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# The NK Cell Response to Mouse Cytomegalovirus Infection Affects the Level and Kinetics of the Early CD8<sup>+</sup> T-Cell Response

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**Natural killer (NK) cells and CD8<sup>+</sup> T cells play a prominent role in the clearance of mouse cytomegalovirus (MCMV) infection. The role of NK cells in modulating the CD8<sup>+</sup> T-cell response to MCMV infection is still the subject of intensive research. For analyzing the impact of NK cells on mounting of a CD8<sup>+</sup> T-cell response and the contribution of these cells to virus control during the first days postinfection (p.i.), we used C57BL/6 mice in which NK cells are specifically activated through the Ly49H receptor engaged by the MCMV-encoded ligand m157. Our results indicate that the requirement for CD8<sup>+</sup> T cells in early MCMV control inversely correlates with the engagement of Ly49H. While depletion of CD8<sup>+</sup> T cells has only a minor effect on the early control of wild-type MCMV, CD8<sup>+</sup> T cells are essential in the control of  $\Delta m157$  virus. The frequencies of virus epitope-specific CD8<sup>+</sup> T cells and their activation status were higher in mice infected with  $\Delta m157$  virus. In addition, these mice showed elevated levels of alpha interferon (IFN- $\alpha$ ) and several other proinflammatory cytokines as early as 1.5 days p.i. Although the numbers of conventional dendritic cells (cDCs) were reduced later during infection, particularly in  $\Delta m157$ -infected mice, they were not significantly affected at the peak of the cytokine response. Altogether, we concluded that increased antigen load, preservation of early cDCs' function, and higher levels of innate cytokines collectively account for an enhanced CD8<sup>+</sup> T-cell response in C57BL/6 mice infected with a virus unable to activate NK cells via the Ly49H–m157 interaction.**

Mouse cytomegalovirus (MCMV) has been extensively used as a model for studying the role of NK cells in virus control. NK cells play a crucial role in the early stage of MCMV infection, prior to the induction of the adaptive immune response (20). However, the contribution of NK cells in the control of early MCMV infection varies among mouse strains (38; reviewed in reference 43). In C57BL/6 mice, the activating NK cell receptor Ly49H mediates resistance to MCMV infection due to the specific binding of m157, a virally encoded protein (6, 46). NK cell activation through Ly49H–m157 interaction is characterized by perforin-mediated cytotoxicity and specific proliferation of Ly49H<sup>+</sup> NK cells (13, 26, 49). Unlike C57BL/6 mice, MCMV-susceptible mouse strains are unable to mount an effective NK cell response to this virus (reviewed in reference 42).

In addition to their direct function, which results in the containment of viral infection, a large body of accumulated data suggests that NK cells play a role also in the shaping of the specific immune response (reviewed in reference 50). However, this topic is still controversial and the subject of intense studies. Robbins and colleagues (40) have shown that NK cell activation via the Ly49H–m157 pathway accelerates the CD8<sup>+</sup> T-cell response *in vivo*. According to the proposed scenario, the activation of NK cells via this axis limits alpha/beta interferon (IFN- $\alpha/\beta$ ) production by plasmacytoid dendritic cells (pDCs) and consequently prevents depletion of splenic conventional DCs (cDCs) and causes a prompt induction of a CD8<sup>+</sup> T-cell response. However, Andrews et al. (3) have shown that an NK cell response mediated through Ly49H–m157 interaction limits the long-term efficacy of specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells and that this is a consequence of an altered frequency and duration of infection of antigen-presenting cells. More recently, Stadnisky and colleagues (47) have demonstrated a role for inhibitory NK receptors in the CD8<sup>+</sup>

T-cell response to MCMV. Although these authors did not exclude a role for MCMV-specific activating NK receptors in their model, they provided first evidence to propose that the recognition of infected cells by licensed Ly49G2<sup>+</sup> NK cells results in a faster recovery of splenic cDCs and an enhanced antigen-specific CD8<sup>+</sup> T-cell response. In BALB/c mice, which do not express Ly49H and thus constitutively lack NK cell activation through Ly49H–m157 signaling, virus replication within the first 4 days postinfection (p.i.) was not notably controlled by either NK cells or CD8<sup>+</sup> T cells, unless deletion of viral immune evasion genes, of gene *m152* in particular, attenuated virus replication by recruiting NK cells and CD8<sup>+</sup> T cells due to elevated expression of the NK cell-activating ligand RAE-1 and enhanced major histocompatibility complex class I (MHC-I) peptide presentation, respectively (7). In addition, we have recently shown that recombinant virus expressing activating NKG2D ligand RAE-1 $\gamma$  is able to activate a strong CD8<sup>+</sup> T-cell response in spite of a significant NK cell- and NKG2D-dependent early attenuation (45).

In the present study, we have assessed the CD8<sup>+</sup> T-cell response in C57BL/6 mice during the first few days of infection with wild-type (WT) MCMV able to engage the Ly49H receptor, or with  $\Delta m157$  virus, which fails to activate NK cells via this receptor. In essence, our results have revealed that Ly49H–m157 signaling drives the NK cell response, whereas the inability of virus control

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via NK cells in the absence of Ly49H–m157 signaling results in a stronger CD8<sup>+</sup> T-cell response. Specifically, the data demonstrate that NK cell engagement through the Ly49H–m157 interaction limits the contribution of CD8<sup>+</sup> T cells to the control of MCMV. In contrast, in Ly49H<sup>+</sup> mice infected with  $\Delta m157$  MCMV, the CD8<sup>+</sup> T-cell response proved to be not only enhanced but also indispensable for virus control. Notably, CD8<sup>+</sup> T cells became essential even in the control of WT MCMV at high doses of infection. A more efficient CD8<sup>+</sup> T-cell response is most likely supported by elevated levels of proinflammatory cytokines able to drive the expansion of antiviral CD8<sup>+</sup> T cells. Altogether, our data suggest that an increased antigen load available for CD8<sup>+</sup> T-cell priming in concert with the preservation of cDCs' function early after infection and with elevated levels of proinflammatory cytokines explains the enhanced CD8<sup>+</sup> T-cell response in mice lacking early NK cell antiviral control.

## MATERIALS AND METHODS

**Mice.** Ly49H<sup>+</sup> mice (C57BL/6, BALB.B6-*Cmv1*<sup>+</sup>) and Ly49H<sup>-</sup> mice (B6.Ly49h<sup>-/-</sup> [15], BALB/c, and 129/SvJ) were housed and bred under specific-pathogen-free conditions at the Central Animal Facility of the Medical Faculty, University of Rijeka, in accordance with the guidelines contained in the *International Guiding Principles for Biomedical Research Involving Animals*. The Ethical Committee at the University of Rijeka approved all animal experiments described in this paper. Eight- to 12-week-old mice were used in all experiments.

**Viruses.** Unless otherwise indicated, mice were injected intravenously (i.v.) with  $2 \times 10^5$  PFU of the tissue culture (TC)-grown virus in a volume of 500  $\mu$ l of diluents. Bacterial artificial chromosome (BAC)-derived MCMV strain MW97.01 has previously been shown to be biologically equivalent to MCMV strain Smith (VR-1399) and is hereafter referred to as wild-type (WT) MCMV (51). MCMV recombinant virus expressing green fluorescent protein (MCMV-GFP), a derivative of the MCMV strain MW97.01, is hereafter referred to as WT MCMV-GFP (34). In addition, the mutant virus  $\Delta m157$  MCMV, which was previously described (8), was used.

**Depletion of lymphocyte subsets and quantitation of viral gene expression and infectivity.** The depletion of NK cells *in vivo* was performed by intraperitoneal (i.p.) injection of 300  $\mu$ g of purified monoclonal antibody (MAb) PK136 (23) 1 day before infection, which was repeated on day 1 p.i. The depletion of the CD8<sup>+</sup> T-lymphocyte subset was done by i.p. injection of 300  $\mu$ g of MAb to CD8 (YTS 169.4) (10) on days 1 and 5 p.i. The efficacy of depletion was assessed by flow cytometric analysis of splenic leukocytes stained with allophycocyanin (APC)-labeled anti-NK1.1 (eBioscience) and phycoerythrin (PE)-labeled anti-CD8 (BD Pharmingen).

Viral transcription in draining popliteal lymph nodes (PLNs) was measured by absolute quantitation of spliced IE1 transcripts using a real-time one-step reverse transcription-PCR (RT-PCR) as described in greater detail previously (7, 44). For quantitating viral infectivity in organs, virus titers were determined by standard plaque assay, as described previously (21).

**Flow cytometry and enzyme-linked immunospot (ELISpot) assay.** Splenic leukocytes were prepared as previously described and, in order to reduce nonspecific staining, Fc receptors were blocked with 2.4G2 MAbs (54). The following Abs were purchased from eBioscience or BD Pharmingen, and cell surface staining was performed specifically for the following antigens: anti-CD3 $\epsilon$  (145-2C11), anti-NK1.1 (PK136), anti-Ly49H (3D10), anti-CD69 (H1.2F3), anti-CD27 (LG.7F9), anti-CD11b (M1/70), anti-CD8 $\alpha$  (53–6.7), anti-IFN- $\gamma$  (XMG1.2), anti-CD19 (1D3), anti-MHC-II (M5/114.15.2), anti-CD11c (N418), and PE-labeled streptavidin (SA-PE). For the *in vivo* cell proliferation assay, mice were i.p. injected with 2 mg of bromodeoxyuridine (BrdU; Sigma) and sacrificed 3 h later. To detect incorporated BrdU, splenic leukocytes were first stained

for surface antigens and then fixed, permeabilized, refixed, treated with DNase I, and intracellularly stained according to the manufacturer's protocol (BrdU flow kit; BD Pharmingen). For the detection of IFN- $\gamma$  expression by NK cells, incubation was performed in RPMI medium supplemented with 10% of fetal calf serum (FCS; Gibco) for 5 h in the presence of 500 IU/ml of interleukin-2 (IL-2) at 37°C with 1  $\mu$ g/ml of brefeldin A (eBioscience) added for the last 4 h of incubation. For detection of IFN- $\gamma$  expression by CD8<sup>+</sup> T cells, incubation was performed in RPMI medium supplemented with 10% of FCS (Gibco) and 1  $\mu$ g of H-2D<sup>b</sup>-restricted M45-derived peptide (<sup>985</sup>HGIRNASFI<sup>993</sup>), H-2K<sup>b</sup>-restricted m139 derived peptide (<sup>419</sup>TVYGFCLL<sup>426</sup>), or H-2K<sup>b</sup>-restricted M57 derived peptide (<sup>816</sup>SCLEFWQRV<sup>824</sup>) for 5 h at 37°C with 1  $\mu$ g/ml of brefeldin A (eBioscience) added for the last 4 h of incubation (35). Custom MCMV-specific H-2<sup>b</sup> class I-restricted antigenic peptide synthesis to a purity of greater than 80% was performed by Jerini Peptide Technologies. After incubation, cells were first surface stained and then fixed and permeabilized using Cytofix/Cytoperm solutions (BD Pharmingen) followed by intracellular IFN- $\gamma$  staining according to the manufacturer's protocol. For DC isolation, splenocytes were digested using collagenase D (Roche) as described previously (40). Flow cytometry was performed on FACSCalibur and FASCAria (BD Bioscience; San Jose, CA), and data were analyzed using the FlowJo software (Tree Star).

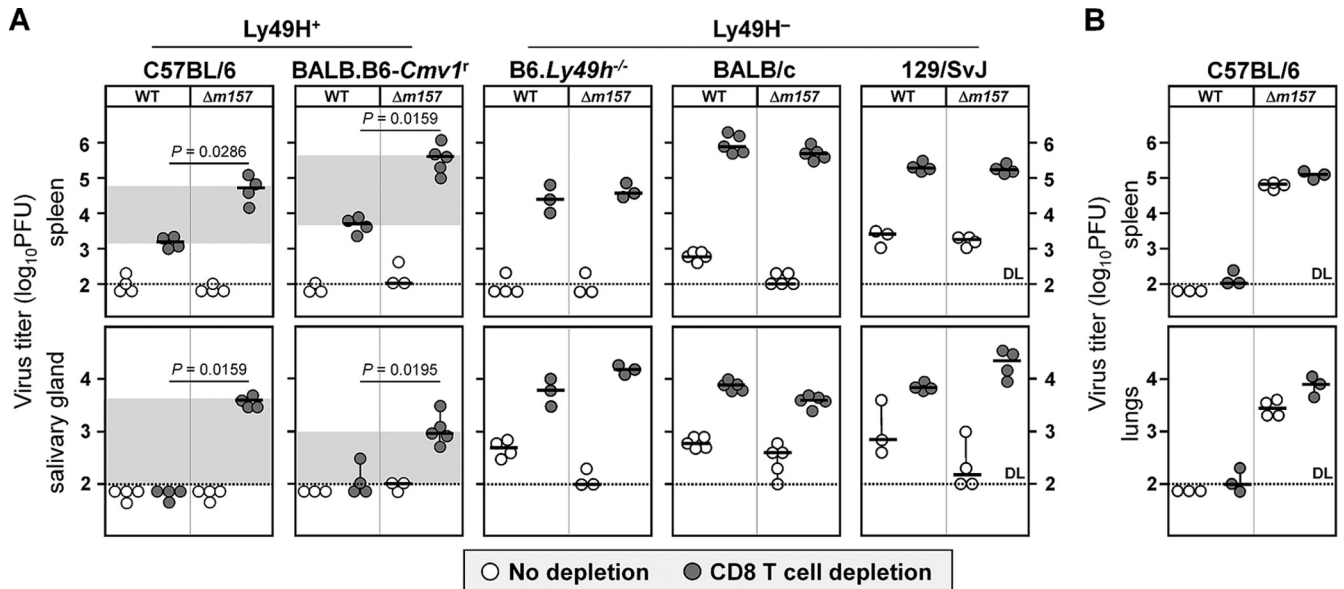
Frequencies of IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells were determined by an ELISpot assay essentially as described in greater detail previously (reference 7 and references therein). Briefly, CD8<sup>+</sup> T cells were isolated from lymph node cells by immunomagnetic cell sorting using the autoMACS system (Miltenyi Biotec Systems, Bergisch Gladbach, Germany) as described previously (27), and graded cell numbers in triplicate cultures were stimulated for 18 h with  $10^5$  EL-4 (H-2<sup>b</sup>) thymoma cells per assay culture and  $10^{-6}$  M synthetic peptides M45, m139, or M57 (see above). After staining for IFN- $\gamma$  and enzymatic color development, frequencies of responding cells and the corresponding 95% confidence intervals were determined from the spot counts by intercept-free linear regression analysis.

**Quantification of serum cytokine levels.** Serum levels of IFN- $\alpha$  were determined by an enzyme-linked immunosorbent assay (ELISA) kit for IFN- $\alpha$  (PBL Biomedical Laboratories) according to the manufacturer's instructions. Serum levels of IL-12, IFN- $\gamma$ , IL-6, tumor necrosis factor alpha (TNF- $\alpha$ ), IL-15, and IL-10 were determined by the Bio-Rad mouse cytokine multiplex assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

**Statistical analysis.** Statistical analysis was carried out using Prism 5 (GraphPad Software, La Jolla, CA). Statistically significant differences between two data sets in cytokine assays, phenotype analyses, and quantitation of viral transcripts (log-transformed numbers) were determined by the unpaired two-tailed Student's *t* test, and *P* values of <0.05 were considered significant. Differences in viral titers were determined by two-tailed Mann-Whitney *U* test.

## RESULTS

**Impact of NK cell control on the dynamics of an early antiviral CD8<sup>+</sup> T-cell response.** MCMV subverts NK cell control by encoding four proteins involved in the downmodulation of ligands for the activating NK cell receptor NKG2D. Specifically, m145, m152, and m155 interfere with cell surface expression of MULT-1, of RAE-1 family members, and of H60, respectively (19, 30). In addition, m138 simultaneously targets MULT-1, H60, and RAE-1 $\epsilon$  (5, 29). However, NK cells can be alternatively engaged by the viral protein m157, a ligand of the activating NK cell receptor Ly49H expressed differentially in mouse strains (6, 46). Accordingly, for studying the impact of NK cells on the early CD8<sup>+</sup> T-cell response, we here used Ly49H<sup>+</sup> mouse strains capable of mounting an NK cell response regardless of the presence of viral inhibitors of NK cells. The effect of CD8<sup>+</sup> T-cell depletion on virus titers



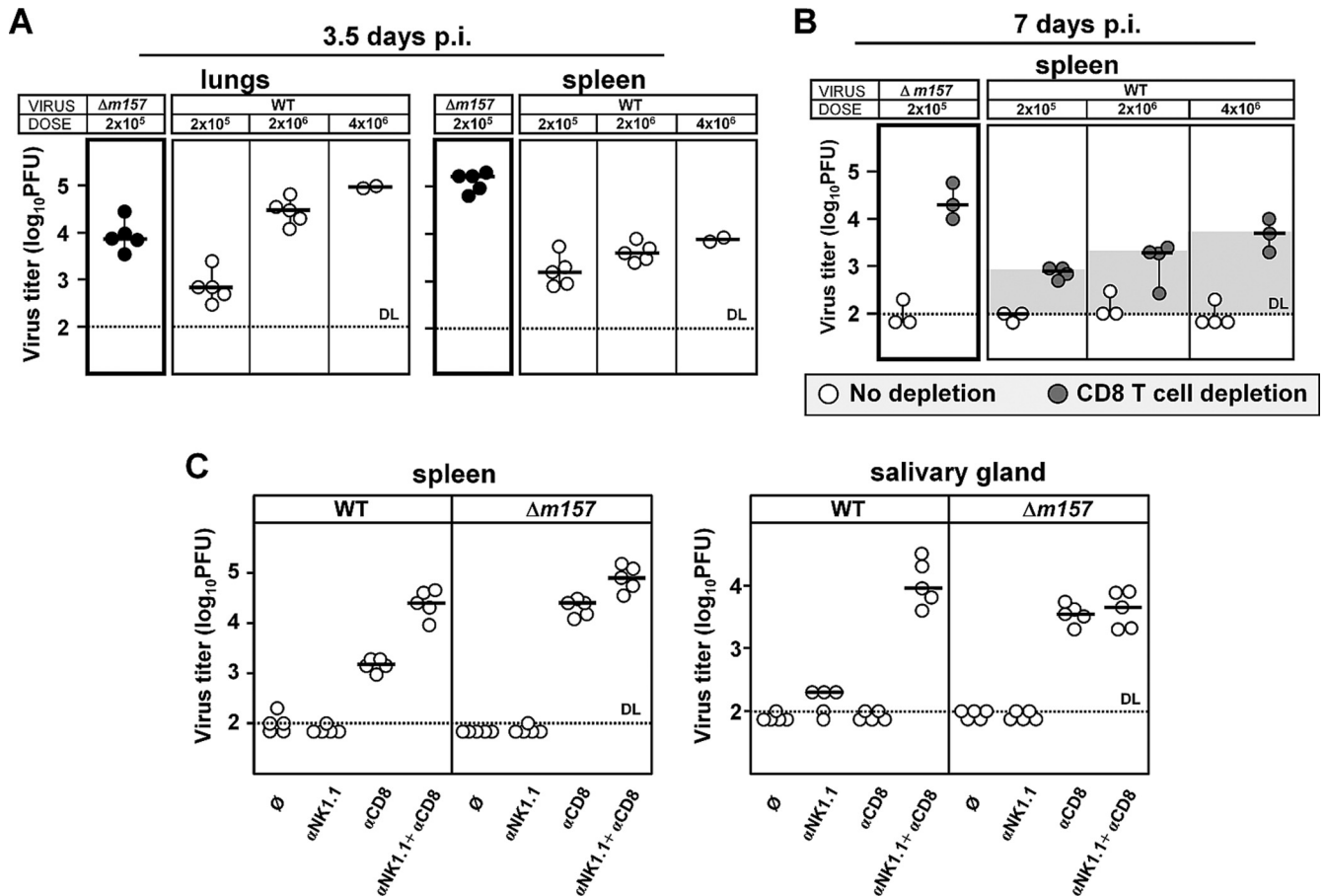
**FIG 1** NK cell-specific recognition of MCMV affects the CD8<sup>+</sup> T-cell response. (A) Indicated strains of mice were i.v. injected with  $2 \times 10^5$  PFU of either WT or  $\Delta m157$  MCMV. On days 1 and 5 p.i., mice were i.p. injected with phosphate-buffered saline (PBS) or depleting anti-CD8 MAb. On day 7 p.i., viral titers in spleens and salivary glands were determined by plaque assay. (B) C57BL/6 mice were i.v. injected with  $2 \times 10^5$  PFU of either WT or  $\Delta m157$  MCMV. On day 1 p.i., mice were i.p. injected with PBS or depleting anti-CD8 MAb. On day 3.5 p.i., viral titers in spleens and lungs were determined by plaque assay. Titers of individual mice (circles) and median values (horizontal bars) are shown. Representative data of at least two independent experiments with three to five mice per group are shown. Differences are regarded as significant for  $P$  values of  $<0.05$ . Dotted line, detection limit.

in different organs 7 days p.i. in Ly49H<sup>+</sup> and, for comparison, in Ly49H<sup>-</sup> mice infected with either WT MCMV or mutant virus lacking the *m157* gene ( $\Delta m157$  virus) was tested (Fig. 1A). On day 7 p.i., in both C57BL/6 and BALB.B6-*Cmv1r* mice (both Ly49H<sup>+</sup>) depleted of CD8<sup>+</sup> T cells, a significant increase of  $\Delta m157$  virus titer was observed, whereas only a minor effect could be seen in WT infected mice (Fig. 1A, left). In contrast, in B6.Ly49h<sup>-/-</sup> mice, as well as in BALB/c and 129/SvJ mice (all three Ly49H<sup>-</sup>), the depletion of CD8<sup>+</sup> T cells significantly increased the viral titers irrespective of the virus used (Fig. 1A, right). However, contrary to the findings on day 7 p.i., the depletion of CD8<sup>+</sup> T cells did not notably affect viral titers in organs of C57BL/6 mice on day 3.5 p.i., thus suggesting that in an early stage of infection CD8<sup>+</sup> T cells do not compensate for the absence of NK cell activity that is caused by lack of Ly49H activation in Ly49H<sup>+</sup> mice infected with the  $\Delta m157$  virus (Fig. 1B). This is in agreement with the interpretation that expression of m152 simultaneously prevents early NK as well as CD8<sup>+</sup> T-cell control by downmodulating RAE-1 and MHC-I, respectively (24, 31, 55). Accordingly, at the same time, NK as well as CD8<sup>+</sup> T cells were found to control  $\Delta m152$  virus (7). Notably, although it is well established that MCMV infection within the salivary glands is controlled primarily by CD4<sup>+</sup> T cells (22; reviewed in reference 9),  $\Delta m157$  virus titers in Ly49H<sup>+</sup> mice as well as titers for both viruses in Ly49H<sup>-</sup> mice indicated that salivary gland colonization in the first 7 days is under CD8<sup>+</sup> T cell control (Fig. 1A).

Next, we tested whether the differential requirements for CD8<sup>+</sup> T cells on day 7 p.i. in control of WT MCMV and  $\Delta m157$  virus (Fig. 1A, left) could be explained by a different virus load at early times when NK cells play a decisive role. To that aim, C57BL/6 mice were i.v. injected with different doses of WT MCMV ( $2 \times 10^5$  PFU,  $2 \times 10^6$  PFU, or  $4 \times 10^6$  PFU) to match the

virus titers in mice that received a single dose of  $\Delta m157$  virus ( $2 \times 10^5$  PFU). On day 3.5 p.i., only in mice that received 10- to 20-fold more WT MCMV, virus titers in organs reached those observed in mice infected with  $2 \times 10^5$  PFU of  $\Delta m157$  virus (Fig. 2A). Even the highest dose of WT MCMV failed to reach the titers observed for  $\Delta m157$  virus in the spleen, thus illustrating the robustness of the Ly49H-m157 pathway in the control of the virus in this organ. In addition, we tested the requirement for CD8<sup>+</sup> T cells on day 7 p.i. in mice that received different doses of WT MCMV and compared this to mice injected with only a single dose of  $\Delta m157$  virus (Fig. 2B). In agreement with results shown in Fig. 1A, the depletion of CD8<sup>+</sup> T cells had only a minor effect on the virus titer in mice infected with a lower dose of WT MCMV (Fig. 2B). However, by increasing the dose of WT MCMV ( $2 \times 10^6$  PFU or  $4 \times 10^6$  PFU), CD8<sup>+</sup> T cells became increasingly more important. Yet, the depletion of CD8<sup>+</sup> T cells in mice infected with a 20-fold higher dose of WT MCMV still failed to reach the titer found in the spleen of the CD8<sup>+</sup> T-cell depleted group infected with  $2 \times 10^5$  PFU of  $\Delta m157$  virus, thus suggesting that in addition to influencing viral load, NK cell signaling via Ly49H-m157 interaction may play also a qualitative role in the regulation of the CD8<sup>+</sup> T-cell response. To test this assumption, we performed combined depletion of NK cells and CD8<sup>+</sup> T cells in MCMV-infected mice (Fig. 2C). On day 7 p.i. in WT MCMV-infected mice depleted of both NK cells and CD8<sup>+</sup> T cells, viral titers in spleen and salivary glands reached those observed in CD8<sup>+</sup> T-cell depleted mice infected with  $\Delta m157$  virus. Thus, this result indicates that enhanced CD8<sup>+</sup> T-cell response in mice infected with  $\Delta m157$  virus positively correlates with the virus load.

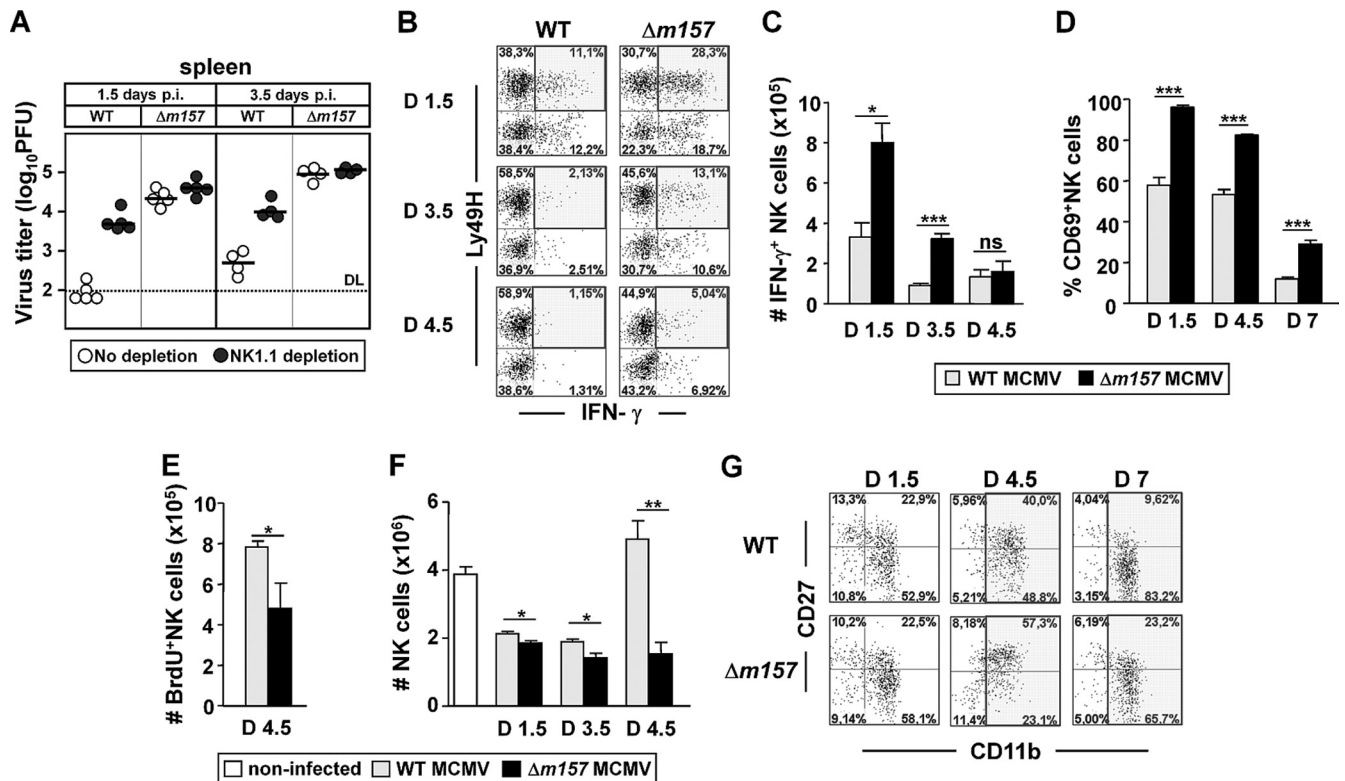
**The impact of Ly49H-m157 interaction on NK cell activation, maturation, and capacity to control virus replication.** There has been great progress in understanding of how the innate



**FIG 2** Virus load affects the CD8<sup>+</sup> T-cell-mediated control of MCMV infection in C57BL/6 mice. (A) C57BL/6 mice were i.v. injected with different doses of WT MCMV ( $2 \times 10^5$  PFU,  $2 \times 10^6$  PFU, or  $4 \times 10^6$  PFU) or with a single dose of  $2 \times 10^5$  PFU of  $\Delta m157$  MCMV. On day 3.5 p.i., viral titers in spleens and lungs were determined by plaque assay. (B) C57BL/6 mice were injected as described above, and on days 1 and 5 p.i. they were i.p. injected with PBS or depleting anti-CD8 MAb. On day 7 p.i., viral titers in spleens were determined by plaque assay. (C) C57BL/6 mice were i.v. injected with  $2 \times 10^5$  PFU of either WT or  $\Delta m157$  MCMV. Indicated groups were depleted of NK cells, CD8<sup>+</sup> T cells, or both NK cells and CD8<sup>+</sup> T cells. On day 7 p.i., viral titers in spleen and salivary glands were determined by plaque assay.  $\emptyset$ , untreated C57BL/6 mice. Data are compiled from two independent experiments with two to five mice per group. Titers of individual mice (circles) and median values (horizontal bars) are shown. Dotted line, detection limit.

immune response influences adaptive immunity (reviewed in reference 50). The results presented above suggest that an early CD8<sup>+</sup> T-cell response in C57BL/6 mice is shaped by the Ly49H–m157 interaction. Next, we decided to test whether the enhanced CD8<sup>+</sup> T-cell response in C57BL/6 mice infected with  $\Delta m157$  MCMV can be related to a differential response of NK cells. In accordance with previous work (8), early virus control in C57BL/6 mice was largely dependent on the Ly49H–m157 interaction. Specifically, deletion of gene *m157* from the virus genome abolished the antiviral control mediated by NK cells during the first 4 days p.i. (Fig. 3A). For characterizing the NK cells, C57BL/6 mice were injected i.v. with  $2 \times 10^5$  PFU of the indicated viruses, and NK cells in the spleen were analyzed for IFN- $\gamma$  production, proliferation, and expression of various markers. A high frequency of Ly49H<sup>+</sup> IFN- $\gamma$ <sup>+</sup> NK cells was observed in mice infected with  $\Delta m157$  MCMV compared to WT MCMV-infected mice as early as day 1.5 p.i. (Fig. 3B and C). This difference was present throughout the observed period. In accordance with the above, the absolute numbers of IFN- $\gamma$ <sup>+</sup> NK cells were significantly higher in mice infected with  $\Delta m157$  on days 1.5 and 3.5 p.i. (Fig. 3C). Similarly to IFN- $\gamma$ , the expression of the early activation marker CD69 on NK

cells was higher in mice infected with the  $\Delta m157$  virus (Fig. 3D). At first glance, these findings were surprising, as one rather might have expected lower levels of NK cell activation and IFN- $\gamma$  production in the absence of Ly49H–m157 signaling. It thus appears that low viral load after efficient virus control in WT MCMV infection limits the numbers of activated NK cells, possibly due to generally lower cytokine levels. Contrary to IFN- $\gamma$  production, however, the proliferation of NK cells was significantly high in mice infected with WT MCMV (13, 16, 26) compared to  $\Delta m157$ -infected mice, indicating a role for Ly49H–m157 signaling in triggering NK cell proliferation (Fig. 3E). This is in line with the absolute numbers of NK cells in the spleen of infected mice (Fig. 3F). Specifically, whereas in WT MCMV-infected mice, after an initial drop, the NK cell number in the spleen returned to the control level on day 4.5 p.i., in  $\Delta m157$ -infected mice the numbers of splenic NK cells remained low. The kinetics of NK cell maturation was analyzed by the expression of CD27 and CD11b markers (17). The maturation of NK cells, characterized by a high frequency of NK cells expressing the most mature phenotype CD27<sup>low</sup> CD11b<sup>high</sup>, was enhanced in WT MCMV-infected mice (Fig. 3G). Compared to the situation in WT MCMV-infected mice, the fre-



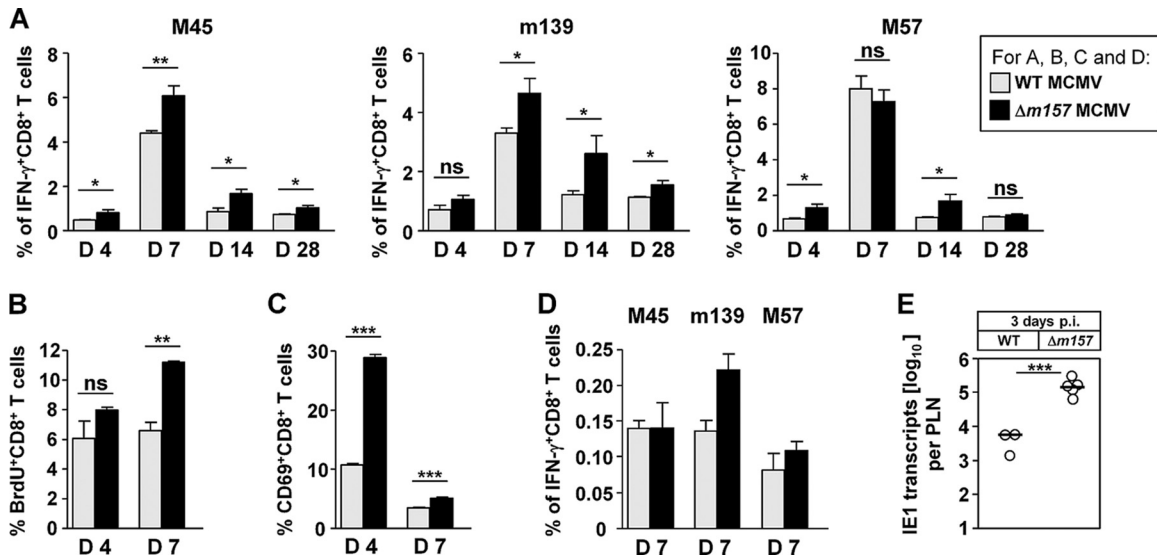
**FIG 3** Impact of Ly49H-m157 signaling on the NK cell response during early MCMV infection. (A) C57BL/6 mice, either NK cell depleted with anti-NK1.1 MAb or left undepleted, were i.v. injected with  $2 \times 10^5$  PFU of either WT or  $\Delta m157$  MCMV. On days 1.5 and 3.5 p.i., viral titers in spleens were determined by plaque assay. Titers of individual mice (circles) and median values (horizontal bars) are shown. (B) On days 1.5, 3.5, and 4.5 p.i., splenocytes were assessed for IFN- $\gamma$  production by Ly49H<sup>+</sup> and Ly49H<sup>-</sup> subsets of CD3 $\epsilon$ <sup>-</sup> NK1.1<sup>+</sup> NK cells. (C to F) The absolute numbers of IFN- $\gamma$ <sup>+</sup> CD3 $\epsilon$ <sup>-</sup> NK1.1<sup>+</sup> NK cells (C), the percentages of CD69<sup>+</sup> CD3 $\epsilon$ <sup>-</sup> NK1.1<sup>+</sup> NK cells (D), the absolute numbers of proliferating bromodeoxyuridine-positive (BrdU<sup>+</sup>) CD3 $\epsilon$ <sup>-</sup> NK1.1<sup>+</sup> NK cells (E), and the absolute numbers of CD3 $\epsilon$ <sup>-</sup> NK1.1<sup>+</sup> NK cells (F) in the total spleen cell population at the indicated time points are shown. (G) The maturation pattern of NK cells on days 1.5, 4.5, and 7 p.i. was determined by expression of cell surface markers CD27 and CD11b. Representative dot plots of splenic NK cells (CD3 $\epsilon$ <sup>-</sup> NK1.1<sup>+</sup>) from two independent experiments with three or four mice per group are shown. (C to F) Data are presented as means  $\pm$  standard error of the mean (SEM) of at least three mice per group from one of two independent experiments. Asterisks denote significant values as calculated by unpaired, two-tailed Student's *t* test: ns, nonsignificant; \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001. Dotted line, detection limit.

quency of CD27<sup>low</sup> CD11b<sup>high</sup> NK cells was lower on day 4.5 p.i. in mice infected with  $\Delta m157$  MCMV, whereas the frequency of CD27<sup>high</sup> CD11b<sup>high</sup> NK cells was higher. By day 7 p.i., the majority of NK cells also had matured to the phenotype CD27<sup>low</sup> CD11b<sup>high</sup>, but, compared to WT MCMV-infected mice, a notable frequency of NK cells in mice infected with  $\Delta m157$  MCMV still remained in the double-positive stage. Thus, these results showed that Ly49H-m157 signaling drives NK cell proliferation and phenotypic maturation, whereas IFN- $\gamma$  secretion and CD69 expression were higher in  $\Delta m157$ -infected mice, putatively as a consequence of a higher virus load in organs.

**Enhanced CD8<sup>+</sup> T-cell response in mice infected with the  $\Delta m157$  virus.** To further evaluate the impact of early NK cell activation on the CD8<sup>+</sup> T-cell response, we measured the frequency of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells in MCMV-infected mice upon stimulation with M45, m139, and M57 peptides *in vitro*. As early as on day 4 p.i., we observed a slight increase in the frequency of CD8<sup>+</sup> T cells specific for the above-mentioned viral epitopes in mice infected with  $\Delta m157$  virus compared to WT MCMV-infected mice. The differences were more pronounced on day 7 p.i. for CD8<sup>+</sup> T cells directed against the epitopes M45 and m139, whereas no significant difference was revealed for the M57 epitope

(Fig. 4A). The high frequency of MCMV-specific CD8<sup>+</sup> T cells in mice infected with  $\Delta m157$  virus, as compared to WT MCMV-infected mice, was present throughout the observed period of 4 weeks (Fig. 4A). The proliferation capacity of CD8<sup>+</sup> T cells was also enhanced on day 7 p.i. in mice infected with  $\Delta m157$  MCMV, compared to the WT MCMV-infected group (Fig. 4B). In addition, on day 4 p.i., the frequency of activated CD8<sup>+</sup> T cells expressing CD69 was significantly higher in mice infected with  $\Delta m157$  MCMV (Fig. 4C) and was still somewhat increased on day 7.

We next investigated if the enhanced CD8<sup>+</sup> T-cell response against  $\Delta m157$  virus is restricted to i.v. infection or applies to other routes of infection as well. To that aim, the frequencies of virus-specific CD8<sup>+</sup> T cells present in the draining popliteal lymph node (PLN) on day 7 after footpad infection were assessed. The results shown in Fig. 4D essentially support the results discussed above for i.v. infection, although the pattern of epitopes that profit from missing NK cell activation was not absolutely congruent with that after i.v. infection. However, taking both routes of infection together, m139 showed consistent results, and all three epitopes had the potential to profit under either of the infection routes. One may speculate that epitope-specific differences depend on the relative contributions of direct antigen pre-



**FIG 4** Absence of Ly49H-m157 signaling enhances the CD8<sup>+</sup> T-cell response in MCMV-infected mice. C57BL/6 mice were infected intravenously (A to C) or at the left hind footpad (D and E) with  $2 \times 10^5$  PFU of either WT or  $\Delta m157$  MCMV. (A to D) On the indicated days p.i., splenic leukocytes (A to C) or immunomagnetically purified CD8<sup>+</sup> T cells from the draining popliteal lymph node (PLN) (D) were stimulated in culture with the indicated peptides followed by intracellular IFN- $\gamma$  staining in cytofluorometric analysis (A to C) or IFN- $\gamma$  secretion in the ELISpot assay (D). (A) The percentages of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells specific for M45, m139, and M57 MCMV peptides among gated CD3 $\epsilon$ <sup>+</sup> CD8<sup>+</sup> T cells are shown. (B and C) The percentages of proliferating BrdU<sup>+</sup> CD8<sup>+</sup> T cells (B) and CD69<sup>+</sup> CD8<sup>+</sup> T cells (C) among gated CD3 $\epsilon$ <sup>+</sup> CD8<sup>+</sup> T cells are shown. (A to C) Data are presented as means  $\pm$  SEM of at least three mice per group from one of at least three independent experiments. (D) Immunomagnetically purified CD8<sup>+</sup> T cells from pools of PLN of five mice per group were stimulated for an IFN- $\gamma$ -based ELISpot assay with EL-4 (H-2<sup>b</sup>) thymoma cells and a saturating concentration ( $10^{-6}$  M) of synthetic peptides corresponding to the epitopes indicated. Bars represent frequencies of responding CD8<sup>+</sup> T cells; error bars indicate the 95% confidence intervals. (E) IE1 transcripts quantitated by RT-qPCR in draining PLN on day 3 after intraplantar infection. Symbols represent PLN of individual mice with the median value indicated. Throughout, asterisks denote significance values as calculated by unpaired, two-tailed Student's *t* test: ns, nonsignificant; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

sentation and cross-presentation to CD8<sup>+</sup> T-cell priming and that this might differ between viral antigenic proteins and routes of infection. Notably, enhanced priming in the PLN in the absence of Ly49H-m157 interaction correlated with an elevated intranodal viral gene expression (Fig. 4E) and thus with a higher viral antigen load.

Altogether, these results indicate that NK cell activation through Ly49H-m157 interaction negatively regulates the CD8<sup>+</sup> T-cell response, which likely relates to a reduced virus replication and thus a reduced antigen load available for CD8<sup>+</sup> T-cell priming.

**Secretion of IFN- $\alpha$  and other proinflammatory cytokines correlates with the CD8<sup>+</sup> T-cell response.** A number of studies emphasized the role of IFN- $\alpha$  and other innate cytokines in supporting CD8<sup>+</sup> T-cell priming through effects on both antigen-presenting cells and T cells (reviewed in reference 18). It has been shown previously that MCMV infection induces high serum levels of IFN- $\alpha$ , IL-12, and IFN- $\gamma$  in B6.Ly49h<sup>-/-</sup> mice on day 1.5 p.i. (15). Based on this finding and in an attempt to explain the enhanced CD8<sup>+</sup> T-cell response in mice infected with  $\Delta m157$  MCMV, we assessed the levels of proinflammatory cytokines in sera of infected mice on days 1.5 and 4 p.i. At the early time point, the serum level of IFN- $\alpha$  was  $\sim$ 4-fold higher in the group of mice infected with  $\Delta m157$  virus than in WT MCMV-infected mice (Fig. 5). A similar pattern was observed for the other three proinflammatory cytokines analyzed, namely, IL-12, IFN- $\gamma$ , and IL-6 (Fig. 5). Yet, the level of all four of these cytokines dropped almost completely by day 4 p.i., suggesting a tight feedback regulation. Contrary to those of the above-mentioned cytokines, the serum

levels of TNF- $\alpha$  were similarly induced with both viruses and only partially downregulated by day 4. The IL-15 level was elevated on day 1.5 p.i. with  $\Delta m157$  virus, compared to WT infected mice, but was strikingly reduced on day 4 p.i. specifically in the group of mice infected with the  $\Delta m157$  virus. Of note is that this correlates with an impaired NK cell proliferation upon  $\Delta m157$  MCMV infection (Fig. 3E). In contrast to proinflammatory cytokines, the concentration of IL-10 was only modestly upregulated in  $\Delta m157$ -infected C57BL/6 mice, but this upregulation lasted until day 4. These results suggest that an early antiviral response, characterized by a high level of proinflammatory cytokines, may be a parameter in determining the outcome of the CD8<sup>+</sup> T-cell response.

To further evaluate the impact of cytokine-mediated CD8<sup>+</sup> T-cell response, we analyzed the requirements for CD8<sup>+</sup> T cells in control of MCMV infection of B6.IFN $\alpha$ / $\beta$ R<sup>-/-</sup> and TNF-Rp55<sup>-/-</sup> mice. Unlike normal C57BL/6 mice, which control  $\Delta m157$  virus on day 7 p.i. via a CD8<sup>+</sup> T-cell-dependent mechanism, B6.IFN $\alpha$ / $\beta$ R<sup>-/-</sup> mice failed to do so, suggesting that type I IFNs play a role in enhancing CD8<sup>+</sup> T-cell response. Contrary to B6.IFN $\alpha$ / $\beta$ R<sup>-/-</sup> mice, TNF-Rp55<sup>-/-</sup> mice efficiently controlled  $\Delta m157$  infection by CD8<sup>+</sup> T cells, indicating that TNF receptor type I signaling does not contribute to enhanced CD8<sup>+</sup> T-cell control of  $\Delta m157$  virus (data not shown).

**The dynamics of splenic DCs correlates with the level of proinflammatory cytokines.** Both the life span and the survival of DCs *in vivo* are critical for the priming of the T-cell immune response. NK cells cross talk with DCs and thus shape the specific immune response (reviewed in reference 2). This includes NK cell killing of immature DCs and the promotion of DC maturation by

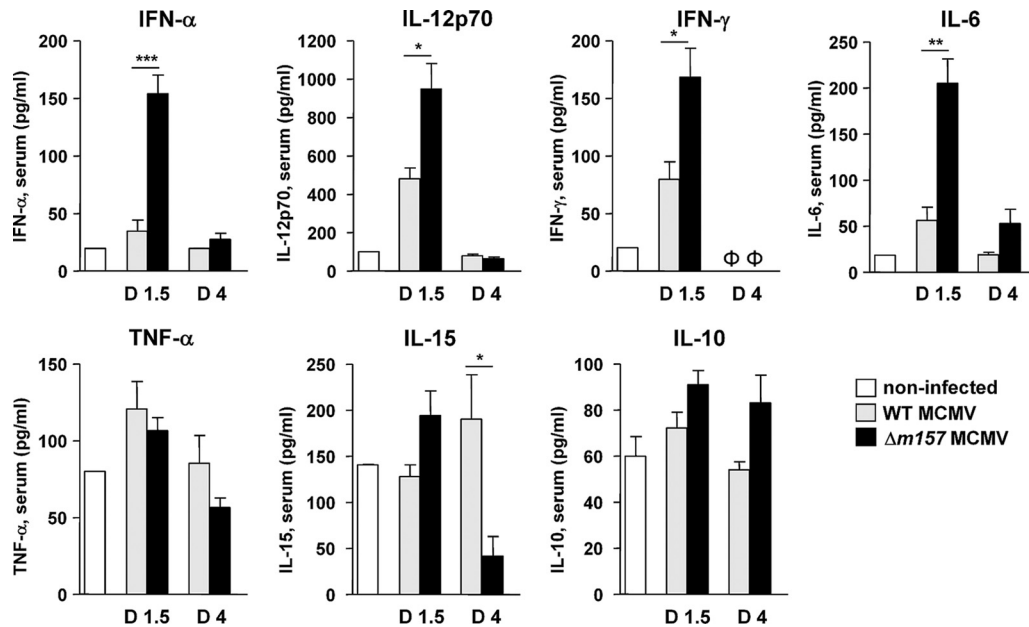


FIG 5 Impact of Ly49H-m157 signaling on cytokine production during early MCMV infection. C57BL/6 mice were i.v. injected with  $2 \times 10^5$  PFU of either WT or  $\Delta m157$  MCMV or left uninfected. On days 1.5 and 4 p.i., serum levels of the indicated cytokines were determined. Data are presented as means  $\pm$  SEM of at least three mice per group from one of two independent experiments. Asterisks denote significant values as calculated by unpaired, two-tailed Student's *t* test: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .  $\Phi$ , not detected.

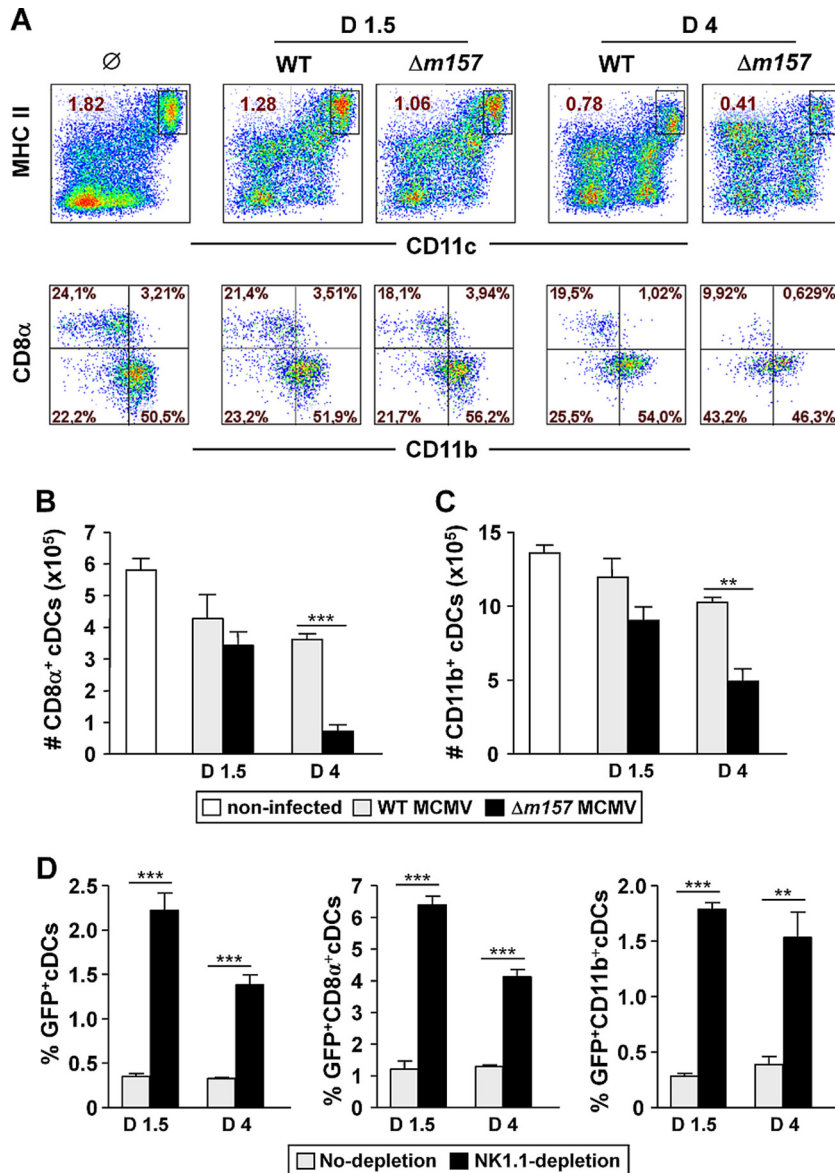
NK cell-derived IFN- $\gamma$  and TNF- $\alpha$ , which can eventually enhance antigen presentation to T cells (reviewed in reference 52). Conversely, DCs are important in NK cell activation and the initiation of the specific immune response (11). Upon MCMV infection *in vivo*, antigen-presenting cells such as conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), and macrophages are key producers of type I IFN and other proinflammatory cytokines (1, 12, 25, 48, 57). However, pDCs seem to play a central role in mounting a protective type I IFN response (25, 57). In order to address the impact of DCs on virus susceptibility to NK cell control and the level of proinflammatory cytokines, we analyzed splenic cDCs during the early phase of MCMV infection (Fig. 6). On day 1.5 p.i., we observed no differences in the frequencies of splenic cDCs (MHC-II<sup>+</sup> CD11c<sup>+</sup>) (Fig. 6A, top) or their CD8 $\alpha$ <sup>+</sup> and CD11b<sup>+</sup> subsets (Fig. 6A, bottom) in the comparison between the two viruses. In addition, at this time point p.i., the absolute numbers of CD8 $\alpha$ <sup>+</sup> (Fig. 6B) and CD11b<sup>+</sup> (Fig. 6C) cDC subsets were similar between the two infected groups of mice. However, on day 4 p.i., a dramatic reduction of splenic cDCs was observed specifically in mice infected with  $\Delta m157$  virus, which may be secondary to the increased level of infection in the absence of an efficient NK cell control. To determine the extent of cDC infection, we infected C57BL/6 mice with recombinant WT MCMV expressing green fluorescent protein (WT MCMV-GFP). Depletion of NK cells significantly increased the frequency of MCMV-infected cDCs and CD8 $\alpha$ <sup>+</sup> and CD11b<sup>+</sup> cDC subsets on days 1.5 and 4 p.i. (Fig. 6D). Therefore, although the frequency of splenic cDCs decreased on day 4 p.i. in mice lacking NK cell control, the high frequency of infected cDCs among the residual population might explain the improved priming of CD8<sup>+</sup> T cells. To conclude, the preservation of cDC subsets on day 1.5 p.i. and the higher frequency of infected cDCs at early time points p.i., as well as the higher level of proinflammatory cytokines, might contrib-

ute to the enhanced CD8<sup>+</sup> T-cell response observed in mice devoid of Ly49H signaling. In addition, our findings imply that the reduction of splenic cDCs observed on day 4 p.i. does not compromise the CD8<sup>+</sup> T-cell response.

## DISCUSSION

CD8<sup>+</sup> T cells play a key role in the control of human cytomegalovirus (HCMV) infections (53). Therefore, the understanding of the cellular and molecular mechanisms involved in priming and shaping of CD8<sup>+</sup> T-cell response is of high relevance. Both host and viral factors contribute to its nature and quality (reviewed in reference 28). There exists evidence to suggest that innate immunity mediated by NK cells is a critical parameter in determining the strength and quality of the primary CD8<sup>+</sup> T-cell response to MCMV (3, 40). MCMV can activate NK cells by encoding protein m157, a ligand of the activating NK cell receptor Ly49H. Here we have specifically investigated the impact of NK cell activation through Ly49H-m157 signaling on CD8<sup>+</sup> T-cell priming and antiviral control. Our results in C57BL/6 mice demonstrated that the requirement for CD8<sup>+</sup> T cells in the virus control during the first week p.i. is determined by the engagement of the Ly49H receptor and the capacity of NK cells to control virus replication during the first 2 days. While CD8<sup>+</sup> T cells proved to be dispensable for early virus control in mice infected with WT MCMV, they became essential in mice infected with a virus strain unable to activate NK cells through the Ly49H receptor, due to deletion of the ligand-coding gene *m157*. Moreover, depletion of NK cells in WT MCMV-infected mice also increased the requirements for CD8<sup>+</sup> T-cell control to a level comparable with the one observed in mice infected with  $\Delta m157$  virus. An enhanced CD8<sup>+</sup> T-cell response after infection with  $\Delta m157$  virus could at least in part be explained by a higher antigenic load, as sug-





**FIG 6** Ly49H-m157 signaling preserves splenic cDCs during MCMV infection. C57BL/6 mice were i.v. injected with  $2 \times 10^5$  PFU of either WT or  $\Delta m157$  MCMV or left uninfected. On days 1.5 and 4 p.i., splenic leukocytes were stained to assess DC subsets cytofluorometrically. (A) Representative dot plots of splenic cDCs ( $CD3^- CD19^- MHC-II^+ CD11c^+$ ) and  $CD8\alpha^+$  and  $CD11b^+$  subsets gated on cDCs are shown. (B and C) The absolute numbers of  $CD8\alpha^+$  cDCs (B) and  $CD11b^+$  cDCs (C) in total splenic leukocytes are shown. (D) C57BL/6 mice, either NK cell depleted or left undepleted, were i.v. injected with  $10^6$  PFU of WT MCMV-GFP. The frequency of infected splenic cDCs ( $CD3^- CD19^- MHC-II^+ CD11c^+ GFP^+$ ) and infected  $CD8\alpha^+$  and  $CD11b^+$  subsets gated on cDCs on days 1 and 4 p.i. are shown. (B to D) Data are presented as means  $\pm$  SEM of at least four mice per group from one of three independent experiments. Asterisks denote significant values as calculated by unpaired, two-tailed Student's *t* test: \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

gested previously from a related model of  $CD8^+$  T-cell priming to MCMV (7; reviewed in reference 28). Here we provide evidence to conclude that, besides antigenic load, elevated levels of proinflammatory cytokines and preservation of cDCs at early times of infection contribute to the enhanced  $CD8^+$  T-cell response in mice in which the lack of Ly49H-m157 signaling precludes NK cell-dependent early virus control.

Our data show that Ly49H-m157 interaction affects the NK cell activation pattern early on. NK cell expression of both IFN- $\gamma$  and CD69 were higher in mice that failed to control virus replication via the Ly49H-m157 pathway (Fig. 3B to D).

This finding also correlates with the level of proinflammatory cytokines, known to induce CD69 upregulation and IFN- $\gamma$  production by NK cells (14, 36). Although NK cells in mice infected with  $\Delta m157$  virus were stimulated to produce more IFN- $\gamma$  as early as 1.5 days p.i., this was obviously not sufficient to limit virus replication in the absence of a specific engagement of the Ly49H NK cell receptor. By the same token, NK cells derived from  $\Delta m157$ -infected mice failed to proliferate, thus confirming that a specific engagement of Ly49H receptor is required for this function (13, 16). This is in accordance with previous reports showing that ligation of Ly49H receptor to

m157 is necessary for induction of NK cell proliferation (6, 46). Furthermore, reduced proliferation capacity of NK cells and their delayed maturation in mice infected with  $\Delta m157$  virus may also be related to the observed sudden decrease in IL-15 secretion, which was not the case in mice infected with WT MCMV (Fig. 3E and G and 5).

Although interesting on their own, the different NK cell maturation and activation patterns in mice infected with WT MCMV or  $\Delta m157$  virus could not directly explain the differences in the CD8<sup>+</sup> T-cell response. Several studies have highlighted the role of proinflammatory cytokines in the activation of the cells of the innate and adaptive immune response (37; reviewed in reference 18). Cytokines produced by NK cells can prime DCs to produce IL-12 and promote their maturation, thereby enabling them to become potent antigen-presenting cells (1; reviewed in references 2 and 56). In addition, IFN- $\gamma$  produced by NK cells promotes antigen processing and presentation to T cells (33; reviewed in reference 39). Here we showed a dramatic rise in proinflammatory cytokines, including IFN- $\alpha$ , IL-12, IFN- $\gamma$ , and IL-6, which may drive a subsequent CD8<sup>+</sup> T-cell response. Furthermore, using mice lacking IFN $\alpha/\beta$  receptor or TNF-Rp55 receptor, we demonstrated that type I IFNs play a role in enhancing CD8<sup>+</sup> T-cell response to the virus unable to activate NK cells, whereas TNF signaling does not contribute to this function. Elevated levels of proinflammatory cytokines on day 1.5 p.i. are in agreement with a previous finding made in MCMV-infected B6.Ly49h<sup>-/-</sup> mice (15). The present study also demonstrated a tight regulation of proinflammatory cytokines, since on day 4 p.i. most of these were close to the levels observed in control groups. We speculate that this tight feedback control is aimed at preventing unleashed proliferation of NK cells and CD8<sup>+</sup> T cells and possibly also immunopathology. At this time point, the serum levels of IL-10 were still high, suggesting its role in negative feedback regulation of immune response (26; reviewed in reference 41).

MCMV infection affects DC maturation, frequently resulting in their depletion from the spleen (4). In agreement with this work, we have shown here that on day 1.5 p.i., cDC subsets were not affected, irrespective of the virus used, and that a dramatic drop was observed 4 days p.i. only if the engagement of the Ly49H receptor was missing (4). Interestingly, in another work, the drop in splenic cDCs in Ly49H<sup>-</sup> mice was observed already on day 1.5 p.i. (40). These differences might be related to different mouse strains or the different source of virus used. In recent work, Stadnisky et al. (47) showed that NK cell activation by the missing-self mechanism also resulted in faster recovery of splenic cDCs after their initial drop and in an improved CD8<sup>+</sup> T-cell response. Thus, the more robust CD8<sup>+</sup> T-cell response in mice infected with  $\Delta m157$  virus looks like a paradox since it is accompanied by a depletion of splenic cDCs. This could be explained by a higher level of virus replication and expression of epitope-encoding viral genes for antigen cross-presentation in  $\Delta m157$  infection, analogous to the model proposed by Böhm et al., which demonstrated that a virus with a more efficient replication gives better priming (7; reviewed in reference 28). Specifically, in that model, WT MCMV was superior to an attenuated immune evasion gene deletion mutant in the priming of CD8<sup>+</sup> T cells due to higher replication and antigen delivery. Our results represent the mirror image of this model by showing that in C57BL/6 mice, more efficiently rep-

licating  $\Delta m157$  virus primes CD8<sup>+</sup> T cells better than WT MCMV does. In addition, the frequency of infected cDCs among residual population on day 4 p.i. was still significantly higher in mice lacking NK cell control. Thus, it is likely that these cells might serve as a source for antigen presentation and cross-presentation. Therefore, we believe that CD8<sup>+</sup> T-cell response is determined by preserved cDCs, higher frequency of infected cDCs, and high levels of proinflammatory cytokines during the first 2 days p.i. and that the subsequent reduction of cDCs has no negative effect on the strength of ongoing CD8<sup>+</sup> T-cell response. Alternatively, in  $\Delta m157$ -infected mice, cDCs might not be physically deleted from the spleen but might rather be recruited to other sites of infection due to a higher level of virus replication. It was suggested that the depletion of cDCs is primarily mediated by NK cell cytotoxicity in a perforin-dependent manner (3). However, we have found a dramatic reduction of the splenic cDC pool during an acute MCMV infection also in mice deficient in perforin (data not shown), suggesting that the depletion of cDCs is not based on this cytolytic mechanism.

The impact of NK cells on the subsequent dynamics and the overall quality of the adaptive immune response may also differ with respect to the activation signaling pathway in NK cells involved in the initial virus control. Indeed, in a recent study we have demonstrated that NK cell activation via the activating NK cell receptor NKG2D after infection of mice with a recombinant virus expressing the NKG2D ligand RAE-1 $\gamma$  resulted in a profound virus attenuation during the first days p.i., yet, notably, without affecting the magnitude of the MCMV-specific CD8<sup>+</sup> T-cell response (45). Challenge experiments indicated that protection after immunization with RAE-1 $\gamma$ MCMV was even superior to the protection observed after immunization with WT MCMV. Even more striking results were observed in C57BL/6 mice infected with RAE-1 $\gamma$ MCMV. As this virus also expresses the m157 protein, the Ly49H NK receptor is engaged and controls the virus, which, as shown here, ought to reduce CD8<sup>+</sup> T cell priming. Surprisingly, however, the response was enhanced at early times after infection with the recombinant virus expressing RAE-1 $\gamma$  in addition to m157. These findings might suggest that different NK cell signaling pathways have different impacts on the subsequent CD8<sup>+</sup> T-cell response. Alternatively, enhancement of priming by expression of an NKG2D ligand on infected cells may involve costimulation by engagement of NKG2D expressed on CD8<sup>+</sup> T cells (32).

Collectively, the currently available body of data clearly demonstrates that the impact of NK cells on the priming of a CD8<sup>+</sup> T-cell response depends on the context of infection conditions and a complex set of host and viral factors, arguing against an easily predictable single mode and fixed direction of NK cell regulation of the primary CD8<sup>+</sup> T cell response. This complexity of parameters and outcomes may explain some of the apparent discrepancies in the literature.

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