Mouse cytomegalovirus-experienced ILC1s acquire a memory response dependent on the viral glycoprotein m12

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Innate lymphoid cells (ILCs) are tissue-resident sentinels that are essential for early host protection from pathogens at initial sites of infection. However, whether pathogen-derived antigens directly modulate the responses of tissue-resident ILCs has remained unclear. In the present study, it was found that liver-resident type 1 ILCs (ILC1s) expanded locally and persisted after the resolution of infection with mouse cytomegalovirus (MCMV). ILC1s acquired stable transcriptional, epigenetic and phenotypic changes a month after the resolution of MCMV infection, and showed an enhanced protective effector response to secondary challenge with MCMV consistent with a memory lymphocyte response. Memory ILC1 responses were dependent on the MCMV-encoded glycoprotein m12, and were independent of bystander activation by proinflammatory cytokines after heterologous infection. Thus, liver ILC1s acquire adaptive features in an MCMV-specific manner.

The ability of an organism to ‘remember’ previous pathogen encounters by mounting a specific and robust secondary response on re-exposure to pathogen-associated antigens is termed ‘immunological memory’. During infection, this memory response is largely coordinated by the selective clonal proliferation and long-term persistence of adaptive lymphocytes that express somatically recombined antigen receptors (for example, T cells) to form antigen-specific memory cells that can epigenetically maintain activation-induced transcriptional changes after the clearance of pathogen1–4. Coordination of stable epigenetic, transcriptional and metabolic changes in adaptive memory cells results in cell-intrinsic increases in cytotoxic potential and proinflammatory cytokine production during secondary pathogen exposure to enhance host protection1–4,5. A robust host-protective memory response requires the coordinated contributions of circulating and tissue-resident memory T cells (TEm). After primary viral infection, circulating virus-specific effector T cells can give rise to TEm cells at the sites of initial pathogen encounter, which rapidly and robustly respond to secondary infection in an antigen-dependent manner5. Although immunological memory is classically defined in an antigen-specific context, recent evidence suggests that circulating and tissue-resident cells of the innate immune system (for example, natural killer (NK) cells, monocytes, group 2 ILCs) can acquire enhanced effector function or stable activation-induced epigenetic changes after exposure to various inflammatory stimuli in an antigen-independent manner6–8. However, whether tissue-resident innate immune cells also have the potential to form antigen-dependent memory responses is unknown.

ILCs are tissue-resident innate immune cells that can be found in non-lymphoid tissues, and are enriched at epithelial barrier surfaces such as the intestine, lung and skin7. ILCs do not express rearranged antigen receptors, but instead express a wide variety of germline-encoded, activating and inhibitory receptors8–10. It is generally believed that ILCs lack the ability to respond to pathogen-derived antigens, but rather respond rapidly to proinflammatory signals within discrete tissue microenvironments in an antigen-independent manner11. ILC1s are tissue-resident sentinels that function to protect the host from bacterial and viral pathogens at initial sites of infection12–14. ILC1s rapidly produce the cytokine interferon (IFN)-γ after the local activation of dendritic cells and the production of the proinflammatory cytokine interleukin (IL)-12 to limit viral replication and promote host survival before the recruitment of circulating lymphocytes into infected tissues12. Studies in parabiotic mice have indicated that ILCs are not continuously replaced by bone marrow-derived precursors during steady-state, or shortly after inflammation, and do not recirculate to other tissues12,15–17. Instead, ILCs are thought to self-renew within tissues, suggesting that pathogen-experienced ILCs would persist after the resolution of inflammation. Given the essential role of ILC1s in host protection, whether ILC1s act as short-lived effector cells or persist and adapt after pathogen exposure remains unclear.

In the present study, it has been demonstrated that liver ILC1s expanded and contracted after infection with MCMV to form a stable pool of memory cells. A subset of memory ILC1s expressing the cytokine receptor IL-18Rα (hereafter called IL-18Rα) displayed enhanced effector function after stimulation of NK1.1 or NKp46

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Liver ILC1s proliferate and persist after MCMV infection. Circulating NK cells can robustly proliferate, contract and persist as long-lived memory cells after infection with MCMV, exhibiting similar responses that have previously been observed in lymphocytes of the adaptive immune system. To investigate the response of ILC1s after primary viral infection, C57BL/6 wild-type (WT) mice were infected with MCMV intraperitoneally (i.p.) and the absolute numbers of ILC1s in the liver decreased on day 2 post-infection, ILC1s in the liver had similar expression of Ki67, but increased incorporation of FLICA compared with ILC1s in the liver of uninfected mice (Fig. 1b,c). Coupled with the decrease in absolute numbers of ILC1s in the liver on day 14 and day 30 post-infection compared with day 7 (Fig. 1a), these observations suggested that ILC1s underwent a contraction phase after resolution of the infection from day 7 to day 30 post-infection. To test whether ILC1s persisted after the resolution of MCMV infection, CD45.2+ ILC1s were sorted from the liver of WT mice and adoptively transferred into CD45.1 WT mice, which were subsequently infected i.p. with MCMV 1 d after transfer or left uninfected. Transferred CD45.2+ ILC1s were recovered from both MCMV-infected and -uninfected hosts 30 d after transfer (Fig. 1f,g), suggesting that ILC1s were maintained long term during homeostasis and after viral infection. These results indicate that ILC1s in the liver went through distinct phases of proliferation, contraction and persistence after infection with MCMV.

ILC1s require proinflammatory cytokine signaling for local proliferation. The proliferation of effector NK cells in response to MCMV is mediated by recognition of the MCMV-encoded glycoprotein m157 by the germline-encoded activating receptor Ly49H (Kra8), proinflammatory cytokines such as IL-12 and IL-18, and the transcription factor Zbtb32 (refs. 6,18–20). We investigated whether these signals were required for the proliferation of ILC1s during MCMV infection from day 7 to day 30 post-infection. ILC1s were recovered in the liver of CD45.1 WT mice injected with MCMV (i.p.) or PBS at 30 d post-infection. Data represent three independent experiments with: a, n = 4 mice and b–g, n = 3 mice per group. Samples were compared using a two-tailed Student’s t-test, and data are presented as the mean ± s.e.m. *P < 0.05, **P < 0.01.
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Liver ILC1s locally proliferate in a proinflammatory cytokine-dependent manner after MCMV infection. a, Relative chimerism shown as ratio of indicated surface receptor or transcription factor-deficient ILC1s (CD45.2⁺) to WT ILC1s (CD45.1⁺) in the liver of reconstituted mBMC mice at indicated time points after MCMV infection (i.p.). b, Histograms of indicated cell surface markers of uninfected WT NK cells, uninfected WT ILC1s and 4 × 10⁴ adoptively transferred CD45.2⁺ liver ILC1s recovered in the liver of a CD45.1 Ly49H-deficient host infected with MCMV (i.p.) at 7 d post-infection (D7 PI). c, Percentage of donor-derived NK cells and ILC1s shown for indicated organs of parabiotic mice that were infected with MCMV (i.p.) at indicated time points after infection. Data represent three independent experiments with: a, n = 3 mice and c, n = 4 parabiotic pairs per group. Samples were compared using the two-tailed Student’s t-test, and data are presented as the mean ± s.e.m. *P < 0.05, **P < 0.01.

Fig. 2 | Liver ILC1s locally proliferate in a proinflammatory cytokine-dependent manner after MCMV infection. a, Relative chimerism shown as ratio of indicated surface receptor or transcription factor-deficient ILC1s (CD45.2⁺) to WT ILC1s (CD45.1⁺) in the liver of reconstituted mBMC mice at indicated time points after MCMV infection (i.p.). b, Histograms of indicated cell surface markers of uninfected WT NK cells, uninfected WT ILC1s and 4 × 10⁴ adoptively transferred CD45.2⁺ liver ILC1s recovered in the liver of a CD45.1 Ly49H-deficient host infected with MCMV (i.p.) at 7 d post-infection (D7 PI). c, Percentage of donor-derived NK cells and ILC1s shown for indicated organs of parabiotic mice that were infected with MCMV (i.p.) at indicated time points after infection. Data represent three independent experiments with: a, n = 3 mice and c, n = 4 parabiotic pairs per group. Samples were compared using the two-tailed Student’s t-test, and data are presented as the mean ± s.e.m. *P < 0.05, **P < 0.01.

Fig. 3 | MCMV-experienced liver ILC1s increase the expression of cytokine receptors in vivo. a, Representative histogram of T cells (TCRβ⁺CD3ε⁺NK1.1⁻) and ILC1s, and quantification of ILC1s for IL-7R mean fluorescent intensity (MFI) in the liver of WT mice uninfected or infected with MCMV i.p. at the indicated time points post-infection (PI). b, c, Representative histogram of NK cells and ILC1s, and quantification of ILC1s for IL-2R MFI (b) and frequency of IL-18R⁺ ILC1s (c) in the liver of WT mice uninfected or infected with MCMV i.p. at the indicated time points post-infection. d, Representative histogram of 4 × 10⁴ adoptively transferred CD45.2⁺ IL-18R⁺ ILC1s recovered in the liver of CD45.1 WT recipient mice infected with MCMV (i.p.) at 7 d post-infection. e, Representative histogram of IL-18R staining on uninfected NK cells, uninfected ILC1 and adoptively transferred sort-purified 4 × 10⁴ CD45.2⁺ IL-18R⁺ ILC1s from the liver of day 30 MCMV-infected WT mice recovered in CD45.1 WT recipients 3 d after transfer. Data represent three independent experiments with: a–c, n = 5 mice and d, e, n = 4 mice per group. Samples were compared using the two-tailed Student’s t-test, and data are presented as the mean ± s.e.m. *P < 0.05, ***P < 0.001, ****P < 0.0001.
derived ILC1s and NK cells at day 7 and day 28 post-infection was analyzed. ILC1s in the liver were ~95% host derived at day 7 and day 28 post-infection, whereas NK cells were derived equally from both parabionts in the spleen, liver and peripheral blood (Fig. 2c). These data suggest that MCMV-induced proinflammatory signals were required to drive the local proliferation of a phenotypically stable lineage of liver ILC1s.

MCMV infection impacts the phenotypic and functional properties of ILC1s. CD8+ memory T cells (Tm; CD4+ Tm cells) and regulatory T cells (Treg cells) have increased expression of Il7r, Il2ra and Il18r1 transcripts compared with naive T cells (see Supplementary Fig. 2)21,22. Tm cells require IL-15 and IL-7 for their survival and persistence during homeostasis24,25, and IL-18 to mediate optimal recall responses during viral and bacterial challenges26,27. It was investigated whether MCMV-experienced liver ILC1s had phenotypic properties similar to those of adaptive memory lymphocytes. ILC1s in the liver had increased cell surface expression of IL-7Rα (hereafter called IL-7R) and IL-2Rα (hereafter called IL-2R) and IL-18R 30 d after MCMV infection compared with uninfected mice (Fig. 3a–c). At this time point after MCMV infection, ILC1s in the liver exhibited a bimodal expression of IL-18R (Fig. 3c). To determine whether naive ILC1s induce the expression of IL-18R after MCMV infection, we transferred IL-18R− ILC1s sorted from the liver of CD45.2 WT mice intravenously (i.v.) into CD45.1 WT mice intraperitoneally (i.p.) in WT mice following plate-bound stimulation with either media alone or αNK1.1 antibody. Infection produced more IFN-γ after ex vivo stimulation with plate-bound antibodies against the activating receptors NK1.1 or NKp46, compared with NK cells or IL-18R− ILC1s isolated from the liver of uninfected or MCMV-infected WT mice 30 d post-infection (Fig. 4a and see Supplementary Fig. 3). To investigate whether IL-18R+ ILC1s displayed enhanced secondary effector responses after pathogen rechallenge in vivo, Rag2−/− mice were immunized i.p. with MCMV or control phosphate-buffered saline (PBS), and then reinfected with MCMV hydrodynamically (i.h.) 28 d after primary infection. Ex vivo IFN-γ production was subsequently measured by flow cytometry after incubation with the Golgi complex inhibitor brefeldin A ex vivo for 4 h. IFN-γ production was similar between IL-18R− ILC1s and IL-18R+ ILC1s isolated from the liver of Rag2−/− mice 24 h after primary MCMV infection (Fig. 4b,c). However, IL-18R− ILC1s, but not IL-18R+ ILC1s, had enhanced production of IFN-γ 24 h after secondary infection (Fig. 4b,c), suggesting that IL-18R+ ILC1s had enhanced effector responses to pathogen rechallenge in vivo. Together, these results demonstrate that MCMV infection induced stable phenotypic and functional attributes in a subset of liver-resident ILC1s.

IL-18R+ ILC1s have a distinct transcriptional and epigenetic landscape. Memory lymphocytes have distinct transcriptional and epigenetic changes compared with their naive counterparts that underpin their enhanced responsiveness to secondary stimulation22,23,28–31. To determine whether IL-18R+ ILC1s showed unique transcriptional and epigenetic signatures after MCMV infection, ILC1s were sorted from the liver of naive mice (called naive ILC1s hereafter) and IL-18R+ ILC1s from the liver of MCMV-infected mice on day 35 post-infection (called day 35 IL-18R+ ILC1s hereafter) and RNA-seq and ATAC-seq performed. RNA-seq analysis identified 308 genes more highly expressed in naive ILC1s compared with day 35 IL-18R+ ILC1s (false discovery rate (FDR)-adjusted P < 0.05, absolute log2(fold change) > 1) and 805 genes more highly expressed in day 35 IL-18R+ ILC1s compared with naive ILC1s (FDR-adjusted P < 0.05, absolute log2(fold change) > 1) (Fig. 5a and see Supplementary Table 1). Day 35 IL-18R+ ILC1s had increased expression of transcripts for Ifng, the transcription factors Rora and Tcf7, and cytokine receptors (Ifngr, Il2ra, Il18r1 and Il7r) compared with naive ILC1s (Fig. 5b). Transcripts with increased expression in day 35 IL-18R+ ILC1s compared with naive ILC1s were compared with genes associated with various Tm cell subsets induced by viral challenge from previously published genome-wide analyses using gene set enrichment analysis (GSEA)32. GSEA results showed a higher association of genes expressed in day 35 IL-18R+ ILC1s, with genes preferentially expressed in effector and resident CD8+ Tm cells compared with naive CD8+ T cells (see Supplementary Fig. 4a).
Next, a core ‘lymphocyte memory’ signature was generated, including genes that were notably increased in day 35 IL-18R$^+$ ILC1s compared with naive ILC1s, and shared with effector, central and resident CD8$^+$ T cells compared with naive CD8$^+$ T cells and ILC1s (FDR-adjusted $P < 0.05$; absolute log$_2$(fold change) $> 1$) (Fig. 5c). This signature of genes included transcription factors (Runx3, Rora), the metabolic enzyme Arg1, the effector molecule Tnf, the autophagy protein Bag3, the Wnt pathway regulator Dkk1, zinc finger family proteins (Zfp523, Zfp579, Zfp692) and cytokine receptors (Il18r1, Il7r, Il2ra). The core lymphocyte memory signature was consistent with the phenotypic analysis of cytokine receptor protein expression on MCMV-primed liver ILC1s (Fig. 3a–c).
ATAC-seq identified an atlas of 23,016 accessible regions, with 373 notably differentially accessible (DA) peaks between naive ILC1s and day 35 IL-18Rγ+ ILC1s (FDR-adjusted P < 0.02) (see Supplementary Fig. 4b,c). Of the 373 DA peaks, 282 peaks were more highly accessible in naive ILC1s compared with day 35 IL-18Rγ+ ILC1s, and 91 peaks were more accessible in day 35 IL-18Rγ+ ILC1s compared with naive ILC1s (FDR-adjusted P < 0.02) (Fig. 5d and see Supplementary Fig. 4e). Day 35 IL-18Rγ+ ILC1s had increased accessibility for transcription factors (Rora, Klf2 and Etv6), cell-adhesion molecules (Il7r and Il6r) and cytokine receptors (IIfnγ and Ilnar) compared with naive ILC1s (Fig. 5e,f). For certain genes (Ilfγ and Rora), there was increased accessibility in the transcriptional start sites in WT mice, which, with a concomitant increase in transcripts in day 35 IL-18Rγ+ ILC1s compared with naive ILC1s (Fig. 5g,h) (see Supplementary Fig. 4d), suggesting that subsets of DA genes were maintained in an enhanced transcriptional state in day 35 IL-18Rγ+ ILC1s compared with naive ILC1s.

The glycoprotein m12 is required for the induction of IL-18Rγ+ ILC1s in the liver. Because lymphocyte memory responses can be antigen dependent or independent33, the recall specificity of IL-18Rγ+ ILC1s was investigated in a heterologous infection model. WT mice were primed intranasally (i.n.) with MCMV or PBS and subsequently challenged i.n. with influenza (PR-8 strain) 28 d after primary MCMV or mock infection. Ex vivo intracellular IFN-γ production of lung ILC1s after brefeldin A incubation measured from MCMV-challenged WT mice infected with influenza 48 h post-infection (Fig. 6a), suggesting that the increased secondary ILC1 IFN-γ responses previously observed in MCMV-challenged WT mice were specific to MCMV reinfection. Next, it was tested whether IL-12 and IL-18 stimulation alone was sufficient to drive heightened IFN-γ responses in MCMV-experienced ILC1s. Ex vivo stimulation for 4 h with IL-12 and IL-18 did not induce enhanced IFN-γ production in NK cells, IL-18Rγ+ ILC1s or IL-18Rγ+ ILC1s isolated from the liver of MCMV-infected WT mice on day 35 post-infection compared with naive ILC1s from the liver of uninfected WT mice (Fig. 6b). These results suggest that cytokine stimulation was not sufficient to drive enhanced IFN-γ production in IL-18Rγ+ ILC1s. Furthermore, WT mice primed i.n. with influenza or left uninfected and subsequently challenged i.n. with either MCMV or Sendai virus 28 d after primary influenza or mock infection had similar ex vivo IFN-γ production measured by flow cytometry in the lung 48 h after secondary intranasal infection (see Supplementary Fig. 5a,b). Taken together, these results suggested that MCMV-primed IL-18Rγ+ ILC1 responses may require MCMV-derived signals for enhanced secondary effector responses in vivo.

As liver ILC1s expressed a limited repertoire of activating and inhibitory receptors (Klrhb1, Klrbc1, Klrbb1) that could be specific for known MCMV-encoded antigens (see Supplementary Fig. 6a), and ex vivo stimulation with antibodies against NK1.1 (Klrbc1) enhanced IFN-γ responses in IL-18Rγ+ ILC1s (Fig. 4a), it was tested whether the NKR-P1 (Klrbb1) family ligand m12 (an MCMV-encoded immunoevasin32) drove the induction of IL-18Rγ+ ILC1s through the stimulation of Klrhb1 on Klrbb1 receptors. WT mice were infected i.p. with MCMV-Smith strain (hereafter called Smith), a MCMV MW97 mutant strain deficient in m12 (hereafter called Δm12) or a MCMV MW97 mutant expressing MCMV-Smith m12 ortholog (hereafter called m12sun2), and the frequency of IL-18Rγ+ ILC1s was measured in the liver at day 30 post-infection. Infection with m12sun2 induced a similar frequency of IL-18Rγ+ ILC1s (~50%) compared with Smith infection (~50%) (Fig. 6c), whereas infection with Δm12 did not generate IL-18Rγ+ ILC1s in the liver (Fig. 6c). Furthermore, only intraperitoneal infection with Smith or m12sun2 MCMV-induced enhanced IFN-γ production after ex vivo stimulation with αNK1.1 antibodies in IL-18Rγ+ ILC1s, but not in IL-18Rγ+ ILC1s or NK cells, compared with WT mice infected i.p. with Δm12 (Fig. 6d and see Supplementary Fig. 6b,c). In addition, IL-18Rγ+ ILC1s generated by primary infection with Smith or m12sun2 in Rag2−/− mice and challenged 28 d later with Smith limited early viral replication in the liver 48 h after secondary challenge to a greater extent than Rag2−/− mice primed with Δm12 (Fig. 6e). Thus, enhanced IFN-γ production and host protection by MCMV-experienced liver ILC1s were dependent on the MCMV-derived glycoprotein m12.

Discussion
In the present study, we show that a subset of MCMV-experienced, liver-resident ILC1s expressing the cytokine receptor IL-18Rγ displayed enhanced effector function after stimulation of the activating receptor NK1.1 ex vivo and reinfected with MCMV in vivo. RNA-seq and ATAC-seq analysis revealed that IL-18Rγ+ ILC1s maintained distinct and stable transcriptional and epigenetic signatures compared with naive ILC1s. Furthermore, enhanced IL-18Rγ+ ILC1

Fig. 6 | The MCMV-encoded protein m12 preferentially drives formation of IL-18Rγ+ ILC1s. a, Quantification of ex vivo intracellular IFN-γ staining by percentage and MFI of ILC1s at 48 h after an influenza/PR-8 challenge in the i.v. CD45 unlabeled fraction of the lung in WT mice, which were injected initially with either PBS or MCMV i.n., and subsequently challenged 28 d later with influenza/PR-8 (i.n.). b, Quantification of IFN-γ+ cells within indicated liver ILC1 populations from WT mice infected with MCMV-Smith i.p. and analyzed 35 d post-infection compared with uninfected controls after stimulation with IL-12 and IL-18. c, Quantification of percentage of IL-18Rγ+ expressing liver ILC1s (c) and quantification of intracellular IFN-γ staining in IL18Rγ+ ILC1s (d) in IL-18Rγ+ ILC1s, compared with naive IL-18Rγ+ ILC1s and infected with indicated MCMV strains i.p. and analyzed 30 d post-infection after stimulation with either media or plate-bound anti-NK1.1 antibody. e, Viral copies of 36 h post-infection liver tissue lysate of Rag2−/− mice primed with either m12-sufficient or -deficient MCMV strains (i.p.) and challenged 28 d later with Smith strain by hydrodynamic injection. Data represent three independent experiments with: a, n = 5 mice and b–e, n = 4 mice per group. Samples were compared using the two-tailed Student’s t-test, and data are presented as the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.
responses were dependent on the MCMV immunoevasive m12, but not observed after heterologous infection.

As a naturally occurring mouse pathogen, MCMV demonstrates evidence of co-evolution with its host through the expression of immunoevasive molecules that antagonize host-activating receptors or stimulate inhibitory receptors14. During MCMV infection, T cells and NK cells each recognize unique MCMV-encoded antigens to generate an effective lymphocyte memory pool15–17. Unlike adaptive CD8+ T cells, which use a somatically rearranged T cell receptor to recognize viral peptides presented on major histocompatibility complex (MHC) class I, NK cell expansion and memory formation are driven by the germline-encoded activating receptor Ly49H through recognition of the MCMV-encoded glycoprotein m157 (refs. 18,19). Studies using hapten-induced contact hypersensitivity models have shown that hepatic ILCs with an ILC1-like phenotype can mediate recall responses to specific hapten in an antigen-specific manner20–23, although the precise mechanisms responsible for the antigen specificity of these cells remained unknown, and the physiological importance of these memory responses remained poorly defined. The present study extends these previous observations by demonstrating that liver-resident ILC1s recognized the MCMV-encoded glycoprotein m12 to drive the formation of an IL-18Rβ+ ILC1 subset, consistent with a memory lymphocyte phenotype. These conclusions are based on the stable acquisition of distinct transcriptional, epigenetic, phenotypic and functional attributes observed in IL-18Rβ+ ILC1s compared with naïve ILC1s, which result in cell-intrinsic increases in IFN-γ production during secondary MCMV exposure to enhance host protection. These traits are consistent with current criteria to define memory lymphocytes1–4.

The shared recognition of MCMV-encoded proteins by NK cells and ILC1s suggests a strong selective pressure for the host to evolve germline-encoded receptors to control subsequent MCMV encounters. Specifically, m12 can be recognized by both activating and inhibitory members of the NKR–P1 receptor family24. The RNA-seq analysis indicated that liver ILC1s expressed Klrk1α (Nkpr1a), Klrk1b (Nkpr1b) and Klrk1c (Nkpr1c, NK1.1), all of which can signal in the presence of m12 in vitro. Stimulation of activating receptor NK1.1 resulted in enhanced IFN-γ production only in MCMV-experienced IL-18Rβ+ ILC1s, which indicates that NK1.1 recognition of m12 and downstream signaling may be required for the generation of memory ILC1s. However, it remains possible that m12-induced Nkpr1b inhibitory receptor signaling in the presence of proinflammatory cytokines can achieve a specific priming signal to generate memory responses in liver ILC1s, or that both m12-induced Nkpr1a and NK1.1 activating signals can overcome Nkpr1b inhibitory signaling to prime liver ILC1 responses. Furthermore, we cannot exclude the possibility that IL-18Rβ+ ILC1s in MCMV-infected mice may have not been exposed to m12, offering a potential explanation for why these cells did not show enhanced IFN-γ production during reinfection. Although future work will be necessary to determine the specific receptors that mediate memory ILC1 responses, the present study defines an essential role for m12 in this process. Thus, these findings leave open the possibility that other tissue-resident ILCs may express germline-encoded receptors with specificity to co-evolved pathogens to modify their effector responses during infection.

Immunological memory is classically defined in an antigen-specific context; however, cells of the innate immune system can acquire stable epigenetic modifications and/or enhanced secondary effector function after exposure to various inflammatory stimuli in an antigen-independent manner25,26. This process, referred to by some as ‘cytokine-induced memory’ and ‘trained immunity’, can be achieved in NK cells and monocytes through stimulation with the proinflammatory cytokines IL-12+IL-18 and Candida albicans-derived β-glucan, respectively27,28. Similarly, papain- or IL-33-stimulated lung ILC2s can display enhanced effector recall responses during challenge with unrelated allergens to enhance allergic pathology29. Although these studies provide evidence that memory-like responses can be observed in various innate immune cell types, the importance of these responses toward host protection during pathogen challenge are still not well understood. The present results demonstrated that liver-resident memory ILC1s are not generated during heterologous infections that induce similar production of proinflammatory cytokines, and did not display enhanced IFN-γ production after ex vivo stimulation with IL-12 and IL-18. These findings are similar to results found with MCMV-specific Ly49H+ NK cells30, and suggest that m12-dependent memory ILC1s are distinct from antigen-independent innate immune memory responses and may be more analogous to adaptive T cell and NK cell memory responses.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0430-1.

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Author contributions

O.E.W. and T.E.O. designed the study. O.E.W., N.M.A., A.D.H., L.R. and T.E.O. performed the experiments. E.S., C.K. and C.S.L. performed RNA-seq and ATAC-seq bioinformatics analysis. J.R.C. and O.A.A. provided reagents. O.E.W., J.C.S. and T.E.O. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Methods

Mice. Mice were bred at University of California, Los Angeles and Memorial Sloan Kettering Cancer Center (MSKCC) in accordance with the guidelines of the institutional Animal Care and Use Committee. The following mouse strains were used this study: C57BL/6 (CD45.2) (Jackson Labs, no. 000664), B6.SJL (CD45.1) (Jackson Labs, no. 002114), Rag2−/− (Jackson Labs, no. 008449), Kba−/− (Ly49H-deficient), Zbtb32−/−, Il12rb2−/− and Il18ra1−/− (MSKCC, J.C.S.). Experiments were conducted using 7- to 8-week-old, age- and gender-matched mice in accordance with approved institutional protocols.

Generation of mBMC mice was performed as previously described7. Paraibiosis surgery was performed as previously described7,14,15. Intravascular labeling of lymphocytes of experimental mice was performed by injecting them (i.v.) with 2.5 μg fluorescein-conjugated CD45 (30-F11) and then they were euthanized 5 min later.

Viruses and in vivo infection models. MCMV (Smith) was serially passed through BALB/c hosts three times, and then salivary gland viral stocks were purified using a discontinuous gradient of 40% over 60% Percoll. To isolate cells from the lung, spleens were dissociated using glass slides and filtered through 100-m strainers and centrifuged, and lymphocytes were removed from the supernatant. Red blood cells in the spleen, lung and liver were lysed using the manufacturer’s protocol. Apoptosis was evaluated by caspase activity staining with approved institutional protocols.

Ex vivo stimulation of lymphocytes. Approximately 3 × 10⁶ liver lymphocytes were stimulated for 5 h in RPMI containing 10% FBS with recombinant mouse IL-12 (20 ng/ml; R&D Systems) plus IL-18 (10 ng/ml; R&D Systems), plate-bound OX-110 (1:100) or plate-bound αNKp46 (29A14). Cells were cultured in media alone as a negative control.

RNA-seq and ATAC-seq. For RNA-seq, RNA was isolated from sorted cell populations of WT mice using TRIzol (Invitrogen) followed by SMARTer amplification and Illumina next-generation sequencing. For ATAC-seq, ATAC-seq libraries were prepared as previously described27. FACs-sorted cell populations (1 × 10⁶ cells) were lysed and accessible chromatin was prepared using Nextera transposase. Transposed DNA fragments were isolated using QIAugen MinElute kit and amplified for five to ten cycles using Nextera PCR primers. ATAC-seq libraries were submitted for paired-end sequencing on Illumina Hiseq.

RNA-seq and ATAC-seq analysis. RNA-seq and ATAC-seq reads were aligned and pre-processed as described previously43. RNA-seq reads were aligned using STAR (2.5.3a-foss-2016b), with filters-outFilterMultimapNmax 1–outFilterMismatchNmax 999–outFilterMismatchNoverLmax 0.02–alignIntronMax 1000000–alignMatesGapMax 1000000, to allow for stringent alignment of unique reads to the mouse (mm10) genome44. Counts were made using BEDtools v2.27.1 coveredage function. Raw count files were processed using DESeq2, removing genes with fewer than 50 counts. For ATAC-seq peak calling, read start sides aligned to the positive strand and negative strands were offset by +4 base pairs and −5 base pairs, respectively, as described by Buenrostro et al.45. Peak calling was performed on each time point (day 0, day 35 IL18R−/−) individually by pooling reads from biological replicates, and using MACS2 (ref.7) R137 with a P value threshold of 0.2. To exclude any peaks identified due to noise, an irreproducible discovery rate (IDR) was calculated7. The IDR is an estimate of the threshold where two ranked lists of results, in this case peak calls ranked by P value, no longer represent reproducible events. Using this measure we excluded peaks that were not reproducible (IDR < 0.05) across replications for each time point. We created a single atlas of accessible sites across the two time points by merging each peak list from each biological replicate. To do this way, an atlas of 23,016 reproducible peaks was created, and each peak was associated with its nearest gene in the mouse genome. Differential expression and accessibility were computed using DESeq2; genes were considered differentially expressed at an FDR-adjusted P of 0.05 and absolute value of log(fold change) < 1, and peaks were considered differentially accessible between time points at FDR-adjusted P < 1.76. For heat maps, the R package heatmap and Broad institute Morpheus was used to plot DESeq2 normalized counts. For visualization of tracks with IGV (v2.3.94), BAM files were indexed with SAMtools/1.5-foss-2016b, and then normalized by sequencing depth and converted to a BigWig file using Python/2.7.13-foss-2016b bamCoverage function. For GSEA, T cell memory raw fastq files from a previously published dataset of lymphocytic choriomeningitis virus (LCMV)-experienced T cell populations sort purified from the liver24 were processed in the the same way as the ILCl1 dataset. A list of differentially expressed genes was created compared with its corresponding naive control. From this list, genes unique to each subset were used to create a gene list for GSEA. The rank list input was created using the differentially expressed gene list and ordering them according to log(fold change) values. A correspondence signature was obtained by looking at differentially expressed genes upregulated between naive and D35 IL-18R−/− ILCs, and identifying shared, upregulated, differentially expressed genes between naive and memory T cell subsets from previously published datasets30.

Statistical analyses. For graphs, data are shown as mean ± s.e.m. and, unless otherwise indicated, statistical differences were evaluated using the Student’s t-test with Welch’s correction to assume a non-normal variance in our data distribution. P < 0.05 was considered significant. Graphs were produced and statistical analyses were performed using GraphPad Prism.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
The RNA-seq and ATAC-seq data are deposited in Gene Expression Omnibus database under the accession number GSE128906. The data that support the findings of this study are available from the corresponding author upon request.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  BD FACSDiva (v.8.0.1) was used to collect flow cytometric data.

Data analysis  FlowJo (v9.9.4) was used for flow cytometric analysis. Prism 7 and R (v.3.5.1) was used for generation of all graphs. STAR, BEDtools 2.27.1, DESeq2 was used for bio-informatic analysis. Please see Methods section in manuscript for further detail.

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All data generated and supporting the findings of this study are available within the paper. The RNA-seq and A TAC-seq data is deposited in gene expression omnibus GSE128906 is currently available.
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Data exclusions
None

Replication
Experimental findings were reproducible across multiple experiments.

Randomization
Experimental groups were not randomized. Age- and sex-matched animals were used for experiments.

Blinding
Experiments were not performed in a blinded fashion. Blinding was not relevant to our study, since we are studying and comparing the property of known cell types.

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Materials & experimental systems

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☐ Animals and other organisms
☐ Human research participants
☐ Clinical data

Methods

n/a Involved in the study

☑ ChiP-seq
☐ Flow cytometry
☑ MRI-based neuroimaging

Antibodies

The following antibodies were used for flow cytometry:

- Marker (Company, Clone, Catalog Number, Dilution)
- K-67 (BD, 1:100)
- NK1.1 (BD, 1:100)
- CD19 (BD, 1:100)
- CD49b/DX5 (BD, 1:100)
- KLRC1 (BD, 1:100)
- CD45.1 (BD, 1:100)
- CD45 (BD, 1:100)
- CD8α (BD, 1:100)
- CD4 (BD, 1:100)
- TCRB (BD, 1:100)
- CD3e (BD, 1:100)
- Ly49H (BD, 1:100)
- Ly6C (BD, 1:100)
- CD200R1 (BD, 1:100)
- CD24a (BD, 1:100)
- IFN-γ (BD, 1:100)
- F4/80 (BD, 1:100)
- CD11c (BD, 1:100)
- CD11b (BD, 1:100)
- Ly49D (BD, 1:100)
- Eomes (BD, 1:100)
- IL-7 (BD, 1:100)

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- CD4 (BD, 1:100)
- TCRB (BD, 1:100)
- CD3e (BD, 1:100)
- Ly49H (BD, 1:100)
- Ly6C (BD, 1:100)
- CD200R1 (BD, 1:100)
- CD24a (BD, 1:100)
- IFN-γ (BD, 1:100)
- F4/80 (BD, 1:100)
- CD11c (BD, 1:100)
- CD11b (BD, 1:100)
- Ly49D (BD, 1:100)
- Eomes (BD, 1:100)
- IL-7 (BD, 1:100)
IL-18ra [Biolegend, P3TUNYA, 48-5183-82, 1:300]
IL-2ra [Biolegend, PE61, 102007, 1:400]

Validation
The antibody validation is provided on supplier website. All antibodies were validated with proper isotype controls by Flow Cytometry.

Animals and other organisms

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Laboratory animals
The following mouse strains were used this study: Balb/c [Jackson Labs, 000651] #CS7Bl/6 (C57Bl/6) (Jackson Labs, #00064), B6.5IL (CD45.1) [Jackson Labs, #0022114], Rag2-/- [Jackson Labs #008449], Kir8-/- (Ly49H-deficient), Zbtb32 -/-, and Il12rb2-/- x Il18ra1-/- (MSKCC, J.C. Sun). Mice were between the ages of 8-12 weeks old, using both males and females. 10 day embryonated chicken eggs (SPAFAS; Charles River Laboratories) was used to passage virus.

Wild animals
We did not use any wild animals.

Field-collected samples
We did not collect and field samples.

Ethics oversight
Mice were bred at UCLA and Memorial Sloan Kettering Cancer Center in accordance with the guidelines of the institutional Animal Care and Use Committee (IACUC).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
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☐ All plots are contour plots with outliers or pseudocolor plots.
☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Spleens were dissociated using glass slides and filtered through a 100-μm strainer. To isolate lymphocytes from liver, the tissues were physically dissociated using a glass tissue homogenizer and purified using a discontinuous gradient of 40% over 60% Percoll. Red blood cells in spleen and liver were lysed using ACK lysis buffer.

Instrument
BD LSRII was used for data collection.

Software
FACSDiva was used for data collection, and FlowJo for data analysis.

Cell population abundance
Cell populations were sorted to >95% purity post sort in pilot experiments, as determined by flow cytometry.

Gating strategy
T cell = TCRβ+, CD3+, NK1.1-
mNK= Lin-NK1.1+ Eomes+CD49b+CD200r-
ILC1 = Lin-NK1.1+CD49b- Eomes- CD200r+ or Lin-NK1.1+CD49b+CD200r+CD11b- Ly49H-
Lin-CD3+ TCRαβ+CD19+ F4/80+

For a detailed ILC1 gating strategy please refer to figure S1 A.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.