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ORIGINAL INVESTIGATION

The specific NK cell response in concert with perforin prevents CD8$^+$ T cell-mediated immunopathology after mouse cytomegalovirus infection

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Abstract Natural killer (NK) and CD8$^+$ T cells play a crucial role in the control of mouse cytomegalovirus (MCMV) infection. These effector cells exert their functions by releasing antiviral cytokines and by cytolytic mechanisms including perforin activation. In addition to their role in virus control, NK cells play an immunoregulatory role since they shape the CD8$^+$ T cell response to MCMV. To investigate the role of perforin-dependent cytolytic mechanism in NK cell modulation of CD8$^+$ T cell response during acute MCMV infection, we have used perforin-deficient C57BL/6 mice (Prf1$^{-/-}$) and have shown that virus control by CD8$^+$ T cells in Prf1$^{-/-}$ mice is more efficient if NK cells are activated by the engagement of the Ly49H receptor with the m157 MCMV protein. A lack of perforin results in severe liver inflammation after MCMV infection, which is characterized by immunopathological lesions that are more pronounced in Prf1$^{-/-}$ mice infected with virus unable to activate NK cells. This immunopathology is caused by an abundant infiltration of activated CD8$^+$ T cells. The depletion of CD8$^+$ T cells has markedly reduced pathohistological lesions in the liver and improved the survival of Prf1$^{-/-}$ mice in spite of an increased viral load. Altogether, the results of our study suggest that a lack of perforin and absence of the specific activation of NK cells during acute MCMV infection lead to an unleashed CD8$^+$ T cell response that is detrimental for the host.

Keywords Mouse cytomegalovirus (MCMV) · CD8$^+$ T cells · Immunopathology · Perforin · NK cells · Ly49H

Introduction

The human cytomegalovirus (HCMV) is a ubiquitous pathogen causing high morbidity risk in immunologically suppressed and immunodeficient patients. Primary cytomegalovirus (CMV) infection is efficiently controlled in immunocompetent hosts, but nevertheless, the viral genome remains in a lifelong state of latency from which periodic reactivation may occur [1]. Due to the strict species specificity, animal CMVs models, particularly mouse CMV (MCMV), are being used in studying HCMV pathogenesis [2, 3].

The host defense against CMV involves the innate and adaptive immune response. Cells of innate immune system including dendritic cells (DCs), macrophages and natural killer (NK) cells are activated rapidly upon CMV infection [4]. The major characteristic of innate immune cells is the secretion of proinflammatory and immunoregulatory cytokines. Previous studies reported that innate cytokines IL-12 and IFN-α/β promote NK and CD8$^+$ T cell effector functions by enhancing the production of cytokines, such as IFN-γ and cytolytic granules including perforin and granzymes [5–8].

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Among the innate immune cells, NK cells are major effector cells, which are involved in the control of primary CMV infection during the time that precedes the induction of the specific immune response [7, 9]. The control of MCMV by NK cells in C57BL/6 mice is primarily dependent on signaling via the activating Ly49H receptor, which specifically recognizes the MCMV protein m157 [10–12]. Therefore, the deletion of the *m157* gene (Δ*m157* MCMV) compromises virus control by NK cells and enhances virus replication in vivo [11]. Evidence has been accumulated that, in addition to early containment of virus replication, NK cells play an important role in shaping the subsequent specific T cell response [13]. This function of NK cells has been extensively studied with regard to their capacity to modulate the antiviral $CD8^+$ T cell response [14–21]. Several studies demonstrated that NK cells can accelerate [22] or enhance the $CD8^+$ T cell response [23, 24]. While the NK cells stimulate $CD8^+$ T cell response inducing the maturation of DCs and consequently T cell polarization, an increasing body of evidence suggests that NK cells may also limit the $CD8^+$ T cell response (reviewed in [13] and [19]). Negative regulation of $CD8^+$ T cell response is important for maintaining homeostasis of lymphocytes, whereby it is especially important in the prevention of an excessive activation of $CD8^+$ T cells, which could eventually lead to immunopathology [15, 17, 18, 20]. Although the mechanisms governing the complex interaction between NK cells and $CD8^+$ T cells are still not been fully elucidated, it is assumed that NK cells may impair $CD8^+$ T cell response by direct killing of DCs [25–28] and/or activated T cells [21], as well as by the secretion of cytokines that have an inhibitory effect [17, 29]. Early upon MCMV infection, in the absence of cytolytic mechanism, expanding NK cells produce IL-10, which is important for limiting overall $CD8^+$ T cell response [17].

In this paper, we have investigated the regulation of $CD8^+$ T cell response to MCMV by NK cells in the absence of cytolytic mechanism by using perforin-deficient C57BL/6 mice ($Prf1^{-/-}$). The lack of perforin and of specific NK cell activation via Ly49H promotes a robust $CD8^+$ T cell response to MCMV, which fails to contain virus replication, but resulted in a severe liver inflammation followed by a high mortality rate of infected mice. The depletion of $CD8^+$ T cells in these mice has proven to have not only positive impact on the resolution of liver inflammation, but also on the survival rate. Specific activation of NK cells after MCMV infection partially compensated for the lack of perforin and had positive effect on the outcome of the infection. Altogether, our results suggest that perforin and specific activation of NK cells are required for homeostatic regulation of the specific $CD8^+$ T cell response to MCMV.

Materials and methods

Mice

C57BL/6 and $Prf1^{-/-}$ mice [30] were housed and bread 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 twelve-week-old mice were used in all experiments.

Viruses, depletion of lymphocyte subsets in vivo and the determination of viral titers and proliferation assay in vivo

Mice were injected intravenously (i.v.) with 2×10^5 PFU of the tissue culture (TC)-grown virus in a volume of 500 µl of PBS. Bacterial artificial chromosome (BAC)-derived MCMV strain MW97.01 has been shown previously to be biologically equivalent to MCMV strain Smith (VR-1399) and is hereafter referred to as wild-type (WT) MCMV [31]. In addition, mutant virus lacking *m157* gene was used [11]. The depletion of $CD8^+$ T lymphocyte subsets was done by intraperitoneal (i.p.) injection of 300 µg of mAb to CD8 (YTS 169.4) either on day 1 and 5 p.i., or on day 1 and every fifth day p.i. The efficacy of depletion was assessed by flow cytometer analysis of splenic leukocytes stained with PE-labeled anti-CD8 (Pharmingen). For determination of viral titers, mice were killed, organs were collected, and viral titers were determined by standard plaque assay as described previously [32]. For in vivo proliferation assay, mice were i.p. injected with 2 mg of bromodeoxyuridine (BrdU, Sigma) 3 h prior they were killed.

Flow cytometry and intracellular staining

Splenocytes were prepared as described previously, and Fc receptor on splenic leukocytes was blocked with 2.4G2 mAbs to reduce nonspecific staining [33]. The following Abs were purchased from eBioscience or BD Pharmingen, and cell-surface staining was performed against the following antigens: anti-CD3ε (145–2C11), anti-CD8α (53–6.7), anti-CD25 (PC61.5), anti-IFN-γ (XMG1.2) and anti-GzmB (16G6). H-$2D^b$-restricted MCMV-specific M45 (^{985}HGIRNASFI^{993}) peptide was synthesized to a purity of >95 % by Jerini Peptide Technologies. The H-$2D^b$-restricted M45 tetramer was synthesized by the National Institutes of Health. For detection of $CD8^+$ T cell IFN-γ and GzmB expression, cells were incubated in RPMI media

supplemented with 10 % of FCS (Gibco) and stimulated with 1 μg of M45 peptide [34] for 5 h at 37 °C with 1 μg/ml of brefeldin A (eBioscience) added for the last 4 h of incubation. After incubation, cells were first surface-stained, then fixed, and permeabilized using Cytofix/Cytoperm solutions (BD Pharmingen) followed by intracellular IFN-γ or GzmB staining according to the manufacturer's protocol. For proliferation assay, liver leukocytes were first surface-stained, then fixed, and permeabilized using Cytofix/Cytoperm solutions (BD Pharmingen) and incorporated BrdU was intracellularly stained according to the manufacturer's protocol (BrdU Flow Kit, BD Pharmingen). Flow cytometry was performed on FACSCalibur and FASCAria (BD Bioscience), and data were analyzed using the FlowJo software (Tree Star).

Preparation of tissue sections for histological analysis

Mice were killed on day 7 p.i., livers were removed, and fixed in 4 % paraformaldehyde. Paraffin-embedded 2-μm liver sections were prepared, stained with hematoxylin, and counterstained with eosin. $CD3^+$ T cells were detected by immunohistochemistry using staining with rabbit anti-CD3 antibodies (Abcam). Biotinylated goat anti-rabbit IgG (Abcam) was used as a secondary antibody, followed by detection with a streptavidin–peroxidase complex (Roche Diagnostics). Diaminobenzidine (Dako) was used as the substrate yielding a brown precipitate. For antigen retrieval, Tris–EDTA buffer (pH 9.0) in microwave oven was used. Double immunohistochemical staining was performed with rabbit anti-mouse caspase-3 antibody, which recognizes a large fragment of active caspase-3 (Cell Signaling Technology). Biotinylated goat anti-rabbit IgG (Abcam) was used as a secondary antibody, followed by a detection with a streptavidin–alkaline phosphatase complex (Roche Diagnostics). Fuchsin (Dako) was used as the substrate yielding a red precipitate. Detection of MCMV IE1 was performed using mAb CROMA101 [35], and goat anti-mouse IgG biotin (BD Pharmingen) was used as secondary antibody followed by the detection with a streptavidin–peroxidase complex (Roche Diagnostics). Diaminobenzidine (Dako) was used as the substrate yielding a brown precipitate. For antigen retrieval, a citrate buffer (pH 6,0) in microwave oven was used. Counterstaining was performed with Shandon hematoxylin. Slides were analyzed on Olympus BX51 microscope, and images were acquired by Olympus digital camera (DP71).

For immunofluorescence visualization of $CD8^+$ T cells, frozen liver cryosections were first stained with rat anti-mouse CD8 mAb (clone YTS 169.4) followed by goat anti-rat TRITC-labeled secondary antibody (Santa Cruz Biotechnology, Inc.). Samples were analyzed with Olympus FV300 confocal laser scanning microscope.

Statistical analysis

Statistical analysis was done using Prism 5 (GraphPad). Statistically significant differences for phenotype analyses were determined by the unpaired two-tailed Student's *t* test, and P values <0.05 were considered significant. Differences in viral titers were determined by two-tailed Mann–Whitney *U* test. Survival was analyzed with the Kaplan–Meier method. Survival curves were compared using the log-rank test.

Results

Lack of perforin affects MCMV-specific $CD8^+$ T cell response

The results of our previous study showed that in C57BL/6 mice, the early activation of NK cells through Ly49H-m157 interaction reduces the $CD8^+$ T cell response to MCMV infection [18]. To further clarify how NK cells exert their immunoregulatory role, we have investigated the extent to which the cytolytic activity of NK cells affects the intensity of $CD8^+$ T cell response to MCMV by using immunodeficient mice lacking perforin. To this aim, control mice (C57BL/6) and $Prf1^{-/-}$ mice were infected either with WT or Δ*m157* MCMV and viral titers in the spleen have been measured. A proportion of mice in each group were depleted of $CD8^+$ T cells. Unlike in C57BL/6 mice, MCMV infection of $Prf1^{-/-}$ mice resulted in higher viral titers on day 3.5 p.i., irrespective of Ly49H-m157 interaction (Fig. 1a). As expected, at this early time point, $CD8^+$ T cells did not exert any antiviral function neither in C57BL/6, nor in $Prf1^{-/-}$ mice. While in C57BL/6 mice, both viruses were well controlled in spleen on day 7 p.i., in $Prf1^{-/-}$ mice, the infection with Δ*m157* virus resulted in significantly higher viral titers, compared with WT MCMV (Fig. 1b). These results suggest perforin-independent antiviral control, but only in conditions when specific activation of NK cells through Ly49H receptor is preserved.

In accordance with our recently published data [18], in C57BL/6 mice, the depletion of $CD8^+$ T cells resulted in a significant increase in Δ*m157* virus titer, while in mice infected with WT MCMV, only a moderate effect on virus titer was observed (Fig. 1b). However, in $Prf1^{-/-}$ mice, $CD8^+$ T cell depletion showed a strong effect on the control of both viruses, although it was generally more pronounced in mice infected with Δ*m157* virus. These data suggest that immunoregulatory role of NK cells in modulation of $CD8^+$ T cell response to MCMV depends on specific activation of NK cells through Ly49H receptor in a perforin-dependent manner.

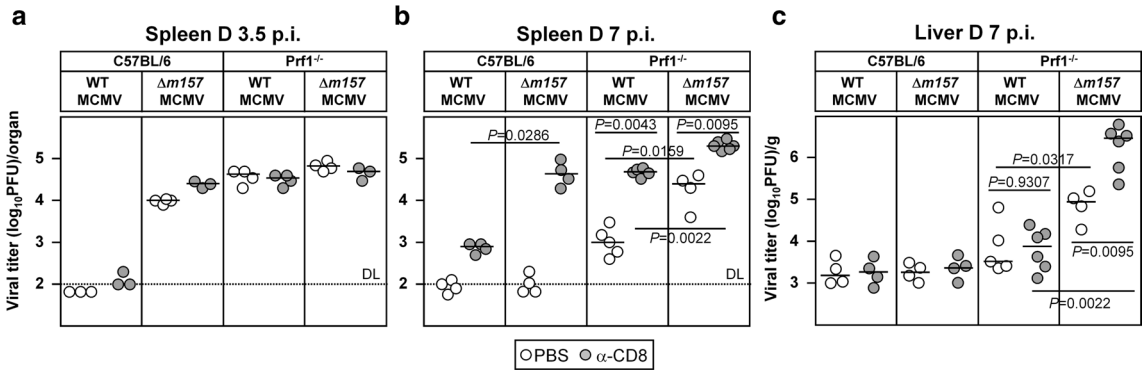

Fig. 1 Perforin-dependant control of MCMV infection. C57BL/6 and Prf1$^{-/-}$ mice were i.v. injected with 2 × 10^5 PFU of either WT or Δ*m157* MCMV, and indicated groups were treated with either PBS or depleting anti-CD8 mAbs on days 1 and 5 p.i. On day **a** 3.5 p.i., and **b** 7 p.i., mice were killed and viral titers in spleen and **c** liver were determined by plaque assay. Titers of individual mice (*circles*) and median values (*horizontal bars*) are shown. Data are representative from two independent experiments with three to six mice per group. *Asterisks* denote significant values: *P < 0.05, **P < 0.01; *n.s.* not significant, *DL* limit of detection

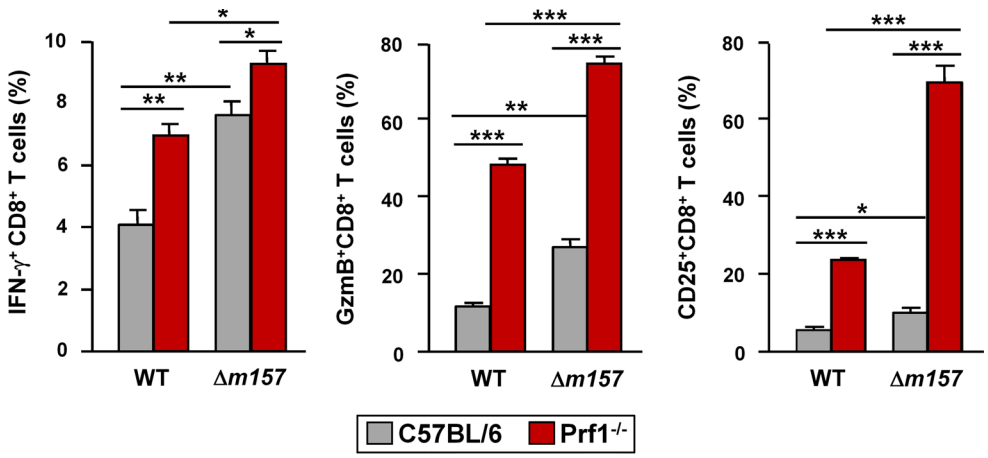

Fig. 2 Augmented CD8$^+$ T cell activation status in the absence of Ly49H signaling and perforin. C57BL/6 and Prf1$^{-/-}$ mice were i.v. injected with 2 × 10^5 PFU of either WT or Δ*m157* MCMV. On day 7, p.i. splenocytes were isolated and stimulated in the culture with MCMV-specific M45 peptide prior to intracellular staining for IFN-γ or GzmB, or processed immediately for staining with anti-CD25 mAbs. The frequencies of IFN-γ$^+$, GzmB$^+$ and CD25$^+$ CD8$^+$ T cells (CD3ε$^+$CD8$^+$) are shown. Data are presented as mean ± SEM of four mice per group from one of two independent experiments. *Asterisks* denote significant values: *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired, two-tailed Student's *t* test)

To further explore the impact of perforin-mediated cytolytic mechanism on the quality of CD8$^+$ T cell response to MCMV, we assessed the phenotype and functional status of CD8$^+$ T cells on day 7 p.i. For this set of experiments, we have used the M45 epitope as an indicator for virus-specific CD8$^+$ T cells [36, 37]. Mice lacking perforin showed increased frequencies of IFN-γ$^+$CD8$^+$ T cells specific for the M45 epitope (Fig. 2). Furthermore, the frequency of M45 epitope-specific CD8$^+$ T cells was significantly higher in both mouse strains infected with the Δ*m157* virus compared with WT-infected groups. Since the expression of granzyme B (GzmB) and CD25 marker characterizes effector T cells, we have analyzed the expression of these markers on CD8$^+$ T cells [38, 39]. The upregulation of both GzmB and CD25 by CD8$^+$ T cells was more pronounced in mice infected with Δ*m157* MCMV. Although the lack of perforin dramatically increased the frequency of GzmB$^+$ and CD25$^+$CD8$^+$ T cells, the differences between WT and Δ*m157* virus were still preserved. Thus, the absence of perforin and of the specific NK cell activation enhances the CD8$^+$ T cell response to MCMV.

CD8$^+$ T cells affect control of Δ*m157* MCMV in the liver of Prf1$^{-/-}$ mice

Having in mind that MCMV can induce hepatitis, which is especially severe in Prf1$^{-/-}$ mice [40], our next aim

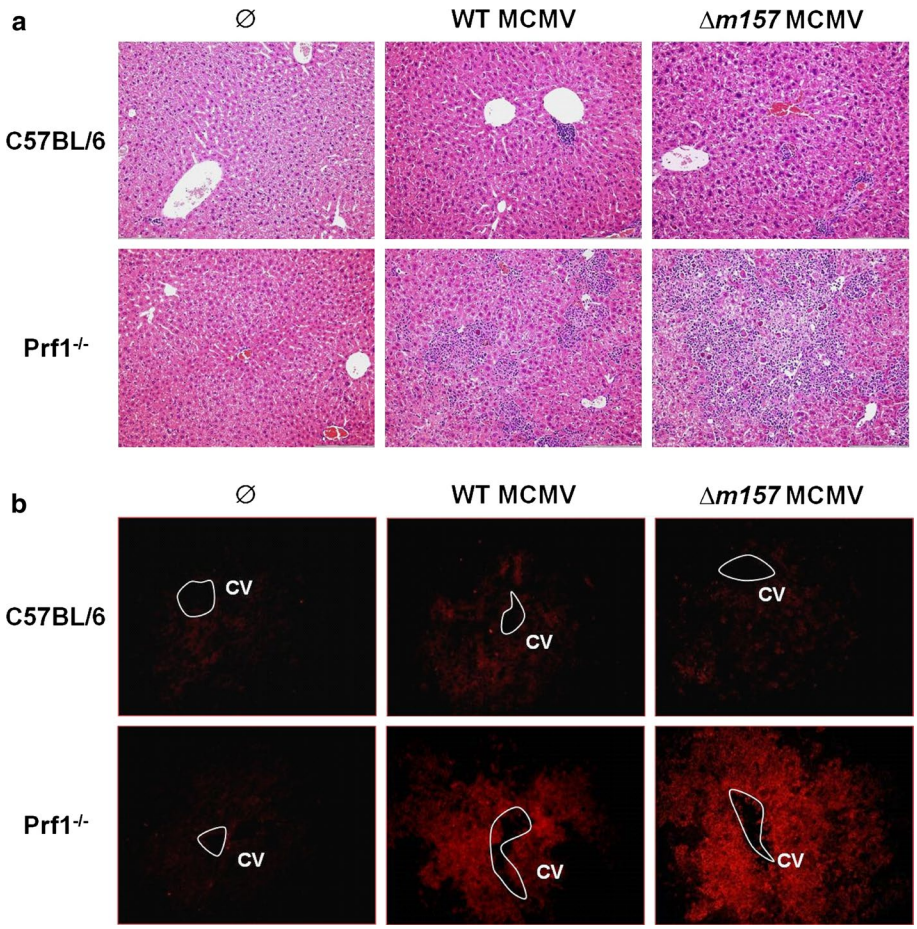

Fig. 3 Pathohistological lesions in liver of Δ*m157* MCMV-infected Prf1$^{-/-}$ mice. C57BL/6 and Prf1$^{-/-}$ mice were i.v. injected with 2×10^5 PFU of either WT or Δ*m157* MCMV, or left uninfected. On day 7, p.i. mice were killed and livers were processed for further analysis. **a** Representative paraffin-embedded liver sections analyzed for the presence of pathohistological lesions are shown (H&E; ×20). **b** Representative images of immunofluorescence detection of CD8$^+$ T cells in liver are shown. Data are representative of two independent experiments with three to six mice per group. *CV* central vein

was to investigate immune surveillance of MCMV infection in the liver of Prf1$^{-/-}$ and control mice. On day 7 p.i., both WT and Δ*m157* MCMV were well controlled in the liver of C57BL/6 mice (Fig. 1c). Unlike in the spleens of C57BL/6 mice (Fig. 1b), CD8$^+$ T cells did not contribute to the control of Δ*m157* virus in the liver. Prf1$^{-/-}$ mice were also able to control WT MCMV in the liver, and the depletion of CD8$^+$ T cells did not affect the control of this virus. However, infection of Prf1$^{-/-}$ mice with Δ*m157* MCMV resulted in significantly higher viral titers compared with WT MCMV. The depletion of CD8$^+$ T cells additionally increased the titer of Δ*m157* virus in liver (Fig. 1c). Altogether, these results suggest that without perforin and specific NK cell activation, CD8$^+$ T cells to some extent compensate virus control via a noncytolytic mechanism.

CD8$^+$ T cells cause a severe hepatic immunopathology in Prf1$^{-/-}$ mice after MCMV infection

Next, we have assessed the impact of NK cell activation via Ly49H and of CD8$^+$ T cell response on hepatitis of MCMV-infected Prf1$^{-/-}$ mice. We have analyzed H&E-stained liver sections prepared from mice which were infected for 7 days. MCMV-infected Prf1$^{-/-}$ mice developed severe pathohistological lesions that included multiple foci of hepatocellular destruction and mononuclear cell infiltrations composed mostly of lymphocytes and histiocytes, whereas in C57BL/6 mice, the lesions were milder and signs of regeneration were observed (Fig. 3a). Of note is that the absence of early NK cell activation through Ly49H-m157 interaction significantly increased liver lesions in Prf1$^{-/-}$ mice. Since previous studies showed an enhanced activation of CD8$^+$ T cells after MCMV infection of Prf1$^{-/-}$ mice [41], we have assumed that a vast proportion of lymphocytes in mononuclear infiltrations might be CD8$^+$ T cells. In order to address this issue, we have performed immunofluorescent staining of frozen liver sections with anti-CD8 monoclonal antibodies. We have observed a massive infiltration of CD8$^+$ T cells in the liver of Prf1$^{-/-}$ mice infected with Δ*m157* virus (Fig. 3b). In WT-infected Prf1$^{-/-}$ mice, the infiltration of CD8$^+$ T cells in the liver was less pronounced but still prominent. Contrary to Prf1$^{-/-}$ mice, MCMV infection did not induce an accumulation of CD8$^+$ T cells in the liver of control C57BL/6 mice. Altogether, these data indicate that absence

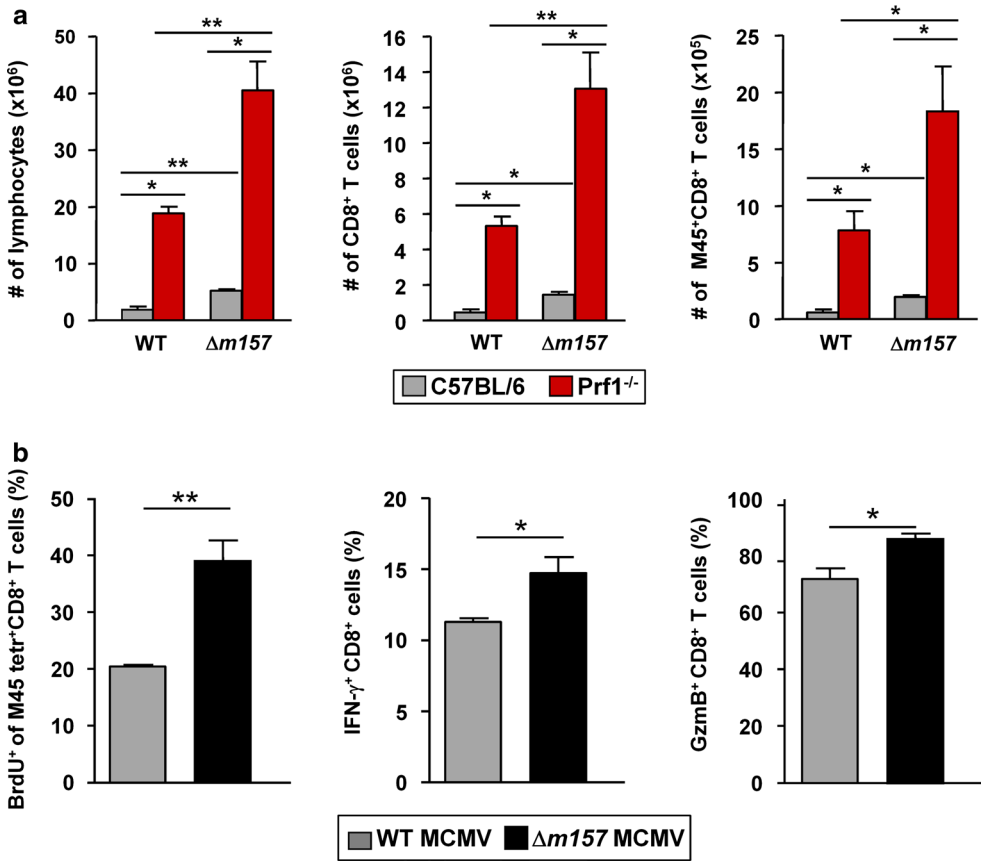

Fig. 4 The strong CD8$^+$ T cell response to Δ*m157* MCMV is further augmented in absence of perforin. **a** C57BL/6 and Prf1$^{-/-}$ mice were i.v. injected with 2 × 10^5 PFU of either WT or Δm157 MCMV. Liver lymphocytes were isolated and stained for M45 tetramer-specific CD8$^+$ T cells. Total number of lymphocytes, total number of CD8$^+$ T cells (CD3ε$^+$CD8α$^+$) and total number of M45-specific CD8$^+$ T cells per liver are shown. **b** Prf1$^{-/-}$ mice were i.v. infected as in **a**. On day 7 p.i. mice were i.p. injected with 2 mg of BrdU and killed 3 h later. For IFN-γ and GzmB assay, liver lymphocytes were stimulated in the culture with MCMV-specific M45 peptide prior to intracellular staining. The frequencies of BrdU$^+$M45 tetramer$^+$CD8$^+$ T cells, IFN-γ$^+$CD8$^+$ T cells and GzmB$^+$CD8$^+$ T cells specific for M45 peptide in total liver lymphocytes are depicted. Data are presented as mean ± SEM of at least three mice per group from two independent experiments. *Asterisks* denote significant values: $*P < 0.05$, $**P < 0.01$ (unpaired, two-tailed Student's *t* test)

of perforin and of the specific NK cell activation results in severe liver damage and a massive infiltration of CD8$^+$ T cells.

Prf1$^{-/-}$ mice infected with Δ*m157* MCMV show an enhanced activation of MCMV-specific CD8$^+$ T cells in the liver

To investigate whether CD8$^+$ T cells in the liver of MCMV-infected Prf1$^{-/-}$ mice are virus specific, we have performed flow cytometry analysis of liver lymphocytes on day 7 p.i. In accordance with the results obtained by immunofluorescent staining (Fig. 3b), infection of Prf1$^{-/-}$ mice with Δ*m157* virus resulted in significantly higher number of CD8$^+$ T cells in the liver compared to Prf1$^{-/-}$ mice infected with WT MCMV and C57BL/6 mice, regardless of the virus used for infection (Fig. 4a). To confirm that these CD8$^+$ T cells are specific for MCMV, we used the MHC class I tetramer filled with M45 peptide specific for the acute phase of MCMV infection. In line with the number of total CD8$^+$ T cells, the number of M45-specific CD8$^+$ T cells was significantly higher in the liver of Prf1$^{-/-}$ mice infected with Δ*m157* virus compared with WT MCMV-infected Prf1$^{-/-}$ mice, as well as C57BL/6 mice, regardless of the virus (Fig. 4a). Furthermore, the proliferation capacity of virus-specific CD8$^+$ T cells was higher in the liver of Prf1$^{-/-}$ mice infected with Δ*m157* virus compared with WT MCMV-infected Prf1$^{-/-}$ mice (Fig. 4b). In addition, the frequency of IFN-γ$^+$CD8$^+$ T cells and GzmB$^+$CD8$^+$ T cells after in vitro restimulation with the M45 peptide was also higher in Prf1$^{-/-}$ mice infected with Δ*m157* MCMV compared to Prf1$^{-/-}$ mice infected with WT MCMV (Fig. 4b). Thus, the lack of perforin and of the specific NK cell activation results in a massive accumulation of activated virus-specific CD8$^+$ T cells in the liver of infected animals.

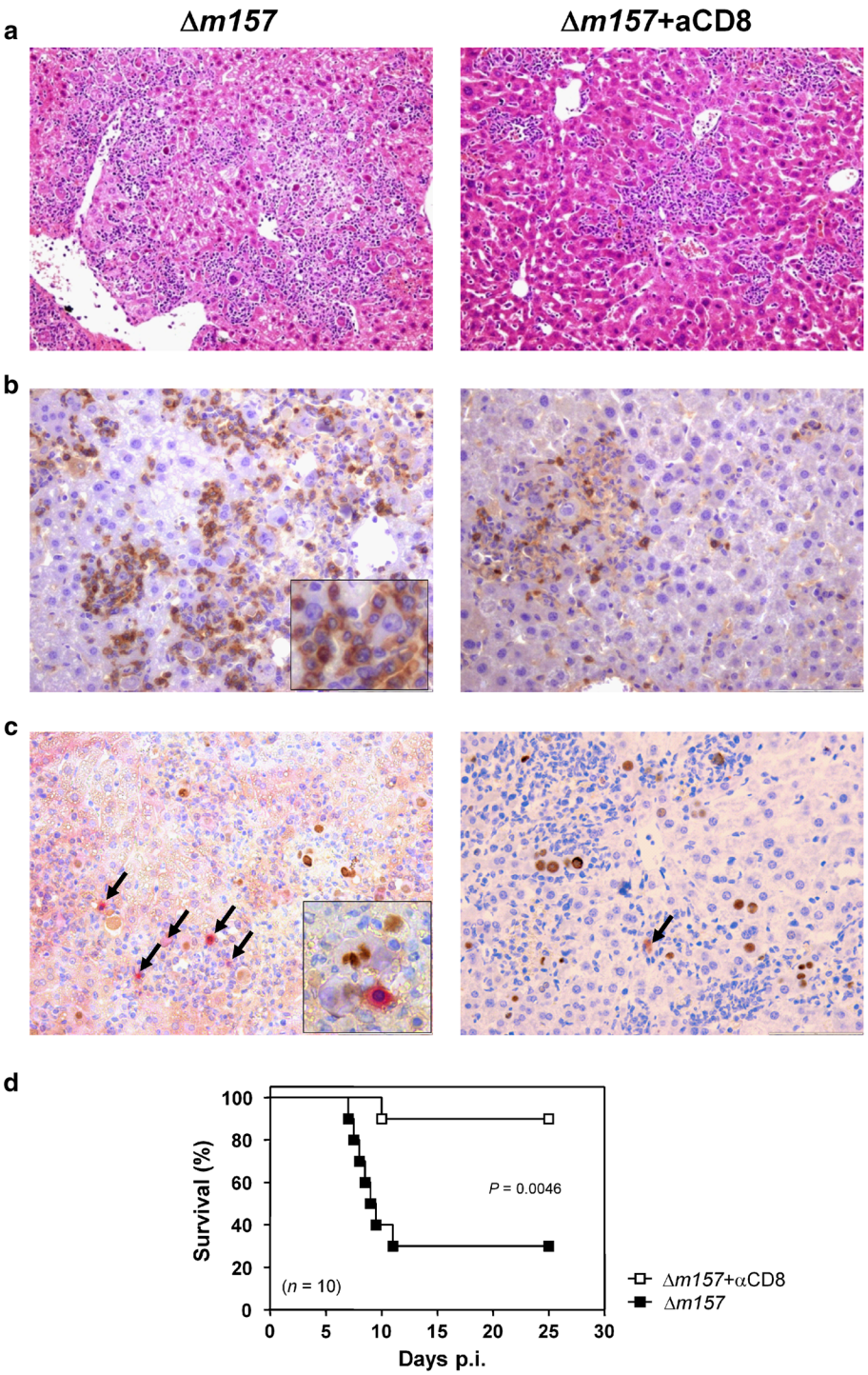

Fig. 5 The CD8$^+$ T cell depletion markedly reduced pathohistological liver lesions and improved survival of Prf1$^{-/-}$ mice infected with Δ*m157* MCMV. Prf1$^{-/-}$ mice were i.v. injected with 2 × 10^5 PFU of Δ*m157* MCMV, and indicated groups were treated with either PBS or depleting anti-CD8 mAbs on days 1 and 5 p.i. Mice were killed at day 7 p.i. **a** Representative paraffin-embedded liver sections analyzed for the pathohistological lesions are shown (H&E; ×20). **b** Immunohistochemistry staining of CD3$^+$ cells on paraffin-embedded liver sections visualized with diaminobenzidine (×40). **c** Double staining for MCMV protein IE1 (*brown*) and active caspase-3 (*red*) performed on paraffin-embedded liver sections. *Arrows* show cells positive for caspase-3 (×40). **d** Prf1$^{-/-}$ mice were i.v. injected with 2 × 10^5 PFU of Δ*m157* MCMV. On day 1 p.i. and subsequently every fifth day, mice were treated with either PBS or depleting anti-CD8 mAbs and monitored daily for survival. Data are representative from two independent experiments. *P* value was determined by the log-rank survival test

Prf1$^{-/-}$ mice infected with Δ*m157* MCMV after depletion of CD8$^+$ T cells show a reduced liver pathology and improved survival

In order to investigate whether the observed massive liver damage in Prf1$^{-/-}$ mice infected with the Δ*m157* virus could be a consequence of CD8$^+$ T cell function, we have analyzed the effect of CD8$^+$ T cell depletion on liver pathology. Depletion of CD8$^+$ T cells significantly reduced pathohistological lesions in liver of Prf1$^{-/-}$ mice on day 7 p.i. compared with the group of undepleted Δ*m157* MCMV-infected Prf1$^{-/-}$ mice (Fig. 5a, b). In addition, in the group of Δ*m157* MCMV-infected and CD8$^+$ T cell-depleted Prf1$^{-/-}$ mice, we have observed much less caspase-3-positive cells compared with the infected undepleted group (Fig. 5c). Double staining of infected tissue

for MCMV IE1 and caspase-3 demonstrated that cells undergoing apoptosis were mostly uninfected. Thus, this finding indicates an immunopathological liver damage by dysregulated $CD8^+$ T cells.

To further analyze the impact of an unleashed $CD8^+$ T cell response on the mortality rate of Δ*m157* MCMV infection in $Prf1^{-/-}$ mice, $CD8^+$ T cell depleted or undepleted Δ*m157* MCMV-infected $Prf1^{-/-}$ mice were monitored daily for survival (Fig. 5d). Although the depletion of $CD8^+$ T cells increased viral titers in the liver of Δ*m157* MCMV-infected $Prf1^{-/-}$ mice (Fig. 1c), the survival rate of these mice was higher than in the group of mice with preserved $CD8^+$ T cells (Fig. 5d). These results indicate that in the absence of perforin and of the specific NK cell activation, unleashed $CD8^+$ T cell response causes detrimental liver damage.

Discussion

We have investigated the role of perforin in NK cell regulation of $CD8^+$ T cell response to MCMV infection. The results of our previous study demonstrated an enhanced $CD8^+$ T cell response in mice infected with the virus, which fails to specifically activate NK cells [18]. Here we show that besides a specific activation through the Ly49H-m157 interaction, this immunoregulatory role of NK cells depends on perforin-mediated cytolytic mechanism. One of the major differences between normal C57BL/6 and $Prf1^{-/-}$ mice in virus control in spleen was that in the absence of perforin, $CD8^+$ T cells were more efficient if NK cells are specifically activated, suggesting that noncytolytic mechanisms contribute to the control of WT MCMV infection of $Prf1^{-/-}$ mice.

A previously published study indicated that perforin is essential in the control of MCMV infection in spleen, whereas the noncytolytic mechanism mediated by IFN-γ is responsible for the control of MCMV infection in the liver [42]. In contrast to these organ-specific differences of the control of MCMV infection, other groups demonstrated that NK cells use perforin and IFN-γ for antiviral control in both organs [43, 44]. Our results confirm and extend these observations, showing the importance of specific activation of NK cells in the control of MCMV infection in $Prf1^{-/-}$ mice. However, noncytolytic mechanisms failed to fully compensate for the lack of activation of NK cells via Ly49H-m157 interaction, resulting in high Δ*m157* viral titers in the spleen and liver of $Prf1^{-/-}$ mice on day 7 p.i. Unlike in the spleen of $Prf1^{-/-}$ mice, where $CD8^+$ T cells play an important role in the containment of WT MCMV infection, they have no role in the liver of the same mice. Yet, in the absence of specific NK cell activation, i.e., in $Prf1^{-/-}$ mice infected with the virus lacking m157, $CD8^+$ T cells took over the virus control, suggesting that noncytolytic compensatory mechanisms are operative only in the absence of a specific activation of NK cells.

Previous work in hematopoietic cell transplantation (HCT) models by Podlech and colleagues demonstrated that the depletion of $CD8^+$ but not $CD4^+$ T cells during immune reconstitution after HCT leads to a disseminated MCMV infection in almost all organs and to a high level of mortality [45, 46]. This model showed that $CD8^+$ T cells do not cause immunopathology, but actually prevent viral pathology. At first glance, our results in MCMV-infected $Prf1^{-/-}$ mice present an opposite scenario, since the depletion of $CD8^+$ T cells was found to be protective, indicating immunopathology rather than viral pathology as the cause of death. However, aberrant $CD8^+$ T cell response that causes liver immunopathology was observed only when specific NK cell activation and perforin were missing in infected mice. This is in line with previously published work showing that a higher cytokine production by expanded $CD8^+$ T cells caused severe immunopathology and lethality of $Prf1^{-/-}$ mice [47, 48]. Moreover, the present study indicates that the activated $CD8^+$ T cells in liver of $Prf1^{-/-}$ mice infected with Δ*m157* MCMV can induce apoptosis of uninfected parenchymal cells in $CD8^+$ T cell-dependent manner. Specifically, the depletion of $CD8^+$ subset significantly prevented liver damage and the frequency of apoptotic cells in these mice, in spite of an increased virus titer, suggesting that tissue damage was mostly a consequence of an immunopathological reaction, rather than a cytolytic effect of the virus. The fact that pathohistological lesions are less pronounced in mice infected with a virus able to activate NK cells indicates a critical role of specific NK cell activity in the prevention of unleashed $CD8^+$ T cell response in the acute phase of infection. We speculate that an unleashed proliferation and activation of $CD8^+$ T cells and a massive infiltration of liver are accompanied by extensive cytokine production, which can be deleterious for healthy cells, but at the same time could still have antiviral capacity. The depletion of $CD8^+$ T cells in these mice eliminates the major source of cytokines and therefore improves the survival in spite of a significantly higher virus load. Using similar model of MCMV infection in $Prf1^{-/-}$ mice, Lee and colleagues showed that NK cells, stimulated through the Ly49H receptor, produce IL-10, which limits the magnitude of $CD8^+$ T cell response [17].

In conclusion, our data further emphasize that NK cells influence the induction and maturation of the $CD8^+$ T cell response to CMV. For this homeostatic regulation of antiviral $CD8^+$ T cell response, perforin-dependent cytolytic mechanism and specific activation of NK cells are required. In their absence, a massive accumulation of activated virus-specific $CD8^+$ T cells occurs, resulting in

liver immunopathology. More detailed studies are needed to reveal the ultimate mechanism of liver damage in this model.

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