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Genetic Labeling Reveals Altered Turnover and Stability of Innate Lymphocytes in Latent Mouse Cytomegalovirus Infection

Andreas Busche,*,1 Susanne Schmitz,†,1 Henrike Fleige,‡ Scott H. Robbins,§,2 Thierry Walzer,§,3 Charles A. Stewart,§,4 Reinhold Förster,† Martin Messerle,∗ and Immo Prinz†,‡

Mouse CMV (MCMV) infection rapidly induces the proliferation of NK cells, which correlates with immunological protection. Whether NK cells are maintained during acute response against MCMV are maintained for the long term is not known. In this study, we used TcrdH2BeGFP mice in which maturing NK cells are genetically labeled with a pulse of very stable histone-2B-eGFP. In this system, we found that the reporter protein was diluted out upon NK cell division during acute MCMV infection. At the same time, mature NK cells in uninfected mice showed only very limited turnover in vivo. Three months after primary infection when MCMV latency was established, the majority of peripheral NK cells still displayed a higher record of proliferation than NK cells in mock-infected controls. This observation included both Ly49H+ and Ly49H− NK cells. Conversely, naive NK cells did not show more proliferation after transfer into latently MCMV-infected mice than that after transfer into mock-infected control mice. This indicated that the observed alterations of the NK cell compartment in MCMV latency were “legacy” (i.e., resulting from prior events during the initial immune response). Together, these results suggest that antiviral immune responses induce sustained alterations of innate lymphocyte populations that extend far beyond the first days of acute infection. The Journal of Immunology, 2011, 186: 2918–2925.

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Address correspondence and reprint requests to Dr. Immo Prinz, Hannover Medical School, Institute for Virology, 30625 Hannover, Germany; and Thierry Walzer, Centre d’Immunologie de Marseille-Luminy, INSERM U631, Centre National de la Recherche Scientifique, UMR 6102, Université de la Méditerranée, 13288 Marseille, France

† A.B. and S.S. contributed equally to this work.
‡ Current address: Immune Design Corporation, Seattle, WA.
§ Current address: Center for Cancer Research, National Cancer Institute, Frederick, MD.

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Address correspondence and reprint requests to Dr. Immo Prinz, Hannover Medical School, Institute for Immunology, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. E-mail address: Prinz.Immo@mh-hannover.de

The online version of this article contains supplemental material.

Abbreviations used in this article: H2B–eGFP, histone-2B–eGFP fusion protein; HCMV, human CMV; MCMV, mouse CMV; MEF, mouse embryonic fibroblast; p.i., postinfection; WT, wild-type.

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role of these cells is the immediate response to infection (20). Latent MCMV in C57BL/6 mice is hierarchically controlled by CD8\(^+\), NK1.1\(^+\), and CD4\(^+\) lymphocytes (21). Failure of such cellular immunosurveillance will ultimately induce viral reactivation, resulting in the production of infectious progeny.

In this work, we studied NK cells 90 to 120 d after MCMV infection when MCMV latency is fully established (17). In this context, an important question was whether residual latent viral gene expression may be sufficient for ongoing NK cell activation. To address this question, we took advantage of TcrdH2BeGFP reporter mice, in which we could easily track \(\gamma\delta\) T cells based on high levels of a very stable histone-2B–eGFP fusion protein (H2B–eGFP) in their chromatin (22). We have previously shown that in these mice, maturing NK cells receive a genetic labeling with the H2B–eGFP reporter (23). We show in this study that NK cells dilute out the genetic H2B–eGFP marker through cell division to minimal levels. Thus, this reporter system serves to monitor the record of cell division (i.e., the “proliferative history” of NK cells without further manipulation). We found that 90 and 120 d after primary infection with MCMV, NK cells displayed a higher record of cell proliferation in the absence of detectable active MCMV infection compared with that of uninfected control mice. This effect is irrespective of Ly49H expression. We conclude that although NK lymphocytes are known to be key players during the first days of viral infection, their population dynamics are still significantly altered in latently infected mice.

Materials and Methods

**Mice**

All mice were on C57BL/6 background. C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). B6.129S7-H2B\(^{tdm1Imx}\)/J, Rag1\(^{-/-}\), and C57BL/6.129-Ly5.1 mice were originally obtained from The Jackson Laboratory. TcrdH2BeGFP mice have been described elsewhere (22). All mice were bred at the central animal facility of the Centre d’Immunologie de Marseille-Luminy or at Hannover Medical School in individual ventilated cages and under specific pathogen-free conditions. All experiments were carried out in accordance with national and institutional guidelines.

**Flow cytometry and intracellular cytokine staining**

Abs directed against CD5 (clone 53-7.3), CD19 (clone 1D3), CD45.1 (clone ABio17B7), and Ly49H (clone 3D10) were purchased from eBioscience. Abs directed against CD11b (clone M1/70), CD117 (clone A2C2), \(\alpha\)-TCR (clone H57-597), CD4 (clone IM7), IL-17A (clone XMG1.2) Ab (Promega), CD8a (clone 53-6.7), and anti-FcR Ab (clone 2.4G2) were produced with rat hybridoma cell lines. Anti-IFN-\(\gamma\) (clone XMG1.2) Ab was purchased from Caltag, and anti-CD122 (clone TM-1), CD27 (clone LG.3A10), and CD49b (DX5) were from Biolegend. Rat serum was obtained from BD Biosciences. Anti-TCR (clone A20), CD45.2 (clone 104), CD196/CCL6 (clone 140706), CD335/CCR6 (clone 140706), CD3 (clone 1D3), CD45.1 (clone A2C2), and magnetic bead-enriched NK cells from 129/Sv background were purchased from AbD Serotec and mouse serum from Invitrogen. For measurements of Ki67 expression, we used a Tissue Lyser II (Qiagen, Hilden, Germany). Infectious virus was determined by serial dilution assays on B95-8 cells in 96-well plates. The generation of the bacterial artificial chromosome-derived MCMV strain MW97.01 has been described (24); the m157 deletion mutant MCMV-2D referred to in this study as Δm157-MCMV will be described elsewhere (A. Busche, unpublished data). Strain MW97.01 has been shown to be as virulent as the MCMV Smith strain and was designated as WT MCMV in this study. The viruses were propagated on primary mouse embryonic fibroblasts (MEFs), which were prepared from the embryos of pregnant BALB/c mice on day 17 of gestation. Virus stocks were produced as described (25).

**Establishment of latent MCMV infection**

Newborn mice were i.p. infected with 10\(^3\) PFU of the MCMV strains in 50 \(\mu\)l PBS. Mice were housed in individually ventilated cages in an area otherwise free of murine pathogens as revealed by microbiological monitoring according to Federation of European Laboratory Animal Science Associations recommendations (26). At designated times p.i., mice were sacrificed, and lymphocytes were isolated from spleen, lymph nodes, liver, and lung by standard procedures. Establishment of latency was verified by the absence of detectable infectious virus in the salivary glands (data not shown) and by reactivation of infectious virus in explanted host tissues (see later).

**Acute MCMV infection**

For acute infection, adult TcrdH2BeGFP mice, or where indicated B6.129S7-H2B\(^{tdm1Imx}\)/J mice, were i.p. infected with 1 \(\times\) 10\(^5\) PFU MCMV-2D or 1 \(\times\) 10\(^3\) PFU salivary gland-derived Smith strain with similar results.

**Detection of infectious virus**

Salivary glands were homogenized with 5-mm stainless steel beads in a Tissue Lyser II (Qiagen, Hilden, Germany). Infectious virus was determined by a plaque assay performed on subconfluent second-passage MEF monolayers. Centrifugal enhancement at 1000 \(\times\) g was applied for 30 min, followed by 2 h of incubation at 37°C. One week p.i., cultures were analyzed for the occurrence of cytopathic effects. Virus reactivation from latently infected host tissue

Salivary glands and lungs were isolated from latently infected mice and cut into small pieces with scissors. The pieces from one organ were randomly distributed into the wells of a 12- or 24-well plate, and the cultures were maintained in DMEM supplemented with 10% FCS and antibiotics. The medium was replaced twice a week, and supernatants were harvested over 5 wk to monitor for the presence of infectious virus by transfer to MEF indicator cells. The cultures were analyzed microscopically for the occurrence of cytopathic effects 1 wk post-transfer.

**Statistics**

All bar graphs are presented as mean ± SEM and were made using GraphPad Prism software (version 4.03). Group data were compared with the two-tailed unpaired \(t\) test.

**Results**

Intrinsic H2B–eGFP labeling of mature NK cells in TcrdH2BeGFP reporter mice is diluted by cell division

Triggering of activating receptors through viral components often induces NK cell proliferation (27–30). To date, this has been ex-
Experimentally demonstrated using specific Abs directed against incorporated BrdU or via adoptive transfer of CFSE-labeled NK cells. However, NK cells may turn quiescent and long-lived after an initial phase of proliferation (31). To monitor the proliferative history of NK cells directly ex vivo without further manipulation, it would be necessary to pulse mature but not yet activated NK cells with a very stable marker. We have previously shown that NK cells in TcrdH2BeGFP reporter mice acquire two levels of the fluorescent H2B–eGFP reporter during maturation of immature DX5– NK cells to mature DX5+ cells (23) through germline transcription at the non-rearranged Tcrd locus (Supplemental Fig. 1B). Gene expression of TcrdH2BeGFP declines to a minimum in mature NK cells, and owing to the H2B–eGFP protein’s half-life of several months, this marker may be lost only by cell division (32, 33). Starting at an age of 2 wk and lasting for at least 18 mo, approximately half of all NK cells defined as CD3–NK1.1+ lymphocytes displayed intermediate H2B–eGFP levels (GFPint) (Fig. 1A, Supplemental Fig. 1B). This difference in very young mice may be due to loss of H2B–eGFP by homeostatic proliferation of NK cells because very young mice (less than 1 wk) are lymphopenic (34, 35). To demonstrate such loss of H2B–eGFP through proliferation directly, purified NK cells from adult mice were cultured in the presence of high levels of IL-2 where they expanded and became lymphokine-activated killer cells. Although proliferating NK cells increased in cell size in vitro, intermediate intensity of the H2B–eGFP marker was diluted out by cell division (Supplemental Fig. 2A). In addition, the stability of the H2B–eGFP fusion protein situated within the chromatin allowed us to perform intracellular staining to show directly correlation of BrdU uptake with loss of fluorescence (Supplemental Fig. 2B). Otherwise, H2B–eGFP reporter levels were remarkably stable. Upon adoptive transfer of purified GFPlow and GFPint NK cells into lymphopenic mice, H2B–eGFP levels remained constant for more than 2 wk, indicating only very limited homeostatic proliferation of peripheral NK cells in the absence of specific stimuli (Supplemental Fig. 3).

To exclude the possibility that differential allelic exclusion effects were responsible for the distinctive two levels of H2B–eGFP labeling, we compared homozygous and heterozygous mice.

**FIGURE 1.** Intrinsic H2B–eGFP labeling of mature NK cells in TcrdH2BeGFP reporter mice. A, Histograms show FACS analysis of H2B–eGFP levels (GFP) in NK cells gated as CD19–CD3–NK1.1+ from TcrdH2BeGFP mice (g/g, solid line) of different ages compared with those of NK cells from WT mice (gray filled histograms). B, Lymphocytes from Rag1–/–TcrdH2BeGFP+/+ (solid line) versus Rag1–/–TcrdH2BeGFP+/– (dotted line) and WT mice (gray filled histogram) were gated as NK1.1+MHC-IIF GR1+. H2B–eGFP fluorescence is shown as histogram overlay. C, CD19+CD3–NK1.1+ from TcrdH2BeGFP mice were gated according to low or intermediate H2B–eGFP levels (left panel); dot plots show surface expression of CD27 and CD11b of GFPint (middle panel) and GFPlow (right panel). Experiments of A–C are representative of at least five independent experiments. See also Supplemental Fig. 1.

**FIGURE 2.** NK cells proliferate during acute MCMV infection and lose their intermediate H2B–eGFP label. TcrdH2BeGFP mice were infected i.p. with MCMV at 3, 6, 9, or 14 d before analysis (n = 3 mice for each group). A, FACS plots showing CD27 and CD11b expression of splenic NK cells gated as CD3–NK1.1+ lymphocytes displayed intermediate H2B–eGFP levels (GFPint), whereas the majority of NK cells derived from 4-d-old mice uniformly showed low H2B–eGFP levels (GFPlow) (Fig. 1A, Supplemental Fig. 1B). This difference in very young mice may be due to loss of H2B–eGFP by homeostatic proliferation of NK cells because very young mice (less than 1 wk) are lymphopenic (34, 35). To demonstrate such loss of H2B–eGFP through proliferation directly, purified NK cells from adult mice were cultured in the presence of high levels of IL-2 where they expanded and became lymphokine-activated killer cells. Although proliferating NK cells increased in cell size in vitro, intermediate intensity of the H2B–eGFP marker was diluted out by cell division (Supplemental Fig. 2A). In addition, the stability of the H2B–eGFP fusion protein situated within the chromatin allowed us to perform intracellular staining to show directly correlation of BrdU uptake with loss of fluorescence (Supplemental Fig. 2B). Otherwise, H2B–eGFP reporter levels were remarkably stable. Upon adoptive transfer of purified GFPlow and GFPint NK cells into lymphopenic mice, H2B–eGFP levels remained constant for more than 2 wk, indicating only very limited homeostatic proliferation of peripheral NK cells in the absence of specific stimuli (Supplemental Fig. 3).

To exclude the possibility that differential allelic exclusion effects were responsible for the distinctive two levels of H2B–eGFP labeling, we compared homozygous and heterozygous mice. Both
CD45.2+CD45.1–CD3–NK1.1+ were analyzed by FACS according to the lymphocytes were prepared from the indicated organs. NK cells gated as day 2 these results implied no strict correlation between NK cell pro-

cellularities contained cells of all maturation stages, and therefore classification, the most immature NK cells are CD27 –CD11b– and

maturation markers CD27 and CD11b (Mac-1) (36, 37). In this manipulated reporter mice according to expression of the NK cell

tensive reporter mice not only serve to track TcrdH2BeGFP reporter mice to

showed the characteristic two H2B–eGFP levels, albeit as expected with lesser intensity in heterozygous reporter mice (Fig. 1B). To address whether H2B–eGFP dilution correlated with cell maturation, we analyzed GFPlow and GFPint NK cells from non-

manipulated reporter mice according to expression of the NK cell maturation markers CD27 and CD11b (Mac-1) (36, 37). In this classification, the most immature NK cells are CD27 CD11b+ and then progress from CD27+CD11b+ to CD27+CD11b+ and further to CD27+CD11b+. Of note, both GFPlow and GFPint NK populations contained cells of all maturation stages, and therefore these results implied no strict correlation between NK cell pro-

liferation and progression of maturation according to CD27 and CD11b (Fig. 1C). However, GFPlow NK cells were on average more mature than GFPint NK cells (Fig. 1C). Taken together, we propose that TcrdH2BeGFP reporter mice not only serve to track GFPlow γδ T cells (22) but also represent a genetic model allowing the direct record of NK cell proliferation by flow cytometry. With this tool, we concluded that there was only very limited proliferation of mature NK cells in adult mice. This is in accordance with previous data obtained by BrdU labeling studies (7, 38). In very young mice, however, it appears that homeostatic cues drive NK cell proliferation and in so doing induce loss of intermediate H2B–eGFP levels. Similar to what is known for expansion of neonatal CD8+ cytotoxic T cells (34, 35), it is likely that mechanisms involving IL-7 and IL-15 underlie the observed augmented proliferation of neonatal NK cells.

**NK cell proliferation in acute MCMV infection**

Next, we took advantage of TcrdH2BeGFP reporter mice to monitor NK cell dynamics during viral infections. We chose acute MCMV infection of adult mice as a model inducing massive NK cell proliferation and followed NK cell dynamics during the first 2 wk of infection. At day 6 postinfection, we observed almost uniform maturation of all splenic NK cells to the most advanced CD27+CD11b+ maturation stage (Fig. 2A), which correlated with the peak of NK cell expansion (Fig. 2B). At later time points, proportions but not absolute numbers of NK cell in the more immature CD27+CD11b+ and CD27–CD11b– stages partially recovered (Fig. 2A, 2B). Intriguingly, loss of intermediate reporter H2B–eGFP levels exactly matched the anticipated progression of NK cell proliferation in this model infection; that is, the lowest frequency of GFP+ NK cells was found at day 6 p.i. (5.1 ± 1.5%) and recovered to 17.5 ± 2.9% at 14 d p.i. (Fig. 2C). To prove formally that the observation noted above was actually due to H2B–eGFP loss of proliferating GFPint NK cells, GFPlow and GFPint NK cells were separated by FACS and adoptively transferred into lymphopenic CD127-deficient recipients prior to MCMV infection (Fig. 3). After 7 d, transferred cells could be recovered from blood, spleen, liver, and lung but not from lymph nodes and identified based on expression of the congenic markers CD45.1 and CD45.2. Analysis showed that 1) GFPlow and GFPint levels of transferred NK cell populations were stable in vivo in mock-infected animals (Fig. 3, black plots), which is in line with the results shown in Supplemental Fig. 3; 2) GFPint NK cells readily lost their reporter fluorescence in vivo in MCMV-infected animals whereas GFPlow NK cells remained GFPint (Fig. 3, orange plots); and 3) loss of the H2B–eGFP marker equally affected Ly49H+ and Ly49H– NK cells. In conclusion, using MCMV infection as a strong mitogenic stimulus for otherwise nonproliferating NK cells, these experiments revealed that loss of H2B–eGFP reporter fluorescence faithfully indicates proliferation of NK cells in vivo. Because we showed previously that GFPlow and GFPint NK cell populations are functionally largely equivalent (23), this suggests that the percentage of GFPint of total NK cells serves as a direct record for the proliferative history of NK cells.

**NK cell proliferation in latent MCMV infection**

Having established above that NK cells from TcrdH2BeGFP mice are a genetic system to analyze the proliferative history of NK cells during acute MCMV infection, we next sought to investigate how NK cell populations would recover or continue to proliferate during latency of MCMV. We chose time points as late as 3 to 4 mo after primary infection when MCMV latency is established (39). Notably, Lanier and co-workers (12) had recently proposed that expansion of NK cell subsets would lead to a form of “NK

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**FIGURE 3.** Ly49H+ and Ly49H– NK cells proliferate during acute MCMV infection and lose their intermediate H2B–eGFP label. NK cells from TcrdH2BeGFP mice (spleen and lymph nodes combined) were enriched by magnetic beads and FACS sorted as CD3+NKp46+ lymphocytes according to low and intermediate H2B–eGFP levels before adoptive transfer into adult congenic CD45.1+ B6.129S7-Il7rtm1Imx/J mice at day −1 before i.p. mock (PBS) or MCMV infection. Seven days later, lymphocytes were prepared from the indicated organs. NK cells gated as CD45.2+CD45.1+CD3–NK1.1– were analyzed by FACS according to the expression of Ly49H and H2B–eGFP reporter fluorescence. One representative of two experiments each with n = 2 mice per group is shown. See also Supplemental Fig. 3.
cell memory." Their hypothesis was based on the observation that the frequency of NK cells expressing the activating NK receptor Ly49H, which is reactive for the MCMV protein m157, was still elevated 50 d after MCMV infection. Therefore, we were curious to see whether the proliferative history of NK cells in this infection model would disclose novel information about such "memory NK cells." Surprisingly, analysis of NK cells in MCMV latency clearly revealed homogenously lower frequencies of GFPint NK cells in secondary lymphoid organs as well as in liver and lung (Fig. 4A, 4B). This suggested that at 90 and 120 d p.i., the large majority of NK cells in latently MCMV-infected mice had proliferated more than in uninfected control mice. At the same time, NK cell maturation stages according to the expression of CD27 and CD11b showed a typical organ-specific pattern with no striking differences between NK cells of MCMV- or mock-infected mice (Supplemental Fig. 4). To address the contribution of Ly49H–m157 interactions to NK cell proliferation in MCMV latency, mice were then infected with either wild-type (WT) MCMV or a mutant of MCMV lacking the expression of m157 (A. Busche, unpublished data). At 45 and at 120 d p.i., we detected a greater record of NK cell proliferation in mice infected with WT MCMV compared with that in mice infected with m157-deficient MCMV (Fig. 5A), and, at least in liver and lungs of these animals, a higher proportion of NK cells expressed the activating Ly49H receptor (Fig. 5B). In conclusion, we confirmed that m157-specific activation positively affected the proportion of Ly49H + NK cells for a long time after primary infection (12). Accordingly, Ly49H + NK cells displayed lower H2B–eGFP levels suggesting that an elevated proportion of these cells had proliferated compared with Ly49H + NK cells (Fig. 5C, 5D). This was independent of whether the primary infection was performed with m157-deficient or -proficient MCMV (Fig. 5D). Therefore, these results suggest that proliferated Ly49H + NK cells also show a sustained stability after MCMV infection.

**NK cells transferred into latent MCMV recipients are not affected**

Next, we aimed at determining whether alterations in NK cell homeostasis in MCMV latency may actually be driven by recognition of residual active virus or by latent viral gene expression, which on a subclinical level may continuously reactivate in tissues such as liver, lung, and salivary glands. Therefore, NK cells from TcrdH2BeGFP mice were purified and adoptively transferred into congenic CD45.1 + recipients that had been mock- or MCMV-infected 90 d beforehand. Fourteen days after transfer, donor NK cells could be recovered from spleen, liver, and lung (but not from lymph nodes) and identified by expression of CD45.2. However, mock- or MCMV-infected mice displayed similar frequencies of GFPint among donor cells (Fig. 6). These data suggest that viral components capable of directly activating NK cells were absent at 90 d after primary infection. Therefore, during MCMV latency, alterations in the turnover and composition of endogenous NK cells should originate from previous activation events when active virus was still present and are thus a legacy of primary infection. This in turn would suggest that after an initial expansion and contraction phase, relatively few NK cells were newly generated during MCMV latency.
deficient MCMV. Statistical significance was tested between mice infected with WT or m157-deficient MCMV. Experiments each with 2 mice per group is shown. ns, not significant.

FIGURE 5. Differential proliferative history of Ly49H⁺ and Ly49H⁻ NK cells in MCMV latency. FACS analysis of NK cells derived from the indicated organs of TcrdH2BeGFP mice latently infected with either WT MCMV (black bars) or m157-deficient MCMV (gray bars) at 45 d (left) and 120 d (right) postinfection (n = 4 mice per group). NK cells were gated as CD3⁻NK1.1⁺ lymphocytes and analyzed for expression of reporter H2B–eGFP and Ly49H. Bar graphs show mean ± SEM of (A) GFP⁺ frequency of Ly49H⁺ NK cell frequency, (B) frequency of Ly49H⁺ NK cells, (C) GFP⁺ frequency of Ly49H⁻ NK cells, and (D) GFP⁺ frequency of Ly49H⁻ NK cells. In A–D, statistical significance was tested between mice infected with WT or m157-deficient MCMV. *p < 0.05, **p < 0.01. ns, not significant.

Functional consequences of innate lymphocyte alterations in MCMV latency

Currently, it is emerging that the role of innate lymphocytes in the course of an antiviral immune response may not be restricted to the first days of infection (40, 41). It is conceivable that sustained alterations of NK cell homeostasis may actually skew cytokine responses to subsequent unrelated infections and even confer immunological cross-protection independent of CD4⁺ and CD8⁺ T cells (42, 43). However, when we compared NK cells from MCMV- or mock-infected mice, we found no significant differences in their potential to produce the antiviral cytokine IFN-γ in response to IL-12/IL-18 stimulation (Fig. 7). Certainly, this result does not exclude that IFN-γ production by NK cells in response to other stimuli may be compromised or augmented during MCMV latency.

Discussion

It is generally assumed that innate NK lymphocytes are an early component of the host response to virus infection. However, it is emerging that the NK cell compartment may also be modified during later phases of antiviral defense, which can be interpreted as immunological memory. In this study, we depict alterations of NK cell turnover as long as 3 to 4 mo after primary MCMV infection. This was made possible by our finding that temporally limited expression of the Tcrd locus during NK cell maturation labeled these cells with a pulse of the very stable H2B–eGFP protein leading to intermediate levels of GFP fluorescence. In mature NK cells, transcriptional activity of the Tcrd locus was maintained only at very low residual amounts (23), and therefore additional cell division led to a dilution of H2B–eGFP to minimal levels. Thus, dilution of nuclear H2B–eGFP fluorescence directly indicated the proliferation of mature NK cells. During acute infection, we observed that NK cells rapidly lost their reporter H2B–eGFP levels and showed a more mature phenotype. As late as 3 mo p.i., when no infectious virus was detectable anymore, the large majority of all NK cells in MCMV-infected mice had proliferated more than NK cells in mock-treated controls, and their phenotype was biased toward a mature CD27⁺CD11b⁺ developmental stage. Notably, these observations were not confined to Ly49H⁺ NK cells, which specifically recognize the MCMV protein m157, but applied even more prominently to Ly49H⁻ NK cells. Our data provide evidence that virtually all NK cells in latent MCMV⁺ mice are affected by the viral infection by showing an increased proliferative history. In future studies, it will be very interesting to investigate the involved mitogenic and activating factors other than Ly49H and m157 in more detail. Notably, two phases of NK cell activation/proliferation have been described in response to MCMV: a first nonspecific cytokine-driven phase (7, 44) and a second m157-driven phase (7, 45). Thus, loss of GFP in transferred cells may result from these two different types of events. However, it is at present not clear whether the observed alterations in NK cell homeostasis are beneficial for the mouse. We would predict a scenario where a combination of elevated NK cell maturation and activation would improve responsiveness to a potential second hit of the same kind. It is conceivable that the same activating cues that initially induced NK cell expansion would thereby have shaped the repertoire of activating NK cell receptors. This process could either depend on specific late “NK epitopes” expressed by MCMV on its way to latency or could be induced by other factors such as homeostatic cytokines. Current

FIGURE 6. No proliferation of adoptively transferred NK cells during CMV latency. NK cells from TcrdH2BeGFP mice (spleen and lymph nodes combined) were enriched by magnetic beads and FACS sorted as CD3⁻NKp46⁺ lymphocytes according to low and intermediate H2B–eGFP levels before adoptive transfer into adult congenic CD45.1⁺ mice at day 90 after i.p. mock (PBS) or MCMV infection. Fourteen days later, input NK cells were derived from the indicated organs and analyzed by FACS for expression of H2B–eGFP fluorescence gated as CD45.2⁺CD45.1⁺CD3⁻NK1.1⁺ lymphocytes. Bar graphs show mean ± SEM of GFP⁺ frequency among all NK cells from mock-infected (white bars) or latently MCMV-infected (black bars) recipients. One representative of three independent experiments each with n = 2 mice per group is shown. ns, not significant.

FIGURE 7. IFN-γ production of NK cells in MCMV latency. Intracellular cytokine staining for IFN-γ production of splenic NK cells from mock-infected or latently MCMV-infected TcrdH2BeGFP mice at 120 d postinfection plotted versus expression of H2B–eGFP fluorescence. Numbers indicate the mean percentage ± SEM of IFN-γ⁺ among all NK cells gated as CD3⁻NK1.1⁺ lymphocytes (n = 3 mice per group).
Innate lymphocytes in MCMV latency

This work introduces TcrdH2BeGFP mice as a novel, versatile genetic tool that next to its genuine function (i.e., tracking of γδ T cells) allows us to monitor NK cell division dynamics without prior experimental manipulation. We expect this system to be particularly useful to study NK cell turnover in models of persistent viral infection, where contributions of innate lymphocytes are clearly not confined to the first days of the immune response. In conclusion, our data show that the homeostasis of NK cells is significantly affected for several months after MCMV infection.

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Disclosures
The authors have no financial conflicts of interest.

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13. Robbins, S. H., M. S. Tessmer, T. Mikayama, and L. Brossay. 2004. Expansion of antiviral CD8+ T cells was frozen and not quickly replaced by newly generated GFPhigh NK cells. Epigenetic imprinting could provide an alternative explanation for stable modifications of peripheral NK cells toward a more mature phenotype; that is, daughter cells would maintain transcriptional profiles similar to the progenitor cell as it was recently reported for activated CD8+ T cells (54). A third, albeit less likely mechanism would be a direct influence of MCMV latency on the transient TcrdH2BeGFP expression of newly developing NK cells thereby leading to regeneration of the peripheral NK cell pool by already GFPlow NK cells. Nevertheless, a small number of reactivation events of latent herpesviruses may in the long run further shape the receptor repertoire of human NK cells as it was shown for CD94/NKG2C in HCMV+ children and adults (40, 41). After virus reactivation or in the course of chronic viral infection, human innate lymphocytes may thus play roles in immune defense resembling those of T and B cells (55, 56).

Initial proliferation and secondary expansion are the factors that decide over the classification of innate or adaptive NK cell responses.