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Research Article

NCR1-deficiency diminishes the generation of protective murine cytomegalovirus antibodies by limiting follicular helper T-cell maturation

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NKp46/NCR1 is an activating NK-cell receptor implicated in the control of various viral and bacterial infections. Recent findings also suggest that it plays a role in shaping the adaptive immune response to pathogens. Using NCR1-deficient (NCR1^{gfp/gfp}) mice, we provide evidence for the role of NCR1 in antibody response to mouse cytomegalovirus infection (MCMV). The absence of NCR1 resulted in impaired maturation, function and NK-cell migration to regional lymph nodes. In addition, CD4⁺ T-cell activation and follicular helper T-cell (Tfh) generation were reduced, leading to inferior germinal center (GC) B-cell maturation. As a consequence, NCR1^{gfp/gfp} mice produced lower amounts of MCMV-specific antibodies upon infection, which correlated with lower number of virus-specific antibody secreting cells in analyzed lymph nodes.

Keywords: Antiviral antibodies · Follicular helper T cells · Germinal center B cells · NCR1 receptor · NK-cell immunoregulation · Viral infection



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Introduction

The major goal of most of the currently used antimicrobial vaccine approaches is to induce long-lived protective immunity based on antibodies. B-cell maturation to long-lived B cells or memory cells which are able to produce high affinity antibodies is a multistep process which includes germinal center (GC) reaction and the help of different cell types populating distinct parts of the lymph node in a coordinated manner in both time and space [1]. T follicular helper (Tfh) cells are a highly specialized subset of the CD4⁺ T

cells which induce GC formation and are indispensable for B-cell maturation. Tfh-cell-mediated help to B cells supports several vital steps during B-cell maturation including: the proliferation and survival of B cells through CD40L-CD40 engagement and IL-21 production, plasma-cell differentiation, somatic hypermutation crucial for B-cell affinity maturation and class-switch recombination [2]. In addition, the expression of adhesion molecules on both Tfh and B cells ensures the longevity of Tfh cell - B cell interaction whereas chemoattraction of B cells by Tfh cells restricts their movement to the germinal center during

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maturation. The importance of Tfh cells in the formation of humoral response is further emphasized by the study of Locci et al. which revealed an increased expression of highly functional population of blood-derived PD-1⁺CXCR3⁻CXCR5⁺ memory Tfh cells in the rare individuals who generate broadly neutralizing antibodies against HIV [3]. A better understanding of the mechanisms by which Tfh cells control the GC reaction and subsequent generation of high-affinity antibodies is essential for designing novel antibody-based therapeutic and prophylactic approaches (including vaccines) aimed at combating microbial infections.

NK cells are innate cytotoxic lymphocytes well known for their essential role in the early defense against viral infections. Activation of NK cells is tightly regulated by a careful balance of signals from the activating and inhibitory receptors expressed on their cell surface [4]. Interaction of NK cells with dendritic cells (DCs) stimulates both cell types to produce cytokines which further enhance their maturation and activation [5]. Besides their well-recognized role in the control of virus infection, NK cells have been shown to possess immunoregulatory functions that can lead to induction or suppression of other immune cells depending on the nature of infection [6]. Recent studies using lymphocytic choriomeningitis virus (LCMV) model of chronic infection have shown an impaired generation of virus-specific long-lived B cells as a result of NK-cell mediated suppression of Tfh-cell activation [7, 8]. Nonetheless, despite large number of studies on this subject, the underlying mechanisms used by NK cells to modulate antibody response are still not elucidated.

NKp46/NCR1 is one of the activating NK-cell receptors which has been shown to be important in the control of various viral [9, 10] and bacterial infections [11]. NCR1 ligand(s) are expressed on DCs and macrophages thus underlining the importance of these receptor–ligand interactions for NK-cell activation during viral infection [12]. Also, recent studies suggested a role of this receptor in NK-cell mediated modulation of the adaptive immune response. Namely, in a mouse model deficient for IL-10 cytokine, NCR1 has been shown to be important for NK-DC crosstalk resulting in an efficient priming of mouse cytomegalovirus (MCMV)-specific CD4⁺ T cells [13].

Here we investigated the role of NK cells and NCR1 in the formation of humoral responses to MCMV. Our results show a higher frequency of CD11b^{hi}CD27^{low}-expressing NK cells in NCR1^{gfp/gfp} mice compared to control mice. Besides their terminal differentiation phenotype, these NK cells showed a reduced ability to migrate to peripheral lymph nodes. In addition, the frequency of CD103⁺ cDCs in lungs of infected NCR1^{gfp/gfp} mice as well as the number of CD44⁺CD62L⁻ effector memory CD4⁺ T cells (TEM) and Tfh cells found in mediastinal lymph nodes (MLN) was lower. Finally, we show that an impaired GC B-cell formation in MLN of infected NCR1^{gfp/gfp} mice results in a lower number of virus-specific antibody secreting cells (ASC) capable of producing virus-specific antibodies of lower affinity and protective capacity compared to control mice.

Results

Reduced antibody response to MCMV in NCR1-deficient mice

To investigate the antibody response to MCMV in NCR1-deficient mice, we have infected BALB/c and NCR1^{gfp/gfp} mice (in which the gene encoding for NCR1 receptor is replaced with a green fluorescent protein (gfp) reporter cassette [9]) and have examined the presence of MCMV-specific antibodies in their sera 14 days post infection (p.i.) by ELISA. Compared to BALB/c mice, NCR1^{gfp/gfp} mice had a significantly lower amount of MCMV-specific antibodies, assessed as either total IgG or IgG2a and IgG1 isotypes (Fig. 1A–C). The observed difference in the antibody production was triggered by the infection as the analysis of total antibody production in the sera of uninfected mice showed no difference between the NCR1-deficient and WT control mice (Supporting Information Fig. 1A). MCMV infection directed the antibody response toward the IgG2a isotype (Supporting Information Fig. 1B) while the total IgG1 antibody production (Supporting Information Fig. 1C) was retained on the same level as in uninfected mice. Next, we used the same sera to perform the *in vitro* neutralization assay on BALB/c mouse embryonic fibroblast (MEF). Sera from NCR1^{gfp/gfp} mice attained a significantly lower neutralization capacity compared to sera derived from control BALB/c mice (Fig. 1D). To assess the protective capacity of virus-specific antibodies *in vivo*, we have used BALB/c newborn mice infected with MCMV within 24 h after birth. Three days later they have received sera from MCMV-infected adult BALB/c, NCR1^{gfp/+} or NCR1^{gfp/gfp} mice, or sera from uninfected BALB/c mice. Virus titers measured in spleen, lungs and liver 8 days p.i. revealed a lower protective capacity of sera derived from infected NCR1^{gfp/gfp} mice while sera derived from NCR1^{gfp/+} mice exhibited an intermediate phenotype (Fig. 1E). To differentiate whether the obtained difference in the protective capacity is a consequence of the lower quality of antibodies in the serum of NCR1-deficient mice or the lower amount of virus-specific antibodies was driving its poorer protection, the experiment was repeated but this time the antibody concentration in sera was determined and mice have received the same amount of corresponding antibodies. The same results were obtained as in the previous experiment indicating a poorer quality of virus-specific antibodies coming from NCR1^{gfp/gfp} mice (data not shown). Thus, the lack of NCR1 resulted not only in a lower abundance of MCMV-specific antibodies but also in their inferior protective capacity compared to NCR1-sufficient mice, suggesting an important role of NCR1 in formation of antibody response.

NK cells and CD4⁺ T cells have essential role in control of MCMV in lungs of infected mice

The analysis of viral titers at early days upon MCMV infection have shown no difference between NCR1^{gfp/gfp}, NCR1^{gfp/+} and WT mice, suggesting that NCR1 is dispensable in the early

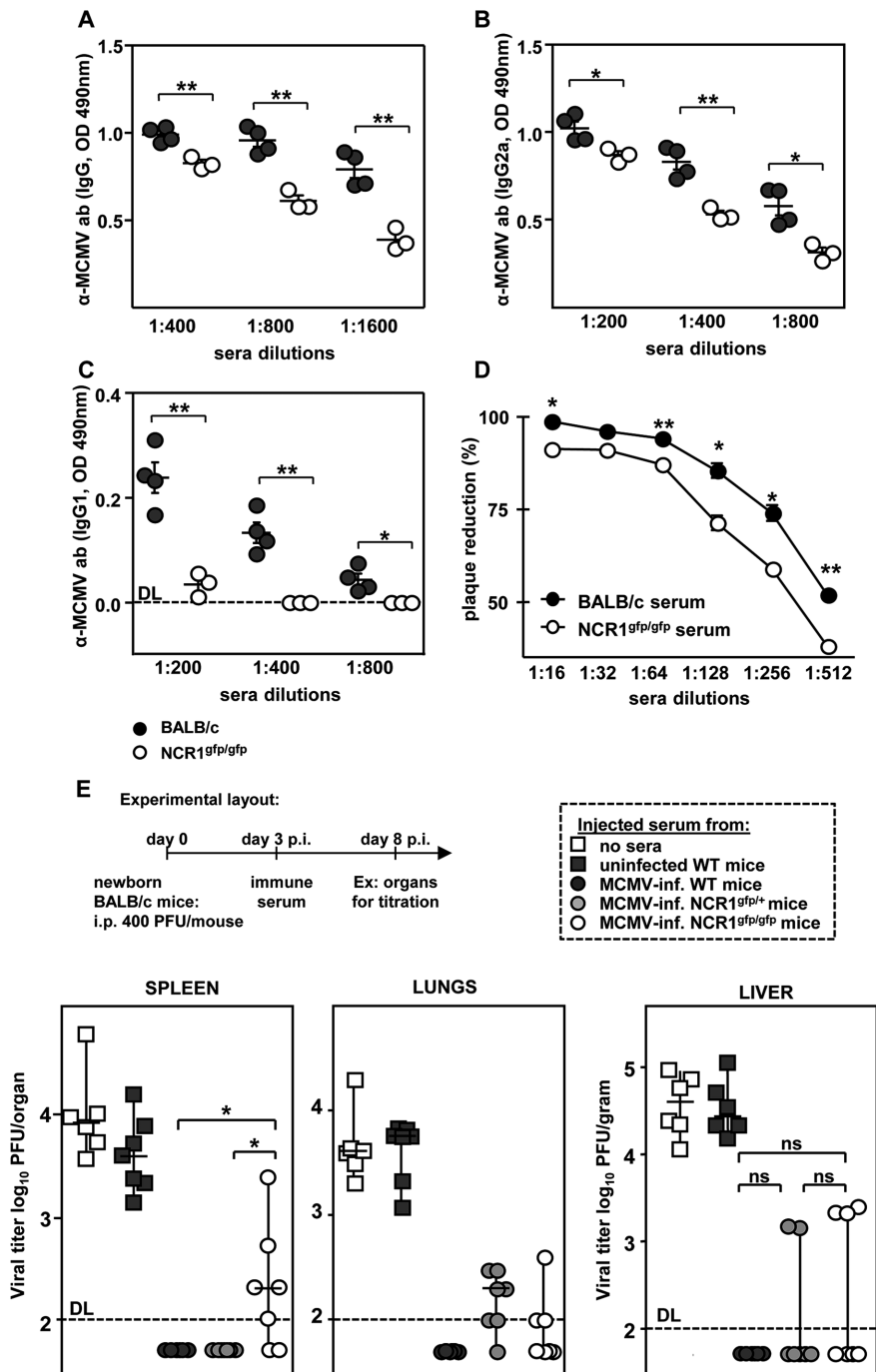


Figure 1. Inferior antibody response to MCMV in NCR1-deficient mice. BALB/c and NCR1^{gfp/gfp} mice were i.p. injected with MCMV. Mice were sacrificed 14 days p.i. and sera were collected for analysis of MCMV-specific antibodies (ab) using the ELISA assay. (A–C) To detect various antibody isotypes, we have used a corresponding secondary ab to IgG (A), IgG2a (B) or IgG1 (C). Results obtained from the ELISA assay are presented in OD values measured at 490 nm. (D) Immune sera collected from MCMV-infected adult BALB/c mice and NCR1^{gfp/gfp} mice 14 days p.i. were used for the neutralization assay. Results are presented as the % of plaque reduction following the infection of BALB/c MEF cells with MCMV preincubated with aforementioned sera and optimal dilution of the rabbit complement. (E) Newborn BALB/c mice were i.p. injected with MCMV within 24 h upon birth. 3 days p.i. pups received immune serum collected from adult uninfected BALB/c mice, MCMV-infected BALB/c, NCR1^{gfp/+} mice or NCR1^{gfp/gfp} mice. One group of infected pups did not receive any sera and served as control. 8 days p.i. pups were sacrificed and viral titers in the spleen, lungs and liver were determined using a standard plaque assay. Data in (A–D) are shown as mean + SEM of 3 or 4 mice per group. In (E) titers of individual mice (circles or squares; 6 or 7 mice per group) and median values (horizontal bars) are shown. The significant differences between tested groups were calculated by the unpaired two-tailed Student's t-test in (A–D) and by two-tailed Mann–Whitney u test in (E). **p* < 0.05, ***p* < 0.01. All the data in Fig. 1 are representative of three independent experiments with three to seven mice per group in a single experiment.

control of MCMV infection [14]. In Figure 1 we show that NCR1 is important in the later stages of MCMV infection when the antibody response to MCMV is formed. Therefore we have investigated how the lack of this receptor reflects on virus control 14 days p.i. We have infected BALB/c and NCR1^{gfp/gfp} mice and assessed viral titers in different organs (Fig. 2A and data not shown). Virus titer measured in lungs 14 days p.i. showed impaired MCMV control in NCR1^{gfp/gfp} mice. Depletion of either NK cells or CD4⁺ T cells resulted in the abolishment of the titer difference, revealing the crucial role of both NK cells and CD4⁺ T cells in the observed phenotype. These results have led us to address the kinetics of

CD4⁺ T cells in lungs of infected mice. Analysis of CD4⁺ T-cell kinetics revealed a significant difference in the number of total CD4⁺ T cells populating the lungs of MCMV-infected NCR1^{gfp/gfp} mice compared to WT control (Fig. 2B). Furthermore, we detected a lower number of CD44⁺CD62L⁻ effector memory CD4⁺ T cells (TEM) in NCR1-deficient mice (Fig. 2C). Finally, the stimulation of lymphocytes derived from lungs of infected NCR1^{gfp/gfp} and control mice with peritoneal macrophages pre-treated with MCMV-infected MEF lysates revealed a lower frequency and absolute number of IFN- γ ⁺ MCMV-specific CD4⁺ T cells in NCR1^{gfp/gfp} mice (Fig. 2D). These findings clearly demonstrate the significance of

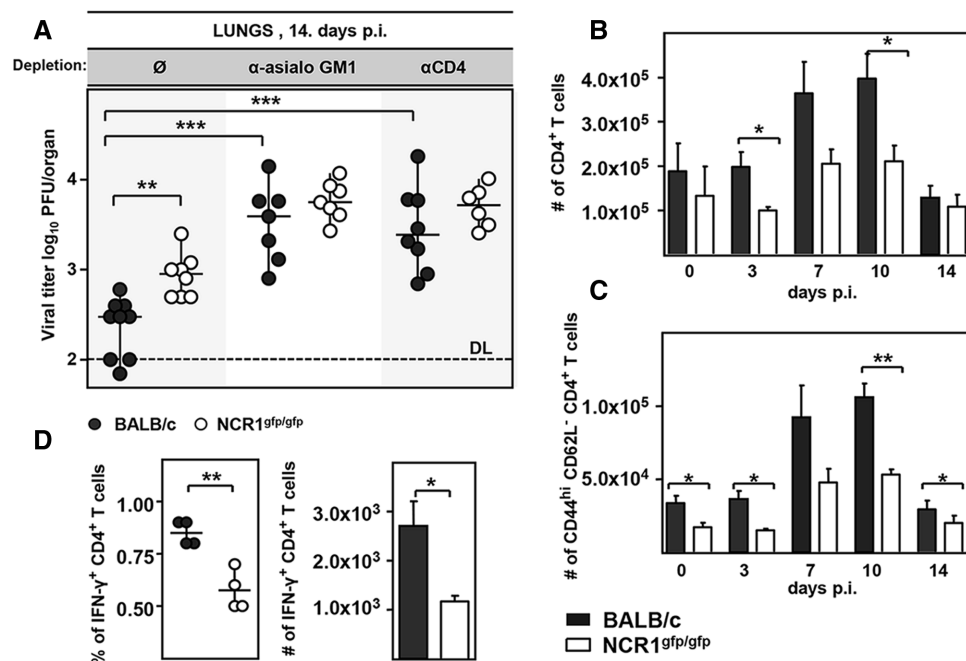


Figure 2. NK cells and CD4⁺ T cells have essential role in control of MCMV in lungs of infected mice. BALB/c and NCR1^{gfp/gfp} mice were i.p. injected with MCMV. (A) Depletion of NK cells and CD4⁺ T cells were performed on days 0, 5 and 10 p.i. Mice were sacrificed 14 days p.i. and viral titer in lungs were determined by a standard plaque assay. (B–C) Lymphocytes were isolated from perfused lungs and the CD4⁺ T-cell response was followed during the indicated time points. The graph represents the total number of CD4⁺ T cells (B) and CD44^{hi}CD62L⁻ CD4⁺ T cells (C). (D) Lymphocytes were isolated from perfused lungs at day 7 p.i. and stimulated o.n. using BALB/c PECs previously incubated with mock or MCMV-infected BALB/c MEF lysates. CD4⁺ T cells were monitored for IFN- γ production by intracellular staining. Frequency of IFN- γ ⁺ virus-specific CD4⁺ T cells (left) and number of IFN- γ ⁺ virus-specific CD4⁺ T cells (right) is shown. In (A) titers of individual mice (circles; 6–9 mice per group) and median values (horizontal bars) are shown. Data in (B–D) are shown as mean + SEM of 3 or 4 mice per group. The significant differences between the tested groups were calculated by two-tailed Mann–Whitney U test in (A) and by unpaired two-tailed Student's t-test in (B–D). * p < 0.05, ** p < 0.01, and *** p < 0.001. Data in (A) represent the pool of two experiments, while data from (B–D) are representative of two or three (D) independent experiments with 3–9 mice per group in a single experiment.

NCR1 in the formation of CD4⁺ T-cell response during MCMV infection.

NCR1^{gfp/gfp} mice have lower number of NK cells and DCs in lungs early days p.i

We have shown the importance of NCR1 in activation of virus-specific CD4⁺ T cells and the production of highly protective MCMV-specific antibodies. Having in mind that NCR1 is almost exclusively expressed on NK cells [15], we have tested how the lack of this receptor impact NK cells during the infection. The analysis of NK cells in the lungs 3 days p.i. revealed a lower number of NK cells in NCR1^{gfp/gfp} mice (Fig. 3A). Furthermore, NCR1^{gfp/gfp} mice also had a lower frequency of IFN- γ -producing NK cells (Fig. 3B). The maturation profile of NK cells revealed a lower frequency of CD11b^{hi}CD27^{hi} NK cells in NCR1^{gfp/gfp} mice, but a higher frequency of CD11b^{hi}CD27^{low} NK cells which corresponds to a more mature NK-cell phenotype (Fig. 3C). This finding was very important as CD11b^{hi}CD27^{hi} NK cells exhibit a greater responsiveness when cultured in vitro with DCs in contrast to terminally mature CD11b^{hi}CD27^{low} NK cells which show reduced responsiveness [16]. NCR1-deficiency affected also the total number of cDCs in lungs of infected mice. Namely, NCR1^{gfp/gfp} mice

had a lower number of cDCs in lungs 3 days p.i. (Fig. 3D). The frequency of migratory CD103⁺ cDCs, cells which play a role in antigen presentation and activation of both CD4⁺ and CD8⁺ T cells in regional lymph nodes [17, 18], was also lower in NCR1^{gfp/gfp} mice (Fig. 3E). On the other hand, NCR1^{gfp/gfp} mice had a higher frequency of CD103⁻CD11b⁺ cDCs in the lungs (Fig. 3F). The lower frequency of CD103⁺ cDCs and the lower total number of cDCs in lungs of NCR1^{gfp/gfp} mice could explain the lower frequency of IFN- γ ⁺ MCMV-specific CD4⁺ T cells observed in lungs 7 days p.i. Namely, CD103⁺ cDCs were shown by other authors to elicit IFN- γ production by CD4⁺ T cells, thus promoting the Th1 immune response [19]. Overall, these results reveal enhanced NK-cell maturation and decreased IFN- γ production by these cells in NCR1-deficient mice. The weaker NK-cell response in NCR1^{gfp/gfp} mice leads to a reduced number of cDCs and an impaired activation of virus-specific CD4⁺ T cells in lungs of infected mice.

Lower expression of CXCR3 chemokine receptor on NK cells from NCR1^{gfp/gfp} mice

In order to have an impact on the formation of B-cell response and antibody production, NK cells need to be in the right place at the right time engaging the cDCs and CD4⁺ T cells. Thus, we have

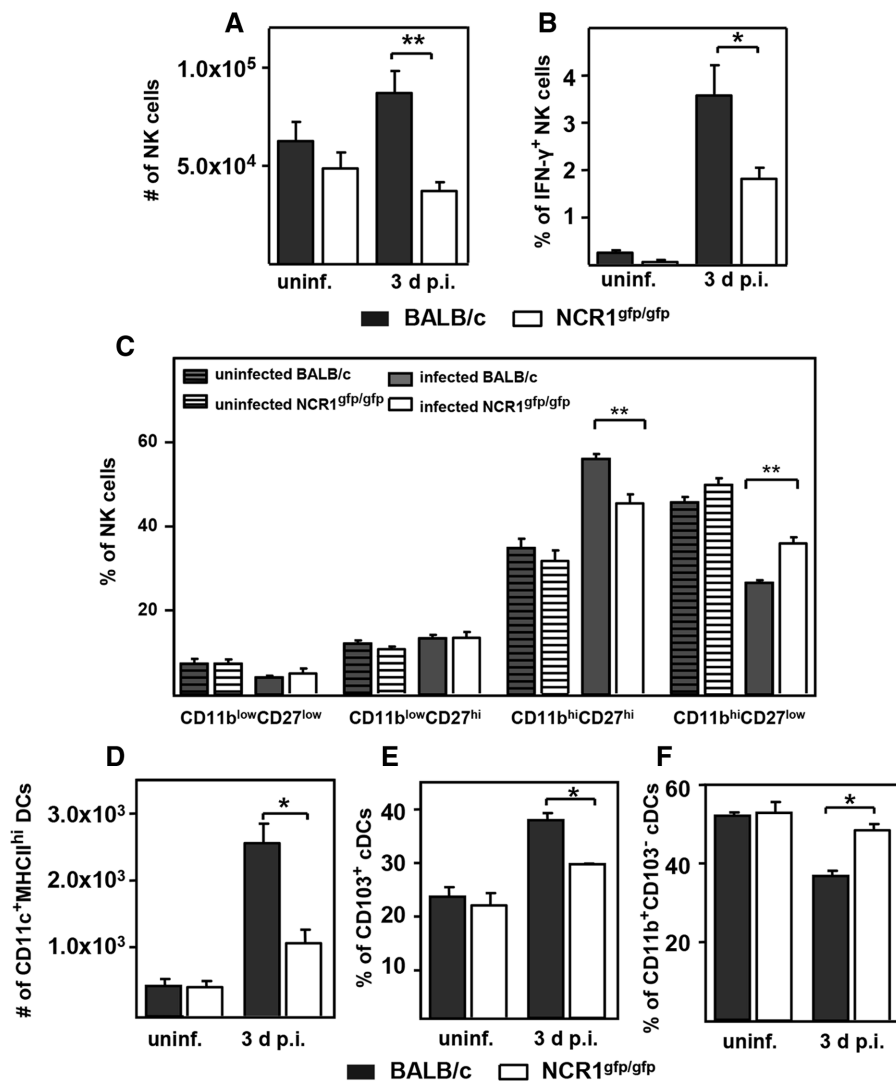


Figure 3. NCR1^{gfp/gfp} mice have a lower number of NK cells and DCs in lungs on early days p.i. BALB/c and NCR1^{gfp/gfp} mice were i.p. injected with MCMV. (A–C) Lymphocytes were isolated from perfused lungs of uninfected and infected mice 3 days p.i. and NK cells were analyzed. NK gate was set on NKp46⁺CD3⁻CD19⁻ or GFP⁺CD3⁻CD19⁻ cells. Total number of NK cells (A), the frequency of IFN-γ-producing NK cells (B) and expression of CD11b and CD27 molecules on NK cells are shown (C). (D–F) Lymphocytes were isolated from perfused lungs of uninfected and infected mice 3,5 days p.i. and stained for cDCs. Gate was set on CD3⁻CD19⁻CD11c⁺MHCII^{hi} cells. Total number of CD11c⁺MHCII^{hi} DCs in lungs (D), the frequency of CD103⁺ cDCs (E) and CD11b⁺CD103⁻ cDCs (F) were measured. Data are shown as mean + SEM of 3 or 4 mice per group. The significant differences between the tested groups were calculated by unpaired two-tailed Student's t-test. **p* < 0.05, ***p* < 0.01. Data in (A, B) are pooled from two experiments, while data from (C–F) are representative of two independent experiments with three or four mice per group in a single experiment.

explored the number of NK cells in MLN at an early time point p.i. when these interactions do take place. Our results showed a significantly lower number of NK cells in MLN of NCR1^{gfp/gfp} mice 1.5 days p.i. (Fig. 4A). Furthermore, the frequency of IFN-γ⁺ NK cells was also lower in NCR1^{gfp/gfp} mice (Fig. 4B). In order to examine this finding in more detail, we have followed the expression of CXCR3 chemokine receptor on the surface of NK cells. The expression of CXCR3 chemokine receptor was shown to be an important signal for NK-cell migration to an adjacent draining lymph node [20]. We have observed a lower frequency of CXCR3⁺ NK cells 1.5 day p.i. in NCR1^{gfp/gfp} mice (Fig. 4C), which could potentially explain their inferior migration to lymph nodes. The lower frequency of CXCR3⁺ NK cells correlated with the terminally mature phenotype of NK cells observed in the lungs (Fig. 3C). To confirm that the observed differences are not due to some unspecific differences between BALB/c and BALB/c NCR1^{gfp/gfp} mice we used littermate mice generated by crossing of BALB/c NCR1^{gfp/+} mice. Significantly lower frequency of CXCR3⁺ NK cells in NCR1^{gfp/gfp} mice were found in all examined organs 24 h p.i. (Fig. 4D). In addition, higher frequency of CD11b^{hi}CD27^{low}

NK cells was found in NCR1^{gfp/gfp} mice. To provide the evidence that the lower expression of CXCR3 is intrinsic property of NK cells from NCR1^{gfp/gfp} mice, we performed bone marrow chimera experiment (Fig. 4E). In short, bone marrow cells have been isolated from WT and NCR1^{gfp/gfp} mice and adoptively transferred in irradiated NCR1^{gfp/gfp} mice. After 8 weeks, FACS analysis was performed on the splenic cells. The results again showed lower frequency of CXCR3⁺ and higher frequency of CD11b^{hi}CD27^{low} NCR1-deficient NK cells compared to their WT counterparts in chimeric mice confirming our original finding. Finally, we have treated BALB/c mice with anti-CXCR3 blocking antibody prior to MCMV infection to formally show that CXCR3 receptor is important for the NK-cell migration to the lymph nodes (Fig. 4F). The results demonstrated that the expression of CXCR3 on NK cells is essential for NK-cell migration to MLN in MCMV-infected mice as the number of NK cells in the anti-CXCR3 ab- treated group dramatically dropped compared to untreated BALB/c mice, reaching the level of NK-cell number found in MLN of uninfected mice. On the other hand, blocking the CXCR3 receptor did not have an impact on the number

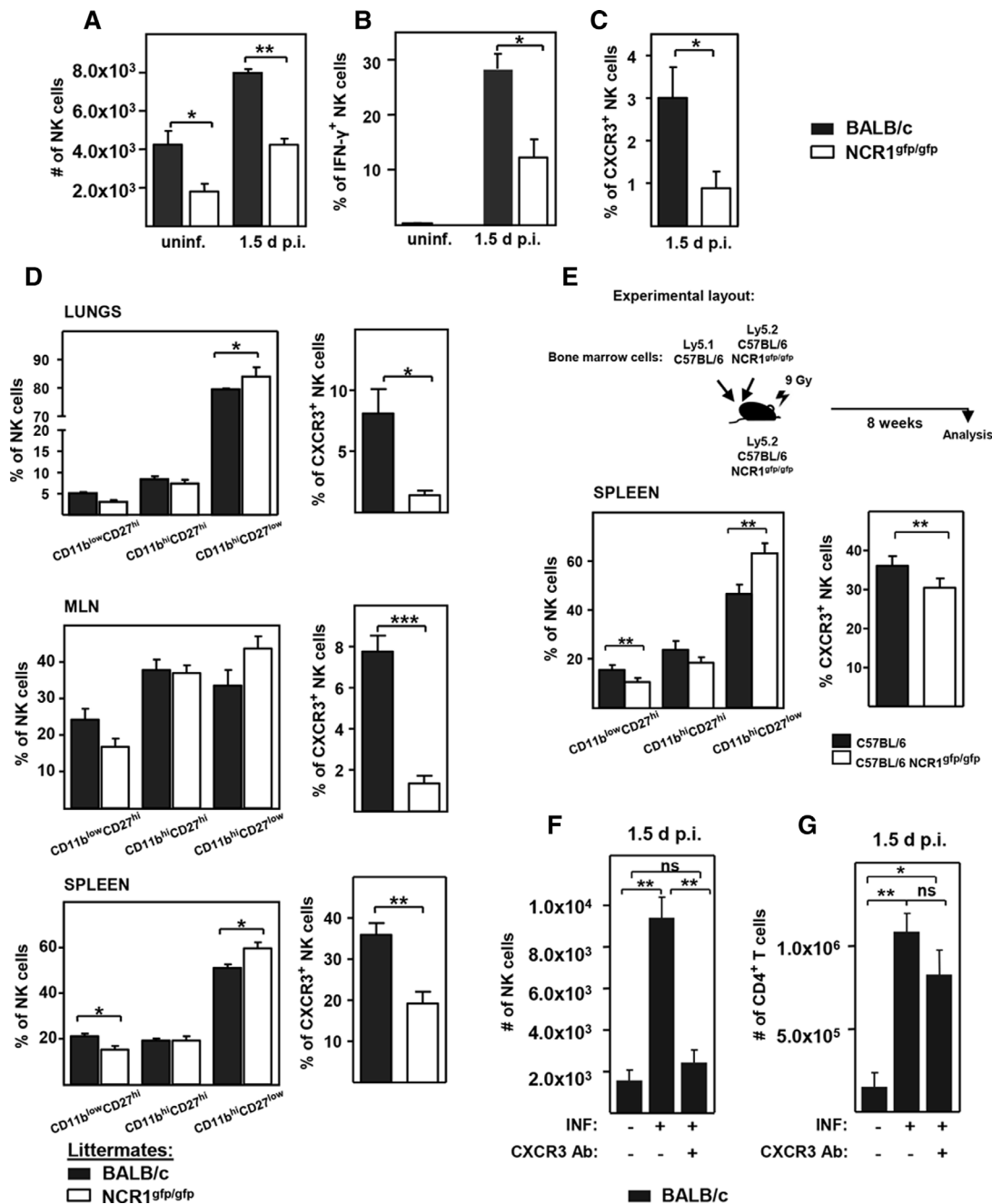


Figure 4. A lower expression of CXCR3 chemokine receptor on NK cells in NCR1^{gfp/gfp} mice results in a lower number of NK cells in MLN. BALB/c and NCR1^{gfp/gfp} mice were i.p. injected with MCMV. (A–C) Lymphocytes were isolated from MLN of uninfected and infected mice 1.5 days p.i. and stained for NK cells. NK gate was set on Nkp46⁺CD3⁻CD19⁻ or GFP⁺CD3⁻CD19⁻ cells. The total number of NK cells in MLN (A), the frequency of IFN-γ-producing NK cells in MLN (B) and the frequency of CXCR3⁺ NK cells from total NK cells present in MLN 1.5 day p.i. (C) are shown. (D) BALB/c WT and NCR1^{gfp/gfp} littermates were i.p. injected with MCMV. 24 h p.i. cells were isolated from lungs, MLN and spleen and stained for NK cells. Expression of CD11b and CD27 molecules on NK cells (left) and the frequency of CXCR3⁺ NK cells from the total NK cells present (right) are shown. (E) Bone marrow cells have been isolated from C57BL/6 (CD45.1⁺) and NCR1^{gfp/gfp} (CD45.2⁺) mice and adoptively transferred in 9 Gy irradiated NCR1^{gfp/gfp} mice. After 8 weeks, cells were isolated from the spleen of the treated mice and stained for NK cells. Gate was on (CD45.1⁺/CD45.2⁺)CD3⁻CD19⁻ (Nkp46⁺/gfp⁺). Expression of CD11b and CD27 molecules on NK cells (left) and the frequency of CXCR3⁺ NK cells from total NK cells present in spleen (right) are shown. (F–G) BALB/c mice were i.p. injected with MCMV or left uninfected. One infected group of mice also received αCXCR3 blocking ab prior to infection. Lymphocytes were isolated from MLN 1.5 days p.i. and stained for NK cells and CD4⁺ T cells. Graphs represent the total number of NK cells (F) and CD4⁺ T cells (G) in MLN. Data are shown as mean + SEM of 3 mice per group (in A, B, F, G), 6 or 7 mice per group (in C) or 5 mice per group (in D and E). The significant differences between the tested groups were calculated by unpaired two-tailed Student's t test (except for data shown in E where two-tailed Mann–Whitney U test was used). **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Data in (A, B, F, G) are representative of two independent experiments with three mice per group in a single experiment, data in (C) represent the pool of two experiments with —three to four mice per group in a single experiment, while data in (D and E) are from a single experiment.

of CD4⁺ T cells in MLN 1.5 day p.i. (Fig. 4G). In summary, we showed that lower CXCR3-dependant migration of NK cells to MLN of NCR1^{gfp/gfp} mice upon MCMV infection resulted in a lower number of these cells in the analyzed lymph nodes. This finding could provide an explanation for a decreased activation of CD4⁺ T cells leading to inferior antibody response to MCMV found in these mice.

Lower frequency of Tfh CD4⁺ cells leads to impaired GC response in lymph nodes of NCR1^{gfp/gfp} mice

Recent studies placed Tfh CD4⁺ cells at the forefront as an essential helper cell population for B-cell activation inside the follicles and their successful differentiation to long-lived plasma cells or memory B cells upon the formation of germinal centers [2]. To determine the role of NCR1 in antibody response to MCMV in more detail, we have assessed the frequency of PD1⁺ICOS⁺ Tfh CD4⁺ cells and GL7⁺ GC B cells in MLN of NCR1^{gfp/gfp} and control BALB/c mice 7 days p.i. when the establishment of mature GC is expected. NCR1^{gfp/gfp} mice had a lower frequency and total number of Tfh CD4⁺ cells in MLN (Fig. 5A–B). Moreover, NCR1^{gfp/gfp} mice also had a lower frequency and total number of GC B cells in MLN (Fig. 5D and E). The frequency of mature, isotype switched, B cells was also lower in NCR1^{gfp/gfp} mice (Fig. 5C).

GC reaction is considered to be a crucial process for the establishment of long-lived plasma cells [1]. Since we have observed a lower formation of GC B cells in NCR1-deficient mice, we wanted to determine if the number of MCMV-specific ASCs will be also affected by the absence of the NCR1 receptor. The ELISPOT assay against MCMV-infected MEF lysate showed a significantly lower number of anti-MCMV-ASCs in MLN of NCR1^{gfp/gfp} mice compared to control mice 14 days p.i. (Fig. 5F). Another indication of a successful GC reaction is the generation of highly specific and affinity matured antibodies in a process known as somatic hypermutation of immunoglobulin sequences [1]. In order to distinguish between low-affinity and high-affinity antibodies, we have performed the avidity assay using urea as a chaotropic agent with sera collected from infected NCR1^{gfp/gfp} and BALB/c mice 14 days p.i. (Fig. 5G). Our results revealed a significantly lower avidity index of anti-MCMV-antibodies derived from sera of NCR1^{gfp/gfp} mice compared to sera from WT (control) mice. These results, together with an inferior neutralization capacity of the sera from NCR1^{gfp/gfp} mice shown in Fig. 1D, directly point toward an impaired GC response in NCR1-deficient mice.

Blocking of CXCR3 receptor results in impaired GC B-cell formation and virus control in vivo

As shown in Fig. 4, the CXCR3 chemokine receptor is vital for the migration of NK cells to MLN. Next, we have analyzed if the blocking of CXCR3 receptor in the initial phase of infection will have an impact on the formation of GC B cells in the lymph node later on. We have observed a significantly lower frequency and total number of GC B cells in NCR1^{gfp/gfp} mice compared to control BALB/c

mice (Fig. 6A–D). The differences were abolished in the groups of mice which received anti-CXCR3 treatment. These results support our finding shown in Fig. 4 and indicate important role of CXCR3-dependant migration of NK cells for GC B-cell formation in the examined lymph nodes. To assess the importance of CXCR3-dependent migration of NK cells for the virus control in vivo, we have treated BALB/c and NCR1^{gfp/gfp} mice with anti-CXCR3 ab prior to MCMV infection and followed the viral titer in the lungs 14 days p.i. The virus titer in anti-CXCR3 ab-treated BALB/c mice reached the titer level found in NCR1^{gfp/gfp} mice pointing to a significant role of this chemokine receptor in the virus control in vivo (Fig. 6E).

Lower antibody titers in the sera of NCR1^{gfp/gfp} mice at later time points after infection

Finally, to assess the consequences of reduced activation of adaptive immune response found in NCR1^{gfp/gfp} mice upon MCMV infection, we examined the production of virus-specific antibodies at later time points, after resolution of primary infection. We found lower amount of virus-specific antibodies in the sera of NCR1^{gfp/gfp} mice 43 days p.i. compared to the antibody amounts found in the sera of WT control mice, assessed as either total IgG or IgG2a and IgG1 isotypes (Fig. 7A–C). ELISPOT analysis of virus-specific ASC cells 43 days p.i. revealed significantly lower number of these cells isolated from MLN of NCR1^{gfp/gfp} mice compared to the number of ASC found in WT controls (Fig. 7D). Furthermore, analysis of sera collected from WT and NCR1^{gfp/gfp} mice one year after primary infection again showed significantly lower amount of MCMV-specific antibodies in the sera of NCR1^{gfp/gfp} mice (Fig. 7E). Overall, our results indicate that the impaired activation of the adaptive immune response in the early phase of MCMV infection shown in NCR1-deficient mice, ultimately lead to inferior long term antibody production in these mice.

Discussion

Several key events must take place in a time coordinated manner to ensure that a high quality B-cell response will be developed. One of the first and most important events in this multistep process is the activation of B cells by antigen-presenting DCs coming from the periphery into the primary follicle of the regional lymph node [1]. This is the necessary signal for B cells to move towards the interfollicular zone where they could interact with activated CD4⁺ T cells. Likewise, activation of CD4⁺ T cells by migratory CD103⁺ cDCs is crucial for the migration of CD4⁺ T cells from the T cell zone to the interfollicular zone. The interaction between activated CD4⁺ T cells and B cells results in a full activation of B cells and the differentiation of CD4⁺ T cells into the Tfh cells, which is vital for the GC formation and the establishment of memory B cells or long-lived plasma cells capable of producing high-affinity antibodies.

Here, we have shown the high importance of NK cells and its activating receptor NCR1 during the formation of humoral

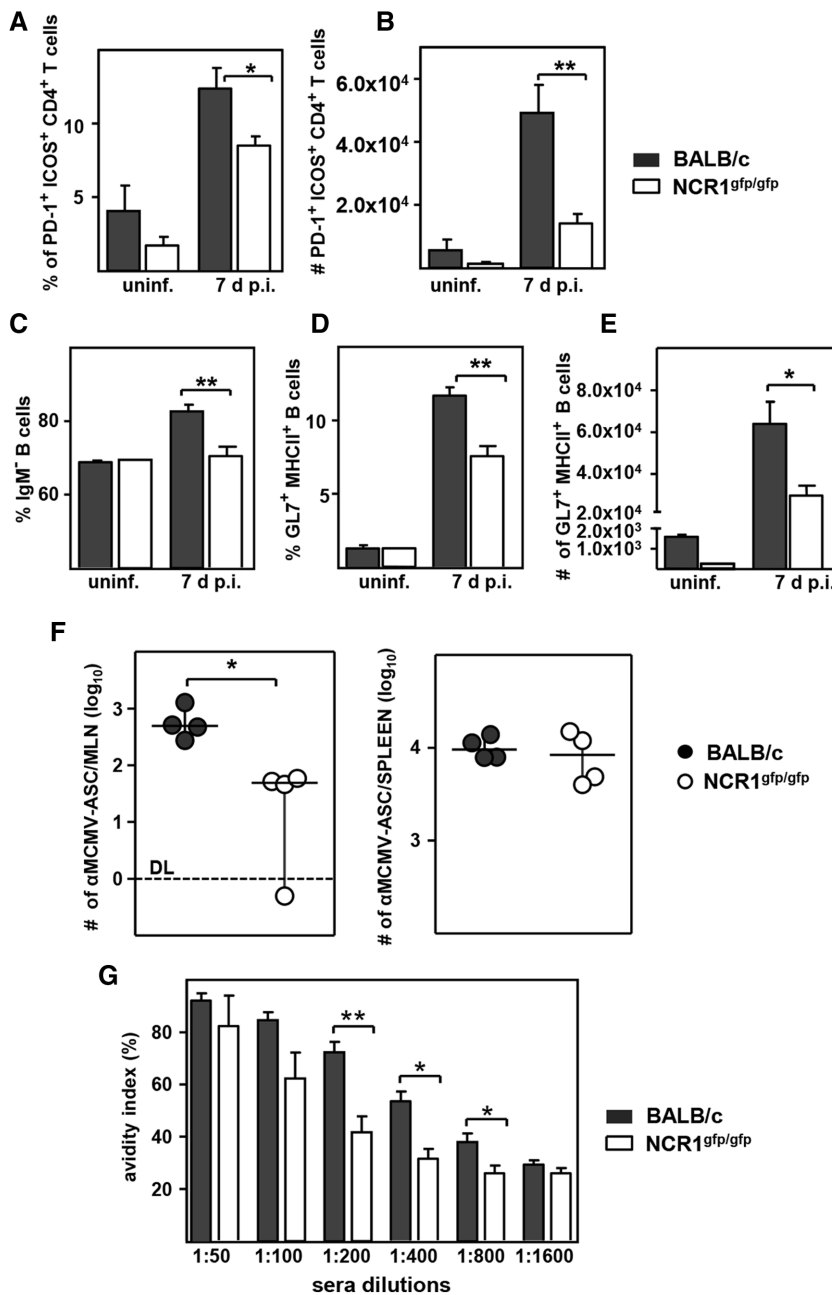


Figure 5. Abrogated frequency of follicular CD4⁺ T cells and germinal center B cells in MLN of NCR1^{gfp/gfp} mice. (A–E) BALB/c and NCR1^{gfp/gfp} mice were i.p. injected with MCMV. Lymphocytes were isolated from MLN of uninfected and infected mice 7 days p.i. and stained for different lymphocyte populations. (A and B) CD4⁺ T cells were analyzed for PD-1 and ICOS receptor expression (Tfh cells). Frequency of PD-1⁺ICOS⁺ CD4⁺ T cells from total CD4⁺ T cells in MLN 7 days p.i. (A) and total number of PD-1⁺ICOS⁺ CD4⁺ T cells in MLN 7 days p.i. in (B) are shown. (C–E) B cells were analyzed for maturation/class switching by following IgM expression (C), frequency and total number of germinal center B cells by MHCII and GL7 expression (D–E). (F) BALB/c and NCR1^{gfp/gfp} mice were i.p. injected with MCMV. 14 days p.i. lymphocytes were isolated from spleen and MLN of uninfected and infected mice and subjected to the ELISPOT assay. Number of MCMV-specific ASC in MLN and spleen of infected mice is shown. (G) Immune sera collected 14 days p.i. from MCMV-infected adult BALB/c mice and NCR1^{gfp/gfp} mice were used for avidity assay. Results obtained from avidity assay are presented as avidity index of MCMV-specific ab. In (F) individual mice (circles; 4 mice per group) and median values (horizontal bars) are shown. Data in (A–E) and (G) are shown as mean + SEM of 3 or 4 mice per group. The significant differences between the tested groups were calculated by two-tailed Mann–Whitney U test in (F) and by unpaired two-tailed Student's t-test in (A–E) and (G). **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Data in (A, B) are pooled from two experiments, data in (C–E, G) are representative of three independent experiments, while data from (F) are representative of two independent experiments with three to four animals per group in a single experiment.

response in MCMV infection. Our data have revealed a reduced frequency of migratory CD103⁺ cDCs and total number of cDCs in the lungs of infected NCR1^{gfp/gfp} mice. Subsequently, the number of Tfh cells and GC B cells in MLN of NCR1^{gfp/gfp} mice analyzed 7 days p.i. was lower compared to control mice. Detailed analysis of humoral response revealed lower number of virus-specific ASCs in MLN as well as lower amount of virus-specific antibodies in sera of NCR1^{gfp/gfp} mice upon infection. These antibodies appeared to be of lower affinity and possess an inferior protective capacity compared to antibodies retrieved from control mice, which directly points toward an impaired GC response in mice lacking the NCR1 receptor. Finally, analysis of antibody titers 1 year after primary infection showed significantly lower

amount of MCMV-specific antibodies in the sera of NCR1^{gfp/gfp} mice, revealing inferior long-term antibody production in these mice.

NK cells are effector lymphocytes recognized for their role in early, innate immune response against transformed or infected cells. Upon their activation, NK cells can act either directly via the formation of immunological synapses with the target cells, followed by its cytolytic activity [21, 22], or indirectly by expressing cytokines to influence the surrounding inflammatory milieu [23, 24]. Moreover, recent studies emphasize their essential immunoregulatory role on a wide range of immune cells during the formation of adaptive immunity [6]. Several studies have already shown positive effects of NK cells on the formation of humoral

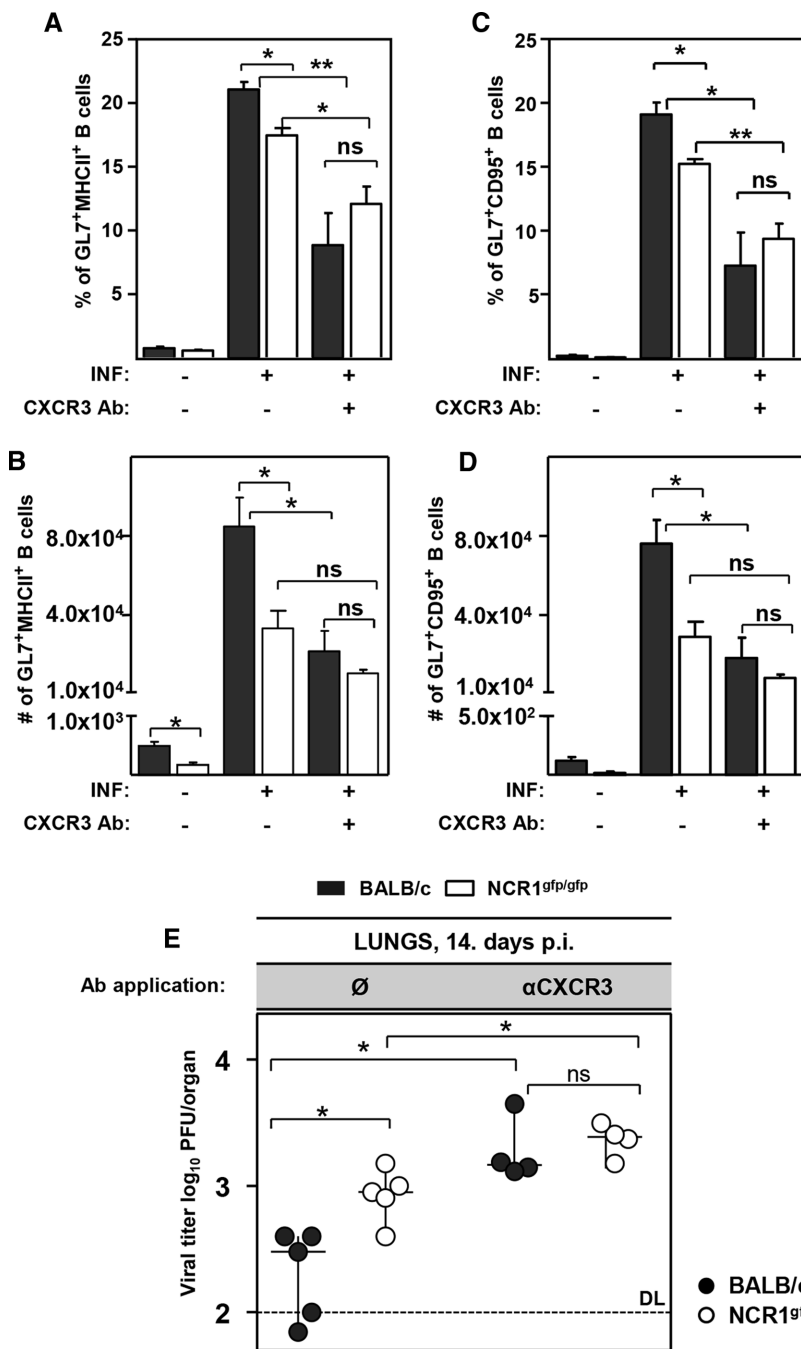


Figure 6. Blocking of CXCR3 receptor results in impaired GC B-cell formation and virus control in vivo. (A–D) BALB/c and NCR1^{gfp/gfp} mice were i.p. injected with MCMV or left uninfected. One infected group of mice was treated with αCXCR3 blocking ab prior to infection. Lymphocytes were isolated from MLN 6 days p.i. and stained for B cells. Frequency of GL7⁺MHCII⁺ B cells (A), total number of GL7⁺MHCII⁺ B cells (B), frequency of GL7⁺CD95⁺ B cells (C) and total number of GL7⁺CD95⁺ cells (D) are shown. (E) BALB/c and NCR1^{gfp/gfp} mice were i.p. injected with MCMV. One group of mice also received αCXCR3 blocking ab prior to infection. Mice were sacrificed 14 days p.i. and viral titer in lungs was determined by the standard plaque assay. Data in (A–D) are shown as mean + SEM of 3 or 4 mice per group. In (E) titers of individual mice (circles; 4 or 5 mice per group) and median values (horizontal bars) are shown. The significant differences between the tested groups were calculated by unpaired two-tailed Student's t test in (A–D) and by two-tailed Mann-Whitney U test in (E). **p* < 0.05. Data in (A–D) are representative of three independent experiments, while data from (E) are representative of two independent experiments with three to five animals per group in a single experiment.

response. NK cells were shown to promote IgG class switching and B cell proliferation indirectly by IFN- γ secretion [25], or directly through cell to cell contact [26]. In addition, direct NK cell-B cell contact was shown to result in an improved antigen presentation of B cells, which ameliorates B-cell interaction with CD4⁺ T cells [27]. Studies assessing NK cell immunoregulatory role in LCMV model are particularly interesting as NK cells do not seem to have any influence on the virus clearance in the early days of infection [28–30]. These studies once again emphasize the great complexity of NK-cell impact on T cells during viral infections spanning from beneficial to detrimental. Namely, study by

Rydzynski et al. using acute LCMV infection showed a perforin-dependent suppression of CD4⁺ T- and Tfh-cell activation by NK cells, which led to a weaker GC response and a diminished generation of virus-specific long-lived B cells [8]. In a similar study using acute LCMV model, NK-cell depletion did not have any influence on either CD4⁺ or CD8⁺ T-cell activation [31]. Increased CD4⁺ T-cell response could be visible only upon depletion of NK cells in $\beta 2m^{-/-}$ mice which would imply that the presence of MHC class I molecules inhibits the immunoregulatory function of NK cells. Furthermore, comparison between acute and chronic LCMV model in a study by Cook et al. revealed significant differences in

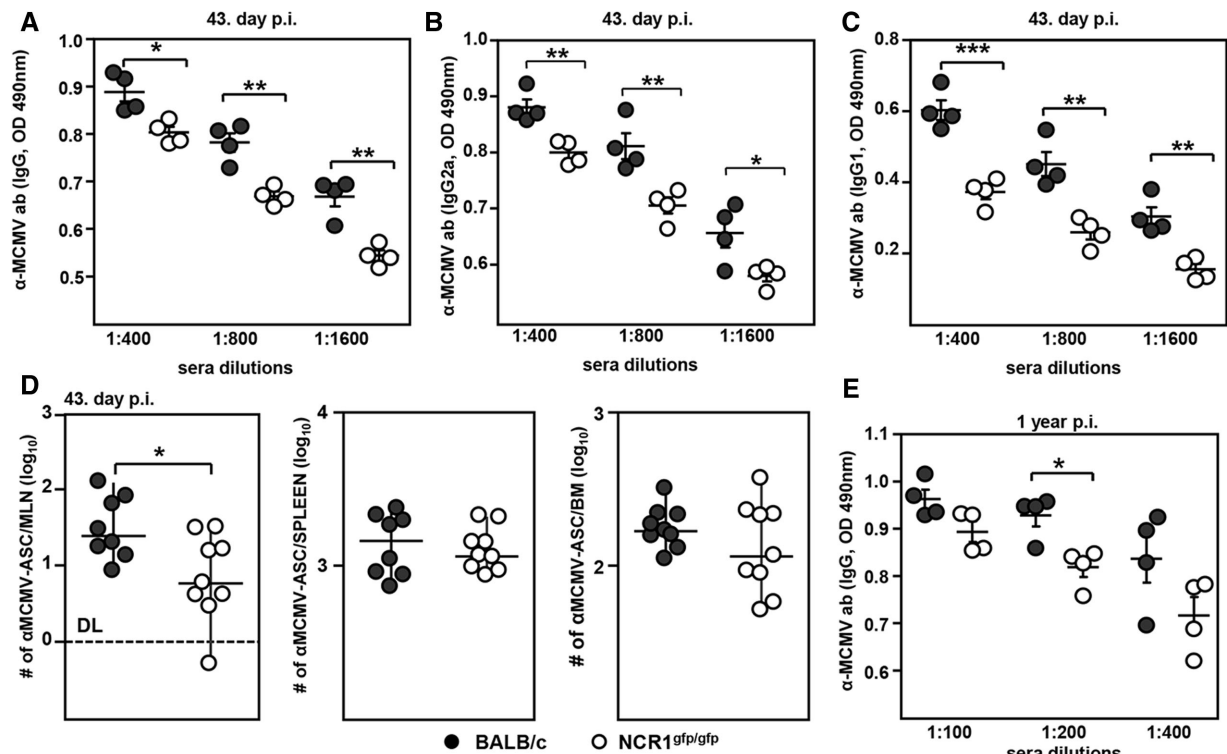


Figure 7. Impaired formation of humoral response results in lower antibody titers in sera of $NCR1^{gfp/gfp}$ mice at later time points after infection. (A–D) BALB/c and $NCR1^{gfp/gfp}$ mice were i.p. injected with MCMV. Mice were sacrificed 43 days p.i. and humoral response was analyzed. Sera were collected for analysis of MCMV-specific ab by ELISA. To detect various antibody isotypes, we have used a corresponding secondary ab to IgG (A), IgG2a (B) or IgG1 (C). Results obtained from the ELISA assay are presented in OD values measured at 490 nm. (D) Lymphocytes were isolated from MLN, spleen and bone marrow (BM) of uninfected and infected mice and subjected to the ELISPOT assay. Number of MCMV-specific ASC in MLN (left), spleen (middle) and BM (right) of infected mice is shown. (E) To analyze late virus-specific ab response, BALB/c and $NCR1^{gfp/gfp}$ mice were f.p. injected with MCMV. Mice were sacrificed one year after infection and sera were collected for the analysis of MCMV specific IgG ab by ELISA. Data in (A–C and E) are shown as mean + SEM of 4 mice per group. In (D) individual mice (circles; 8 or 9 mice per group) and median values (horizontal bars) are shown. The significant differences between the tested groups were calculated by unpaired two-tailed Student's *t*-test in (A–C and E) and by two-tailed Mann–Whitney U test in (D). **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Data in (A–C) are representative of three independent experiments with four animals per group in a single experiment. Data in (D) are pooled from two experiments. The data in (E) are from a single experiment.

eliciting humoral response depending greatly on the dose and the type of the infection [7]. In mice infected acutely with LCMV, levels of virus-specific antibodies reached their peak soon after the infection was resolved, while chronically infected mice showed continued increase of antibody levels exceeding the ones found after acute infection. Moreover, it has been shown that even though NK-cell depleted mice had increased Tfh-cell numbers in the early phase of chronic LCMV infection, the improved virus control in these mice led to a reduced Tfh-cell numbers and virus-specific antibody levels in comparison to control mice just 1 month p.i. Hence, NK-cell depletion had opposite effects on B-cell response over the course of the infection. It seems that the elevated virus load at later time point is needed in order for B cells to still receive antigen stimulation, whereas the controlled infection prevents long-term production of virus-specific antibodies. NK cells play an essential role in the early phase of MCMV infection by limiting viral burden through perforin-mediated cytotoxicity and IFN- γ release. However, the efficacy of NK cells in virus control varies between various mouse strains due to the differences in the composition of NK cell receptors. Viral immunosubversion of NK-cell response in MCMV-sensitive mouse strain delays viral clearance and results in

a higher viral load than in mice with a controlled infection. This inflammatory environment may very well be the reason why in our model, NK cells from BALB/c mice were induced to stimulate the formation of virus-specific T- and B-cell response. Moreover, analysis of antibody titers at later time points upon infection revealed higher amount of MCMV-specific antibodies in the sera of BALB/c mice compared to the values found in $NCR1^{gfp/gfp}$ mice 1 year after the primary infection has been resolved. Our results show that NCR1 is important for the regulation of adaptive immune response by NK cells. The analysis of the maturation profile of NK cells from lungs of infected $NCR1^{gfp/gfp}$ mice revealed a lower frequency of CD11b^{hi}CD27^{hi} NK cells, but a higher frequency of CD11b^{hi}CD27^{low} cells which corresponded to a more mature NK-cell phenotype. In addition, we have found lower frequency of IFN- γ -producing NK cells in lungs and MLN of infected $NCR1^{gfp/gfp}$ mice. Faster maturation and lower infiltration of NK cells in addition to weaker production of IFN- γ by NK cells in $NCR1^{gfp/gfp}$ mice clearly had a negative impact on their ability to interact with cDCs since the number of these cells was lower compared to the number of cDCs in control mice. Moreover, the total number of NK cells in MLN of infected $NCR1^{gfp/gfp}$ mice was lower which corresponded

well with the lower frequency of CXCR3⁺ NK cells detected in the same organ. Thus, it could be that in NCR1^{gfp/gfp} mice the lower number of NK cells available for NK/DC crosstalk, together with their decreased functionality, result in a lower number of cDCs which ultimately affects cDC-dependent stimulation of CD4⁺ T cells.

Numerous studies have already shown the importance of the crosstalk between NK and DC cells in the shaping of the adaptive immune response [6]. Robbins et al. have shown that a strong NK-cell response to MCMV in Klr8 mice (mice on a BALB/c background congenic for the C57BL/6 NKC) severely decreased the activation of plasmacytoid DCs and their production of IFN α/β , enabling the survival of cDCs and eventually resulting in enhanced initiation of an antiviral CD8⁺ T-cell response [32]. NK cells were also shown to play a critical role in the initiation and shaping of the T-cell response after influenza A infection by supporting the recruitment of CD8⁺ T cells and DCs to the MLN [33]. In addition, it has been shown that NK cells influence the uptake and transport of influenza A virus by DCs in an IFN- γ and perforin-dependent manner. Another study showed that CXCR3-dependent recruitment of NK cells to corresponding draining lymph node upon their stimulation by LPS-matured DCs in mice provided CD4⁺ T cells with the necessary source of IFN- γ for Th1 polarization [20]. Finally, in a mouse model deprived of IL-10 cytokine, NK-DC crosstalk resulted in efficient priming of MCMV-specific CD4⁺ T cells [13].

The question which remains is why is NCR1 so important for the observed immunoregulatory role of NK cells? Does it have some yet unrevealed function during NK-cell maturation which otherwise forces NK cells from NCR1-deficient mice to rapidly shift to more mature phenotype unable to take part in the modulation of the adaptive immune response? The expression of CXCR3 on NK cells is closely related to their maturation status being highly expressed on CD11b^{hi}CD27^{hi} NK cells and downregulated on CD11b^{hi}CD27^{low} NK cells [16]. This enables only CD11b^{hi}CD27^{hi} NK cells to migrate from blood to the site of infection and the corresponding draining lymph node. The phenotype we have observed in NCR1-deficient mice is at least in part a result of an impaired migration of terminally mature NK cells to the regional lymph nodes, which affects the development of the adaptive immune response. The other possibility is that NCR1 has a more direct role in interaction of NK cells with cDCs, CD4⁺ T cells or even B cells. Study by Crouse et al. revealed a mechanism by which T cells can evade the potent cytolytic activity of NK cells through regulation of NCR1 ligand expression upon LCMV infection [34]. Namely, an elevated expression of NCR1 ligands on Ifnar1-deficient T cells triggered NCR1-mediated NK cell killing of these cells. Our own in vitro data showed the downregulation of the NCR1 ligands on bone marrow derived DCs just several hours after MCMV infection (data not shown), thus discarding the possibility of NK-cell mediated killing of infected cells through NCR1 engagement. In Figure 4B, we have shown lower frequency of IFN- γ ⁺ NK cells in MLN of infected NCR1^{gfp/gfp} mice. As already mentioned, IFN- γ production by NK cells have the beneficial role in B-cell proliferation [25]. Thus, one can speculate that the lower frequency of

IFN- γ ⁺ NK cells present in the MLN of NCR1-deficient mice could have negative effect on the B-cell maturation process. Further studies will be necessary to investigate the possibility of a direct role of NCR1 in the formation of adaptive immune response.

Overall, our data reveal a high importance of NCR1 in the formation of Tfh cells and subsequently GC B-cell development inside germinal centers. CXCR3-dependant migration of NK cells to regional lymph nodes, a place where they could interact with cDCs and CD4⁺ T cells, appears to be important for the successful development of humoral response leading to the production of highly protective antiviral antibodies.

All the data published so far, and the fact that different results are obtained by using different mouse or virus infection models, imply that there is no unique pathway in NK-cell regulation of the adaptive immune response. Depending on the nature of the infection, its intensity and longevity, as well as the fitness of the host response, immunoregulatory role of NK cells can vary greatly from being beneficial or detrimental to the host adaptive immunity. All of this needs to be taken into account when contemplating of new vaccine strategies targeting chronic virus infections.

Materials and methods

Mice

BALB/c, BALB/c NCR1^{gfp/+}, BALB/c NCR1^{gfp/gfp}, C57BL/6, and C57BL/6 NCR1^{gfp/gfp} mice [9] were housed and bred under specific pathogen free conditions at the Faculty of Medicine, University of Rijeka. WT and NCR1^{gfp/gfp} littermates were generated by crossing of BALB/c NCR1^{gfp/+} mice. Bone-marrow chimeras were prepared using 8-week-old mice as donors and recipients. Briefly, C57BL/6 NCR1^{gfp/gfp} (CD45.2⁺) recipient mice were γ -irradiated with 9 Gy. After 24 h, recipient mice were injected intravenously (i.v.) with 10⁷ of donor bone-marrow cells containing a 1:1 mixture of C57BL/6 (CD45.1⁺) and C57BL/6 NCR1^{gfp/gfp} (CD45.2⁺) bone-marrow cells. Recipient mice were maintained on antibiotic water containing enrofloxacin for 2 weeks following irradiation. Chimerism was evaluated and chimeras were used in experiments after 8 weeks of reconstitution. All animal experiments described in this paper were performed in accordance with the guidelines contained in the International Guiding Principles for Biomedical Research Involving Animals and approved by the Animal Welfare Committee at the Faculty of Medicine University of Rijeka. Eight-to-twelve week-old mice were used in all experiments with the exception of experiments assessing the protective capacity of antibodies which were done on newborn BALB/c mice.

Viruses

Mice were injected intraperitoneally (i.p.) with 2 × 10⁵ PFU of tissue-cultured grown virus diluted in a volume of 500 μ L of phosphate buffer saline (PBS). In some experiments mice were footpad (f.p.) injected with 1 × 10⁵ PFU of MCMV to analyze late virus-specific ab response. For the evaluation of protective

capacity of MCMV-specific antibodies, newborn BALB/c mice were i.p. injected with 400 PFU of MCMV in a volume of 50 μ L of PBS. We have used bacterial artificial chromosome (BAC)-derived MCMV strain MW97.01 as wild type (WT) MCMV. This virus has previously been shown to be biologically equivalent to the MCMV strain Smith (VR-1399) [35]. In addition, a previously described mutant virus Δ m138 MCMV [36], was used for the infection of BALB/c mouse MEF cells to obtain the lysates used in ELISA and ELISPOT assays as detection antigens.

Antibody administration

In vivo depletion of CD4⁺ T cells and NK cells were performed by i.p. injection of 400 μ g/mouse of anti-CD4 mAb (YTS 191.1) and 20 μ L/mouse of anti-asialoGM1 serum (Wako Chemicals) diluted in PBS to a final volume of 500 μ L at the day of infection and repeated on days 5 and 10 p.i. The 500 μ g/mouse of anti-CXCR3 antibody (Clone CXCR3-173; Bio X Cell) was administered in a single dose at the day of infection to block CXCR3 receptor.

Assessment of virus-specific ab from sera of MCMV-infected mice

Preparation of Δ m138 MCMV lysates

We have used Δ m138 MCMV-infected MEF lysate to avoid unspecific antibody binding by virus Fc receptor m138. MEF cells were grown in large culture dishes (diam. 150 mm) with the addition of 3% DMEM media. 11×10^6 of MEF cells/large culture dish were used for the infection. Cells were infected with 0.01 PFU/cell of Δ m138 MCMV. Infected MEFs have been kept in the incubator for 4–5 days until high infection was reached. Cells were then detached by adding cold 2 mM EDTA which was followed by washing with PBS. Lysates were obtained by sonication of these cells in bicarbonate buffer. Lysates were stored at -20°C or used immediately for coating plates.

ELISA

MCMV-specific IgG titers were determined by ELISA as previously described. In short, 96-well plates (Greiner, cat.no. 655061) were coated overnight at 4°C with Δ m138 MCMV lysates in bicarbonate buffer. Plates were then blocked for 2 h at room temperature. Serial dilutions of sera retrieved from MCMV-infected mice 14 days p.i. were made in PFT (PBS containing 1% FCS and 0.05% Tween 20) and incubated on the prepared plates for 2 h at room temperature. Plates were washed with PBS, after which HRP-conjugated IgG, IgG1, or IgG2a antibodies were incubated for 1 h at room temperature. The OPD substrate was used to develop the plates. To stop the reaction, 50 μ L of stop solution (1M H₂SO₄) has been added. The absorbance of the samples was read using optic reader at 490 with 630 nm used as the reference wavelength.

Avidity assay

To determine the avidity of the MCMV-specific antibodies, we have used the same procedure as in the ELISA protocol with one additional step. Before detection of antigen-captured serum IgGs with the corresponding secondary antibody, we have incubated the plates with PBS or 8M urea for 5 min at room temperature. The 8M urea was used as a chaotropic agent to elute low-avidity antibodies. The avidity index of the MCMV-specific antibodies was determined by the ratio of the amount of antibodies bound after elution with 8M urea relative to the amount of antibodies bound in the absence of 8M urea, expressed as a percentage.

ELISPOT

The quantification of MCMV-specific ASCs was done according to the Mabtech Mouse IgG ELISPOT protocol (for coated antigen). In short, PVDF plates (MSIP, Millipore) used in the ELISPOT assay were first activated by ethanol treatment, washed extensively with sterile water and then coated overnight at 4°C with Δ m138 MCMV lysate (50 μ g/mL). Next day the plates were washed with PBS and blocked for 1 h on room temperature using 10% RPMI media. 2.5×10^5 splenocytes or lymphocytes from MLN of MCMV infected mice were added per well and incubated overnight at 37°C. Cells were removed from the plates by washing extensively with PBS. Next, the plates were incubated for 2 h with detection anti-IgG-biotin antibody at room temperature, washed, and incubated with streptavidin-ALP for another hour at room temperature. After subsequent washing, spots were visualized using BCIP/NBT substrate (Mabtech) and reaction was stopped using tap water. The total number of ASC per organ was determined by multiplying the amount of counted spots per well with the ratio of the absolute cell number relative to the amount of plated cells.

Neutralization

Serum from MCMV-infected mice was decomplemented by heating in a water bath at 56°C for 30 min. Different dilutions of serum starting from 1:8 were prepared with the addition of rabbit complement previously diluted with DMEM media in a 1:12 ratio. Prepared sera were incubated for 60 min with 100 PFU of WT MCMV at 37°C. The sera-virus mix was transferred to 48-well plates containing a confluent layer of MEF cells and further incubated for next 60 min at 37°C. Cells were then covered in carboxymethyl cellulose-containing medium. Plates containing virus plaques were read after 3 days. Results were presented as the percentage of plaque reduction determined by a ratio of the amount of the plaques counted in the sample wells relative to the amount of plaques found in wells containing the serum of uninfected mouse used as a negative control.

Flow cytometry and IFN- γ assay

Single-cell leukocyte suspensions were prepared from perfused lungs and mediastinal lymph nodes as described before [13]. In order to reduce nonspecific staining, Fc receptors were blocked with 2.4G2 mAb. NK cell-surface staining was performed for the following antigens: anti-NKp46 (29A1.4), anti-CD49b (DX5), anti-CD19 (1D3), anti-CD3e (145-2C11), anti-CD11b (M1/70), anti-CD27 (LG.7F9), anti-CD183/CXCR3 (CXCR3-173). CD4⁺ cells were characterized using: anti-CD4 (GK1.5), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD279/PD-1 (J43), anti-CD278/ICOS biotin (7E.17G9) and SA-PerCP Cy5.5. Lung DCs were surface stained using anti-CD103 (2E7), anti-CD19 (1D3), anti-CD3e (145-2C11), anti-CD11c (N418), anti-CD11b (M1/70) and anti-MHC II (I-A/I-E) (M5/114.15.2). Phenotypization of B cells was done using: anti-CD19 (1D3), anti-CD3e (145-2C11), anti-IgM (II/41), anti-GL7 (GL7), anti-MHC II (I-A/I-E) (M5/114.15.2) and anti-CD95 (15A7). All the above-mentioned abs were purchased from eBioscience. IFN- γ production by NK cells was examined by intracellular staining using anti-IFN- γ (XMG1.2) ab. Incubation of NK cells was performed in RPMI medium supplemented with 10% of FCS for 5 h in the presence of 500 IU/ml of IL-2 at 37°C and 1 μ g/mL of brefeldin A (eBioscience). Brefeldin A was added to the cell suspension for the last 4 h of incubation. For the detection of IFN- γ expression by MCMV-specific CD4⁺ T cells, we have used the protocol described in [37]. In short, lymphocytes were stimulated using BALB/c peritoneal macrophages (PECs) incubated with mock or Δ m138 MCMV-infected BALB/c MEF lysates. PECs were retrieved by rinsing the peritoneum of BALB/c mice with sterile PBS 3 days after they have been injected with 500 μ L 3% thioglycolate solution. PECs were then put to overnight incubation with mock or Δ m138 MCMV-infected BALB/c MEF lysates, adjusting the concentration of lysates to 100 μ g/mL and PEC number to 0.5×10^6 cells. The next day, lymphocytes were isolated from perfused lungs of mice infected with WT MCMV for 7 days. The number of lymphocytes was adjusted to achieve 1:1 ratio of PECs to CD4⁺ T cells. Lymphocytes were stimulated with the prepared PECs overnight with the addition of brefeldin A. The analysis of IFN- γ -producing CD4⁺ T cells by flow cytometry was conducted the following morning. Flow cytometry was performed on FACSAria (BD Bioscience, San Jose, CA) and data were analyzed using FACSDiva or FlowJo software (Tree Star, Ashland, OR).

Gating strategies for all flow cytometry data shown in the manuscript are provided as a part of Supporting information file (Supporting Information Fig. 2–8).

Statistics

Statistical significance was determined by GraphPad Prism5 Software. The differences in viral titers were determined by a two-tailed Mann–Whitney *u* test. Statistically significant differences between two data sets in phenotype analyses were determined by the unpaired two-tailed Student's *t*-test, and *p* values less than 0.05 were considered significant. The significant differences between

tested groups are indicated with star symbols as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

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Author contributions

A.M., A.K., and S.J. designed research; A.M., B.P., M.L., I.B., T.T., K.M., and A.K. performed research; O.M. provided new reagents/analytic tools; A.M. analyzed data; and A.M., A.K., and S.J. wrote the paper.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: ASC: antibody secreting cells · DCs: dendritic cells · f.p.: footpad · GC: germinal center · gfp: green fluorescent protein · IFN-γ: interferon-gamma · i.p.: intraperitoneally · i.v.: intravenously · LCMV: lymphocytic choriomeningitis virus · MCMV: mouse cytomegalovirus · MEF: mouse embryonic fibroblast · MLN: mediastinal lymph nodes · p.i.: post infection · Tfh cells: T follicular helper cells · WT: wild type

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