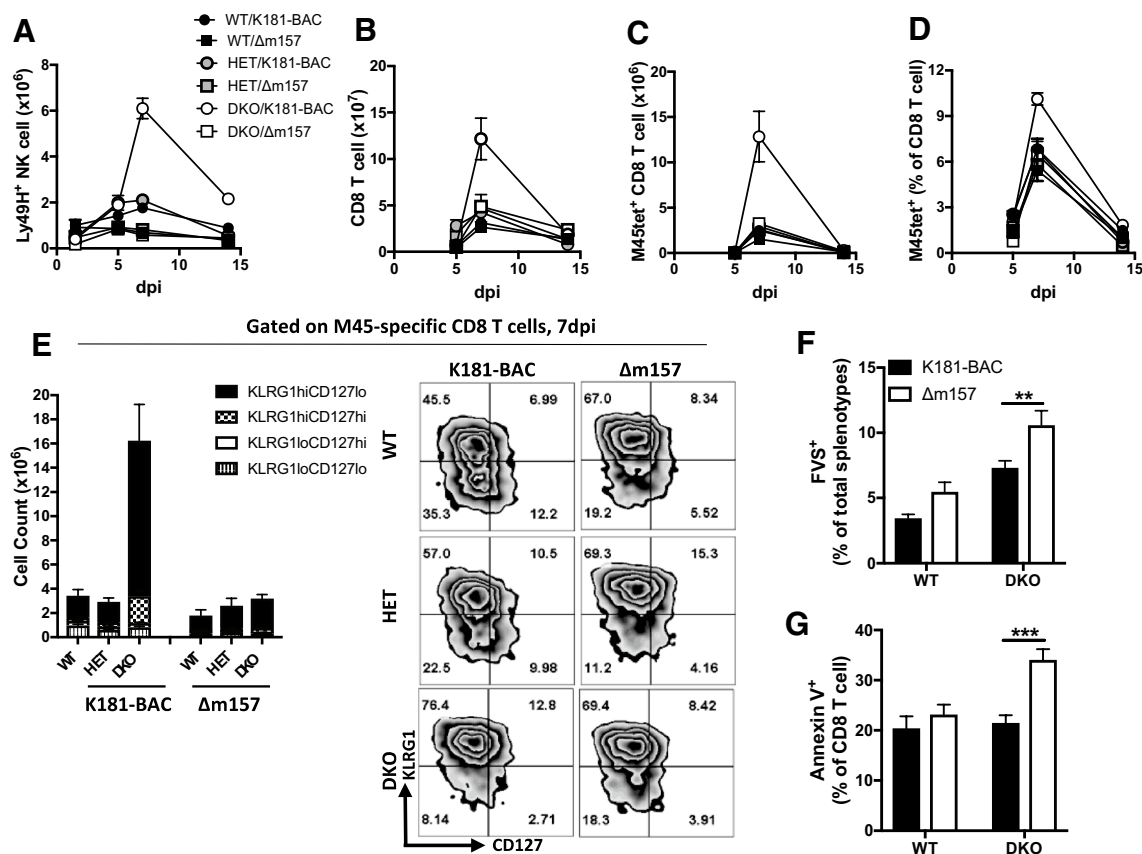
Fig. 5 Impact of m157-driven NK cells on antiviral CD8 T cell responses in DKO mice. WT, HET and DKO mice were inoculated i.p. with 1×10^6 PFU/mouse of K181-BAC or Δm157 virus and mean numbers $\pm$ S.E.M. of Ly49H$^+$ NK cells were determined in spleens on days 1.5, 5, 7, and 14 pi (**a**). Expansion, contraction, and phenotype of splenic CD8 T cell response to infection, showing mean numbers $\pm$ S.E.M. of total CD8 T cells (**b**) as well as numbers (**c**) and percentages (**d**) of M45-specific cells on days 5, 7, and 14 post infection. Bar graphs comparing numbers $\pm$ S.E.M. of different effector subsets (**e**, left panel) and representative zebra plots showing KLRG1 and CD127 expression (**e**, right panel) following gating on M45-specific CD8 T cells at 7 dpi. Cell death analysis on day 7 post infection with K181-BAC or Δm157 virus, comparing mean frequencies of FVS$^+$ dead cells in spleens of WT and DKO mice (**f**), mean percentages of annexin V$^+$ cells within CD8 T cell subset (**g**) when FVS$^+$ dead cells were excluded from this analysis. Data are presented as mean $\pm$ S.E.M. and represent two independent experiments with $n \geq 4$ mice per group

lower numbers and percentages of Ly49H$^+$ NK cells than WT controls (Fig. 7), despite hyperaccumulation during the acute phase of infection. Enhanced percentages of KLRG1^{hi} Ly49H$^+$ NK cells were observed in both CASP8-deficient settings when compared with controls. The proportion of Ly6C$^+$ NK cells was comparable regardless of genotype, suggesting that robust memory NK cell development is independent of CASP8 or RIPK3 function. Consistent with this observation, over 90% of the cells had a mature NK cell phenotype of CD27^{lo}CD11b^{hi} regardless of genotype. Thus, CASP8 is dispensable for memory NK cell formation, where Bim control of intrinsic apoptosis likely plays the central role [20]. The amplified Ly49H$^+$ NK cell response and pattern of enhanced terminal differentiation do not lead to better viral control [27], again indicating that the quality of *Casp8$^{-/-}$Ripk3$^{-/-}$* NK cells may be compromised in some ways.

Discussion

Recent studies have highlighted the adaptive immune features of NK cells in mice, humans, and nonhuman primates [2, 39]. Like the virus-specific CD8 T cell proliferation, signals through Ly49H receptor, cytokines and co-stimulatory molecules together drive efficient NK cell proliferation, differentiation and memory development. The extent to which cell death machinery contributes to NK cell responses has not been fully explored [21]. In the current study, we show that CASP8, a critical mediator of extrinsic death pathway [23], restricts NK cell expansion during the acute phase of MCMV infection but is dispensable for memory NK cell formation. These observations seem to parallel the cell-autonomous role of CASP8 in restraining CD8 T cell proliferation; however, additional studies will be needed to clarify this area, particularly given that regulation is independent
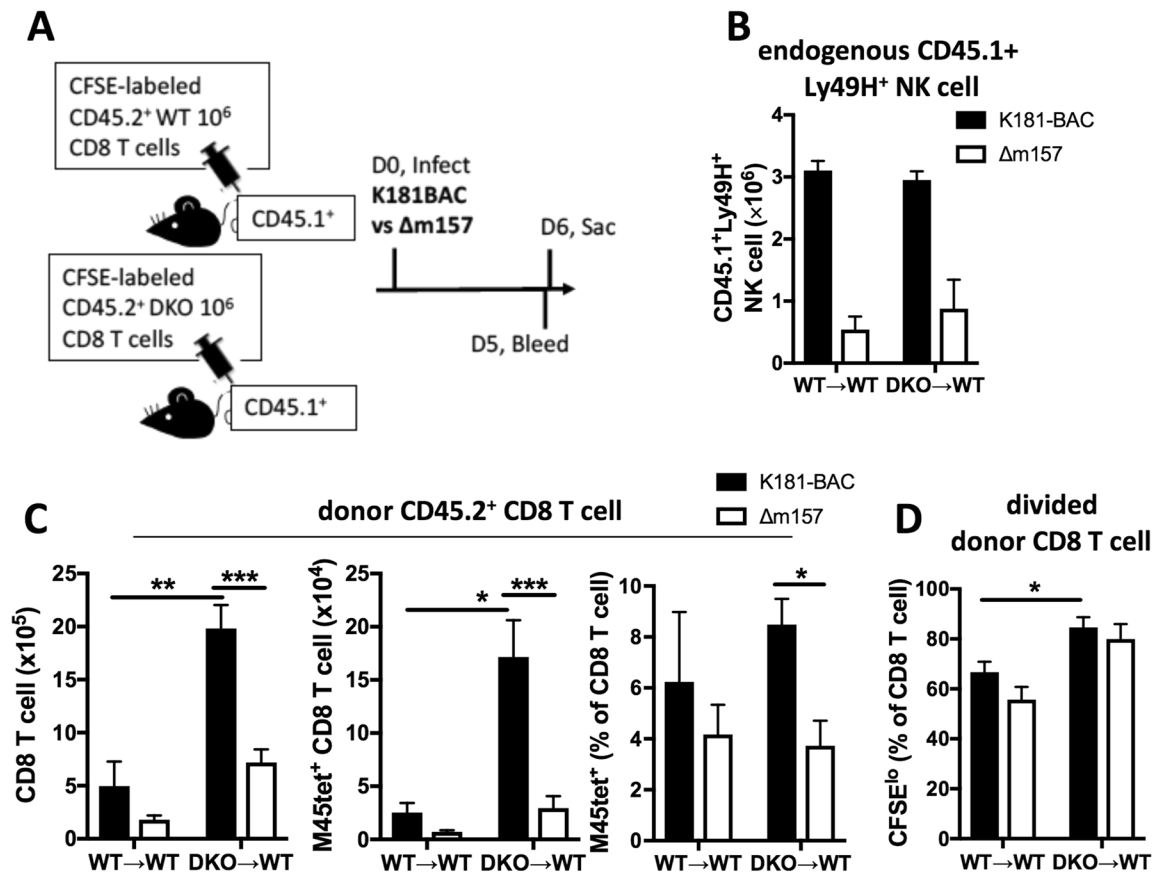
Fig. 6 Impact of WT m157-driven NK cells on antiviral DKO CD8 T cell responses. CFSE-labeled total splenocytes containing 1×10^6 NK cells from naive WT (CD45.2$^+$) or DKO mice (CD45.2$^+$) mice were transferred into CD45.1$^+$ WT mice, followed with infection of 1×10^6 PFU/mouse K181-BAC or Δm157 the next day followed by flow cytometric evaluation of splenocytes at 6 dpi (**a**). Numbers of endogenous CD45.1$^+$ Ly49H$^+$ NK cells in the recipients (**b**). Bar graphs comparing numbers of donor CD45.2$^+$ total CD8 T cells, and numbers as well as percentages of CD45.2$^+$ M45-specific cells (**c**). Bar graphs showing the percentages of CFSE^{lo} divided cells within the transferred donor CD45.2$^+$ CD8 T cells in the recipients (**d**)

Fig. 7 Phenotype of NK cells in long-term-infected CASP8-deficient mice. WT, DKO, $C8^{-/-}R3^{K51A}$ mice were infected and splenocytes were analyzed at 60 dpi. Bar graphs comparing the numbers (**a**) and percentages (**b**) of Ly49H$^+$ NK cells. Graphs comparing the percentages of KLRG1^{hi} (**c**) and Ly6C^{hi} (**d**) cells within the Ly49H$^+$ NK population. Representative flow plots depicting CD27 versus CD11b expression on the NK cell subset (**e**). Data are presented as mean $\pm$ S.E.M. and represent one experiment with mice $n = 5$ in each group
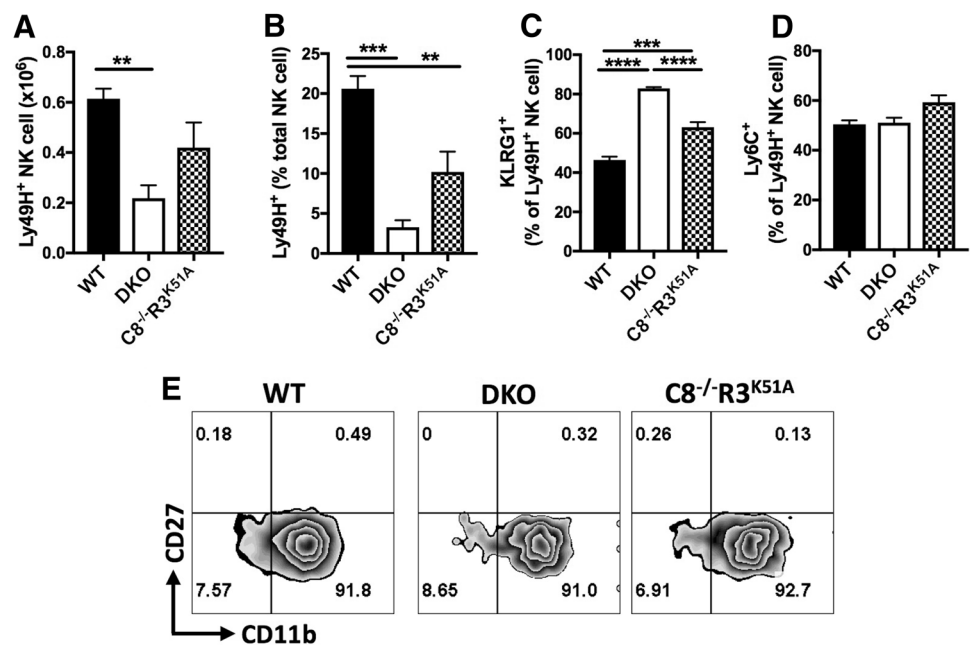
of pro-necroptotic RIPK3 but dependent on RIPK1. In addition to driving hyperaccumulation of Ly49H+ NK cells in *Casp8−/−Ripk3−/−* mice, the resulting environment supports CASP8-deficient T cell survival during expansion, but does not appear to directly drive proliferation. Rather, NK and CD8 T cell-autonomous CASP8 play different, but interdependent roles once the consequences of RIPK3 necroptosis are removed from the host.

Elimination CASP8 together with RIPK3 enhances the magnitude of Ly49H+ NK cell responses, a result reminiscent of observations in Bim-deficient mice [20]. Where Bim-deficiency impairs memory NK cell development, *Casp8−/−Ripk3−/−* mice do not show defects in NK memory. The impact of Bim in T cell memory is likely to be due to the importance of intrinsic apoptosis, which is intact in the mice we have studied here. Bim alters terminal differentiated effector subsets as well as memory precursors accumulating during the acute phase of virus infection [40], whereas combined deficiency in CASP8 and RIPK3 only affects the terminal differentiated effector subsets without evident impact on the memory precursors [28]. In addition, differences may be attributable to a death-independent role of CASP8 in regulating cell differentiation. CASP8 is likely to crosstalk with Akt–mTOR pathway [41], which is important for NK cell activation [42, 43]. Thus, disruption of CASP8 may interfere with NK or other adaptive immune cell differentiation through the Akt–mTOR signaling axis, areas that are ripe for further investigation.

Studies of CASP8 in NK cells were initially performed in patients with a CASP8 catalytic mutation [29, 30] which exhibited a defect in NF-κB activation following CD16 or 2B4 ligation. Whether this result may be compared to results of T cell-specific disruption of CASP8 in mice remains to be resolved. Importantly, studies in T cell-specific CASP8-deficient mice certainly suffered from unleashed RIPK3-mediated necroptosis that was not recognized at the time but was resolved in 2011 [44–46]. The dual elimination of CASP8 together with RIPK3 removes risk of death and results in enhanced Ly49H+ NK cells response upon MCMV infection. Thus, as in T cells [28], CASP8 restricts NK cell proliferation once the risk of unleashed necroptosis is eliminated. Similar to TCR signal transduction, Ly49H receptor activation triggers the formation of the CARMA–MALT1–BCL10 complex that subsequently drives NF-κB activation [47]. Studies of T cell signal transduction have shown that this complex recruits FADD and CASP8 through the DD interactions between FADD and MALT1 [30, 47, 48]. Given the similarity between TCR- and Ly49H-dependent pathways, a complex may form directly in association with Ly49H, although this may also be influenced by Fas DR activation of CASP8 and contribute [49] to Ly49H signal transduction.

By comparing WT and Δm157 virus, we show that a robust Ly49H+ NK cell response supports survival of proliferating *Casp8−/−Ripk3−/−* CD8 T cells, mainly affecting the KLRG1hi subsets that contribute to a higher peak CD8 T cell response. Indeed, the normal peak of *Casp8−/−Ripk3−/−* CD8 T cell response following Δm157 virus infection is reminiscent of patterns observed with LCMV-infected mice once T cell-specific CASP8 deficiency was placed on a RIPK3-deficient background (*Cd4CreCasp8flox/floxRipk3−/−*) [46]. In our work, Ly49H+ NK cells promote *Casp8−/−Ripk3−/−* CD8 T cell survival by either reducing viral antigen levels in a critical site or by otherwise altering the environment. Curiously, the increased accumulation of *Casp8−/−Ripk3−/−* CD8 T cells following TCR stimulation in culture does not require help from other immune cells [28], including NK cells. NK cells may support *Casp8−/−Ripk3−/−* CD8 T cell survival by metering antigen–TCR interactions in vivo, such as by maintaining DC numbers and increasing the amount of viral antigen available for cross-presentation by DCs. Previous studies show that NK cell function influences DC levels during MCMV infection [35–38]. *Casp8−/−Ripk3−/−* CD8 T cell hyperaccumulation may result from the more sustained antigen stimulation that occurs in vivo but through some de facto quality of in vitro stimulation assays [35–38]. Sustained DC levels would be expected to drive terminal differentiated KLRG1hiCD127lo cell accumulation, which depend more on sustained TCR stimulation than their KLRG1lo counterparts [18]. Therefore, hyperaccumulation is an inherent feature of *Casp8−/−Ripk3−/−* T cells following TCR stimulation and is maintained via a NK cell-dependent survival mechanism during herpesvirus infection.

The function of Casp8 in NK cell regulation beyond suppressing necroptosis remains unknown, although this intensely studied protease has many uncharacterized substrates, oligomerization states and interaction partners [25]. We show that Casp8 restricts Ly49H+ NK cell expansion with no obvious impact on Ly49H− NK cell levels. Strikingly, NK cell responses are ablated once RIPK1 is eliminated along with Casp8 and RIPK3. Thus, Casp8 and RIPK1, likely via a FADD bridge in a ripoptosome-like complex, collaborate to influence NK cell responses following infection.

Compliance with ethical standards

Ethical approval Animal experiments were approved according to the United States Public Health Service National Institutes of Health and Emory University Institutional Animal Care and Use Committee guidelines.

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