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Adaptive Immune Features of Natural Killer Cells

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Abstract

In an adaptive immune response, naïve T cells proliferate during infection and generate long-lived memory cells that undergo secondary expansion following re-encounter with the same pathogen. Although Natural Killer cells traditionally have been classified as cells of the innate immune system, they share many similarities with cytotoxic T lymphocytes. In a mouse model of cytomegalovirus (MCMV) infection, we demonstrate that, like T cells, NK cells bearing the virus-specific Ly49H receptor proliferate 100-fold in the spleen and 1000-fold in the liver following infection. Following a contraction phase, Ly49H$^+$ NK cells reside in lymphoid and non-lymphoid organs for several months. These self-renewing "memory" NK cells rapidly degranulate and produce cytokines upon reactivation. Adoptive transfer of these NK cells into naïve animals followed by viral challenge results in a robust secondary expansion and protective immunity. These findings reveal novel properties of NK cells previously attributed only to cells of the adaptive immune system.

Key gene/proteins

Ly49H; DAP12; m157; interferon-gamma; NK1.1

During an infection, naïve T cells proliferate in response to encounter with cognate ligand and mediate effector functions1–4. This first phase of the adaptive immune response is known as the expansion phase. In many infectious models of *in vivo* T cell priming, pathogen-specific T cells become activated and expand in number over the course of one week, undergoing greater than 10 divisions, and give rise to thousands of daughter cells capable of effector functions5–7. In the second phase, known as "contraction", the activated T cells undergo apoptosis and a precipitous drop in cell numbers (90–95%) is observed in all tissues6,8. The third or "memory maintenance" phase3,9–11 is where stable populations of long-lived memory T cells reside in lymphoid and non-lymphoid tissues12,13, patrolling against previously encountered pathogens. Lastly, a fourth phase, the secondary or recall response, occurs when memory T cells re-encounter their cognate antigen and again robustly expand in numbers to fight against pathogen challenge1,4. These four phases are ascribed to

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cells of the adaptive immune system, and the latter two phases have not been previously documented in NK cells.

NK cells have many traits in common with $CD8^+$ T cells14–16. The existence of immunological memory in NK cells has recently been suggested in a model of chemical hapten-induced contact hypersensitivity17; however, the precise mechanism and identity of the antigen-specific receptors responsible for mediating the recall responses were not defined. Here, using the well-established model of MCMV infection in which NK cells provide host protection, we demonstrate that NK cells undergo all four phases of an immune response against a pathogen.

NK Cell Expansion and Contraction Phase

The early virus-specific immune response against MCMV in C57BL/6 (B6) mice is dominated by NK cells expressing the Ly49H receptor, which recognizes the virally encoded m157 protein on the surface of infected cells, and these NK cells confer protection against infection18–22. Over the first week of MCMV infection, $Ly49H^+$ NK cells undergo a 2–3 fold expansion in the spleen and ~10-fold increase in the liver, as previously described23,24 (Supplementary Fig. 1). Because $Ly49H^+$ cells constitute ~50% of total NK cells in a naïve B6 mouse, we hypothesized that a “ceiling” for NK cell expansion (measured at 80–90% of total NK cells) is rapidly achieved during infection and inhibits further proliferation in a normal host. Therefore, we sought to investigate the proliferation potential of NK cells by experimentally decreasing the initial precursor frequency of the $Ly49H^+$ cell population. We reconstituted lethally irradiated mice with 1:1 mixed bone marrow from wild-type ($CD45.1^+$) and DAP12-deficient (*Tyrobp*−/−) ($CD45.2^+$) B6 mice (which are defective in Ly49H receptor expression and function25), thus decreasing the initial frequency of fully functional, wild-type $Ly49H^+$ cells in the host to ~25% of the total NK cells (Fig. 1a). Following infection of mixed chimeric mice, wild-type NK cells preferentially expanded over 7 days, and become the predominant NK cell subset in the spleen (Fig. 1a). Although the chimeric mice contained a lower precursor frequency of $Ly49H^+$ NK cells compared to wild-type mice, by day 7 after infection, the chimeric mice had absolute numbers of $Ly49H^+$ NK cells comparable to infected wild-type mice (data not shown). The $Ly49H^+$ cells within the $CD45.1^+$ NK cell fraction upregulated expression of the maturation marker KLRG1 (Fig. 1a) and preferentially incorporated BrdU over 7 days after infection (Fig. 1b), confirming proliferation of this specific NK cell subset and excluding the possibility that DAP12-deficient NK cell populations were diminishing. Indeed, the absolute number of DAP12-deficient NK cells increased only slightly over the course of the infection (data not shown). The percentage of $Ly49H^+$ NK cells began to decline after day 7 post-infection (PI), but continued to be elevated at 15 and 28 days PI, when compared to uninfected animals, with frequencies returning to those measured in uninfected animals by 37 days PI (Fig. 1c). Similar expansion and contraction of $Ly49H^+$ NK cells were measured in the liver and lymph nodes of chimeric mice during infection (Supplementary Fig. 2). The $Ly49H^+$ NK cell response was specific for the m157 viral ligand because we did not observe preferential expansion of the Ly49H subset during infection with MCMV lacking m157 (Fig. 1d). In 1:5 wild-type:DAP12-deficient bone marrow chimeric mice, where the starting percentage of wild-type $Ly49H^+$ cells was ~10%

of total NK cells, the Ly49H$^+$ NK cells also preferentially expanded over 7 days after infection, allowing the wild-type (CD45.1$^+$) NK cells to constitute the dominant population observed at the peak of expansion (Supplementary Fig. 3a). This expansion of Ly49H$^+$ NK cells following infection observed in the spleen and liver was even greater than the proliferation measured in the 1:1 chimeras (Supplementary Fig. 3b). Together, these findings highlight the ability of a small number of precursor NK cells to robustly expand into effector cells and mount a primary response against viral infection.

We next sought to address whether maximal proliferation of Ly49H$^+$ NK cells could be achieved by adoptively transferring mature wild-type NK cells (CD45.1$^+$) into DAP12-deficient mice (CD45.2$^+$) and infecting with MCMV (Fig. 2a). Although DAP12-deficient mice have defective Ly49H$^+$ NK cells, CD8$^+$ T cell responses to MCMV are normal and mice clear infection within two weeks (data not shown). We were able to recover the transferred NK cells from recipient spleen (Fig. 2b) and thereby showed that early after infection (d1.5 PI), they were capable of becoming activated (CD69$^+$ and NKG2D^{hi}) and producing IFN-γ (Fig. 2b). Seven days after infection, we observed a dramatic increase in transferred NK cell numbers, with preferential expansion observed in the Ly49H$^+$ NK cell subset (Fig. 2c). Adoptive transfer of CFSE-labeled NK cells confirmed that only the Ly49H$^+$ NK cells were dividing, fully diluting their CFSE during the 7 days following infection, while the Ly49H$^-$ NK cell subset remained CFSE^{hi} (Fig. 2d). NK cells transferred into DAP12-deficient mice expanded as much as 100-fold in the spleen following infection (Fig. 2e). This expansion was not seen when NK cells were adoptively transferred into wild-type B6 mice, which contain competing endogenous Ly49H$^+$ NK cells and thus restrict proliferation of the transferred cells (Fig. 2e). The expansion of Ly49H$^+$ NK cells was not unique to DAP12-deficient recipients because we also observed robust expansion of Ly49H$^+$ NK cells when they were adoptively transferred into C57BL/6 mice that do not possess the *Ly49h* gene (data not shown).

The amplitude and kinetics of the MCMV-specific NK cell response measured in our system mirrors analogous responses in primary T cells7,26, as well as with adoptive transfer of TCR-transgenic T cells5,27. Furthermore, adoptive transfer of NK cells permits us to track the congenic CD45.1$^+$ cells late after infection, allowing us to distinguish between antigen-experienced NK cells and naïve NK cells recently exiting the bone marrow. Using this experimental approach, we were able to recover a long-lived “memory” pool of NK cells that can persist in lymphoid as well as non-lymphoid tissues such as the liver (Fig. 3a–b). We examined whether lowering the precursor frequency allowed for greater expansion of NK cells by measuring the amplitude of the Ly49H$^+$ NK cell response in mice after transfer of 10^5 or 10^4 cells, and determined that both the kinetics and fold expansion were comparable in spleen (~100-fold) and liver (~1000-fold) of MCMV-infected mice irrespective of initial transfer numbers (Fig. 3a–b). Thus, a lower threshold exists where small precursor NK cell numbers no longer lead to enhanced overall responses. A summary of Ly49H$^+$ NK cell fold-expansion in B6 mice, the different mixed chimeric mice, and the adoptive transfer system is found in Fig. 3c, highlighting the previously underappreciated ability of antigen-specific NK cells to undergo a profound expansion and persist following viral infection.

NK Cell Memory Phase

In our adoptive transfer model, the contraction phase observed during the NK cell response to MCMV infection (Fig. 3a–b) appears to emulate the prolonged decline in effector cell numbers observed with antigen-specific $CD4^+$ T cell responses26, rather than the rapid and more precipitous decline observed with most $CD8^+$ T cell responses8. Following the contraction phase, we were able to detect "memory" NK cells at 70 days PI (Fig. 4a), and sought to determine whether these cells were still functional. These "memory" NK cells were indeed functional, as they produced IFN-γ *ex vivo* in response to plate bound antibody to NK1.1 or Ly49H (Fig. 4a). In fact, their response was heightened, compared to the response by naïve NK cells, in that a greater percentage of "memory" NK cells responded to plate-bound antibodies as well as m157-expressing target cells, and the amounts of IFN-γ produced by individual cells were higher (Fig. 4b and Supplementary Fig. 4). Degranulation by "memory" NK cells was also enhanced, as assessed by LAMP-1 (CD107a) expression following *ex vivo* stimulation with anti-NK1.1 (Fig. 4c).

"Memory" $Ly49H^+$ NK cells could be distinguished from naïve $Ly49H^+$ NK on the basis of several surface antigens. Notably, "memory" NK cells expressed higher levels of the Ly49H receptor, but not other activating receptors, compared with naïve NK cells (Fig. 4d), perhaps permitting "memory" NK cells to have a lower threshold for activation. "Memory" NK cells also demonstrated higher expression of KLRG1, CD43, and Ly6C, and a decreased expression of CD27, suggesting they are more mature than naïve NK cells (Fig. 4d). A similar phenotype was also observed on long-lived NK cells isolated from non-lymphoid tissues such as the liver (Supplementary Fig. 5). "Memory" NK cells also constitutively expressed higher levels of IFN-γ transcripts when compared with naïve NK cells (Fig. 4e), as assessed by studies using the IFN-γ reporter *Yeti* mice28. Altogether, these data suggest that "memory" NK cells possess characteristics similar to memory T cells in both their phenotype and ability to robustly produce effector cytokines.

NK Cell Recall Phase

Antigen-specific memory T cells undergo secondary expansion, and we investigated whether "memory" NK cells also possessed this adaptive immune characteristic. Because mice previously immunized with MCMV contain neutralizing antibodies and memory CTL, we could not directly challenge with a second dose of virus to examine recall responses. Therefore, following the primary expansion and contraction phase in our adoptive transfer model, we enriched for "memory" $Ly49H^+$ NK cells ($CD45.1^+$) at 40–50 days PI, adoptively transferred these cells into a second DAP12-deficient mouse ($CD45.2^+$), and infected with MCMV (Fig. 5a). Although the transferred "memory" NK cells were present in small numbers in uninfected recipients and at d1.5 after infection, an expanded population of transferred $Ly49H^+$ NK cells could be readily detected at 7 and 14 days PI (Fig. 5b). Thus, like memory T cells, "memory" NK cells are self-renewing, long-lived, and can mount a secondary response after viral challenge, with expansion measured at greater than 100-fold in the spleen (Fig. 5c). When CFSE-labeled naïve and "memory" NK cells were transferred into separate DAP12-deficient recipient mice and proliferation kinetics compared following MCMV infection, we observed a similar rate of CFSE dilution at day 3 PI, and

found that both groups of NK cells were CFSE-negative (indicating at least 10 divisions) by day 6 PI (Fig. 5d). Although large numbers of naïve NK cells were easier to obtain and thus more cells were transferred into recipient animals than "memory" NK cells, the kinetics and magnitude of expansion between naïve and "memory" NK cell populations were comparable (Fig. 5d). Overall MCMV-specific NK cell responses following adoptive transfer of normalized naïve and "memory" NK cell numbers showed no significant differences in the expansion and contraction phases (Fig. 5e). Several careful studies of $CD8^+$ T cell responses have also revealed similarities in naïve and memory T cell proliferation rates29–33, suggesting that the increased memory T cell responses could be attributed to higher precursor frequency rather than a differential rate of proliferation. Although phenotypic and functional differences exist between naïve and "memory" NK cells, it is not surprising that the kinetics of expansion between these populations do not differ, given the property of naïve NK cells as "ready-to-respond" effectors14. In fact, memory $CD8^+$ T cells have often been compared to NK cells for their ability to rapidly mediate effector functions14.

Lastly, we wanted to determine whether "memory" NK cells are more protective than naive NK cells. We adoptively transferred equal numbers of naive and "memory" $Ly49H^+$ NK cells into neonates, which are MCMV-susceptible due to the lack of mature NK cells34, challenged with MCMV, and monitored survival. Neonatal mice receiving "memory" NK cells showed significant protection against viral infection compared to an equivalent number of naive NK cells ($p=0.0024$) (Fig. 5f). At least ten-fold more naïve NK cells were required to mediate protection against MCMV infection compared with the "memory" NK cells. When neonatal recipients were treated with an antibody that blocks the Ly49H receptor, protection was abrogated ($p=0.03$) (Fig. 5f). Thus, like T cells, NK cells have the ability to "remember" previously encountered pathogens and are able to mediate more efficient protective immunity against subsequent infection.

Conclusions

Because the innate immune system is considered evolutionarily older than the adaptive immune system, NK cells bearing receptors that recognize MHC and MHC-like molecules may have arisen as a predecessor to T cells, which express clonally selected receptors for the recognition of a limitless antigen repertoire in the context of MHC. Alternatively, NK cells may have co-evolved with T cells. As an evolutionary bridge, NK cells might conceivably possess attributes of both innate and adaptive immunity, and we are only beginning to unearth characteristics of the latter. The categorization of NK cells within the immune system is beginning to undergo a paradigm shift, which will have major implications in our approach to vaccination strategies for the generation of immunological memory against pathogens.

METHODS

Mice and infections

C57BL/6 and congenic $CD45.1^+$ mice were purchased from the National Cancer Institute. DAP12-deficient mice25, backcrossed 13 generations onto C57BL/6, were bred and maintained in accordance with IACUC guidelines. Dr. Richard Locksley (UCSF) generously

provided *Yeti* mice28. Mixed bone marrow chimeric mice were generated as described35. Mice were infected by intraperitoneal injections of Smith strain MCMV (5×10^4 PFU) and MCMV-Δm157 (10^5 PFU) (generously provided by Dr. Ulrich Koszinowski) 36. Neonate mice were infected with 2×10^3 PFU MCMV.

NK cell enrichment and adoptive transfer

NK cells were isolated using an NK cell Isolation Kit (Miltenyi Biotec) and injected intravenously into adult recipients or intraperitonally into neonatal recipients one day before viral infection. Neonatal mice were given 50 μg of anti-Ly49H mAb 3D10 one day prior to infection.

Ex vivo stimulation of NK cells

DOTAP-treated (Sigma) tissue culture plates were coated with anti-NK1.1 or anti-Ly49H (generously provided by Dr. Wayne Yokoyama) and cells were incubated for 5 hours at 37°C in the presence of Golgiplug (BD Pharmingen), followed by staining for intracellular cytokines. NK cells were cocultured with Ba/F3 cells or m157-transduced Ba/F318 prior to intracellular cytokine staining.

Flow cytometry

Fc receptors were blocked by using 2.4G2 mAb prior to surface staining with indicated antibodies or isotype-matched control Ig (BD or eBiosciences). BrdU staining was done using a BrdU Flow Kit (BD). CFSE-labeling of cells was performed according to manufacturer's instructions (Invitrogen). Samples were acquired on a LSRII (BD) and analyzed using FlowJo software (TreeStar).

Statistical methods

The Mann-Whitney nonparametric U test was used to compare survival between groups of mice. A value of 25 days was assigned to survivors that lived more than 25 days after MCMV infection. The Student's t-test was used to compare groups in *ex vivo* stimulation experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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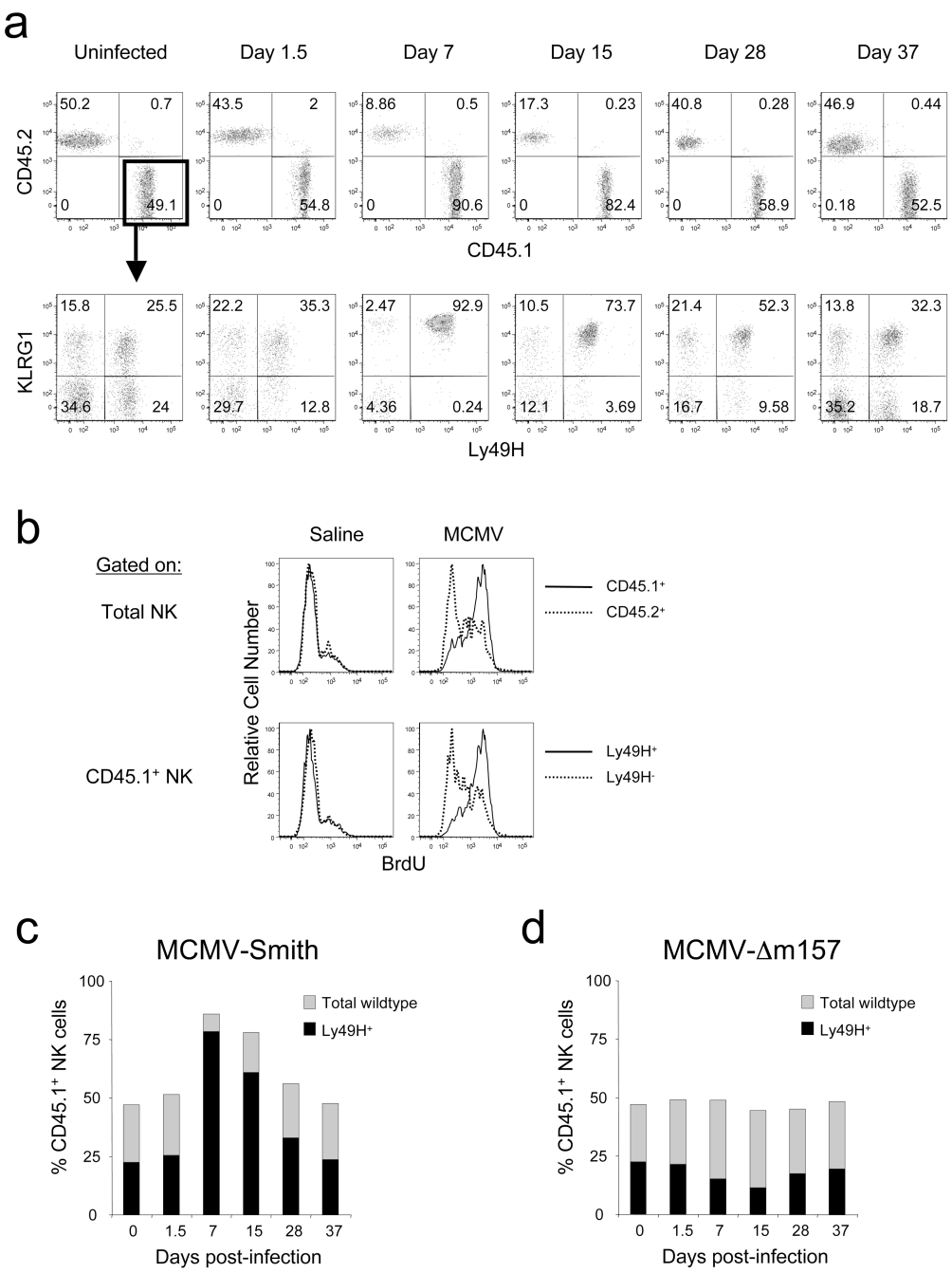


Figure 1. Preferential expansion of wild-type but not DAP12-deficient NK cells during MCMV infection

Mixed bone marrow chimeric mice (1:1 mixture of wild-type (CD45.1$^+$) and DAP12-deficient (CD45.2$^+$) cells) were infected with MCMV. **a**, Upper, percentages of wild-type and DAP12-deficient NK cells (gated on CD3$^-$, NK1.1$^+$). Lower, Ly49H and KLRG1 on wild-type NK cells. **b**, Upper, BrdU incorporation (day 7 PI) of wild-type (solid lines) and DAP12-deficient (dotted lines) NK cells. Lower, BrdU incorporation by Ly49H$^+$ (solid lines) and Ly49H$^-$ (dotted lines) wild-type NK cells. **c**, **d** Percentages of Ly49H$^+$ cells

within the wild-type NK cell population following infection with MCMV (**c**) or MCMV-Δm157 (**d**). Data are representative of 3 experiments with 3–5 mice per time point.

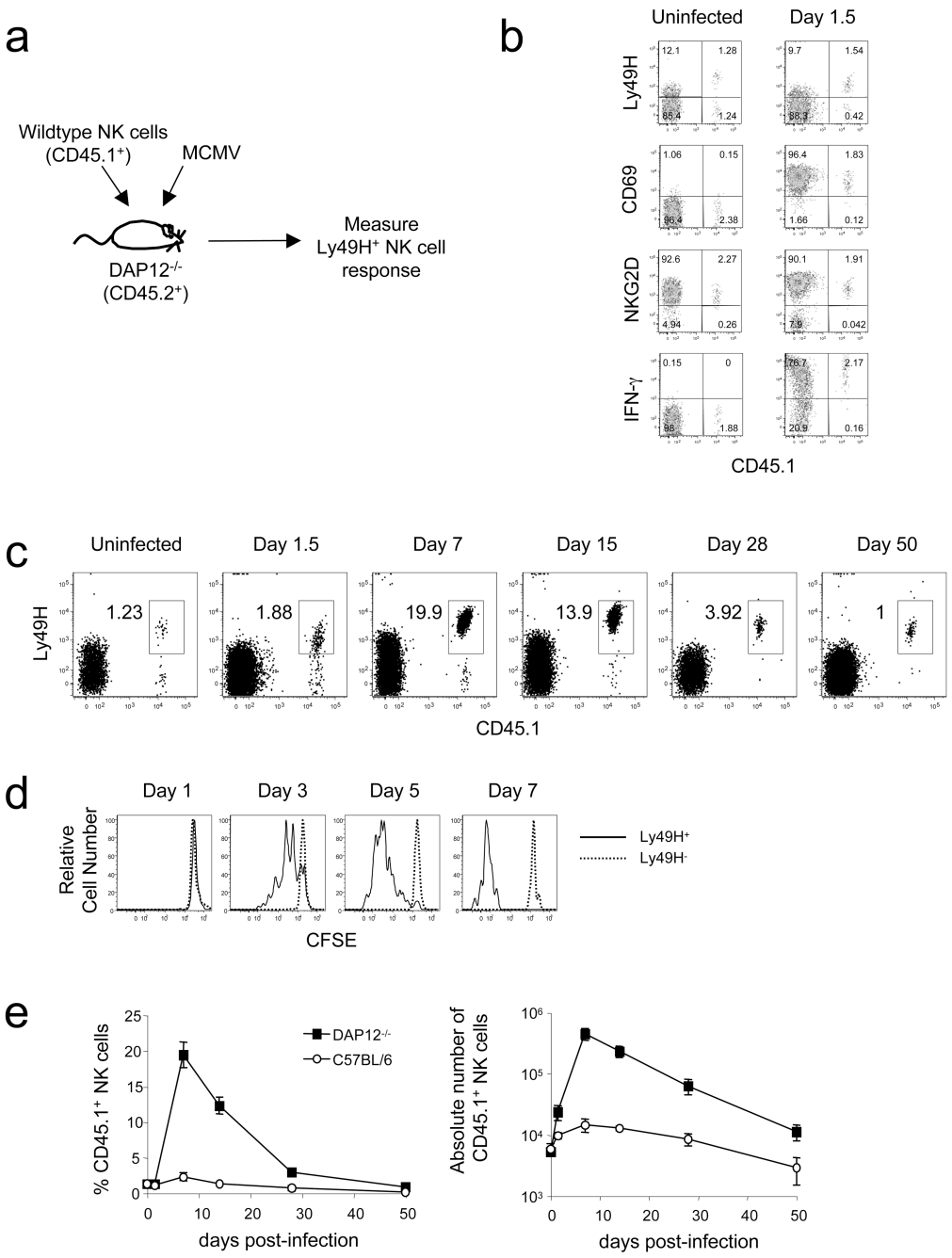


Figure 2. Robust proliferation of adoptively transferred wild-type NK cells in DAP12-deficient mice following MCMV infection

a, 10^5 wild-type NK cells (CD45.1$^+$) were transferred into DAP12-deficient mice (CD45.2$^+$) and infected with MCMV. **b**, Transferred NK cells (CD45.1$^+$) within the total CD3$^-$ NK1.1$^+$ gated population analyzed for Ly49H, CD69, NKG2D, and intracellular IFN-γ. **c**, Percentages of transferred CD45.1$^+$ Ly49H$^+$ NK cells within the total CD3$^-$ NK1.1$^+$ population after infection. **d**, CFSE-labeled wild-type NK cells (5×10^5) were transferred into DAP12-deficient hosts. Ly49H$^+$ and Ly49H$^-$ NK cells were analyzed after infection. **e**,

Percentages (left graph) and absolute numbers (right graph) of transferred CD45.1$^+$ NK cells in DAP12-deficient or wild-type B6 recipients after infection. Error bars display s.e.m. (n = 3–5). Data are representative of 5 experiments.

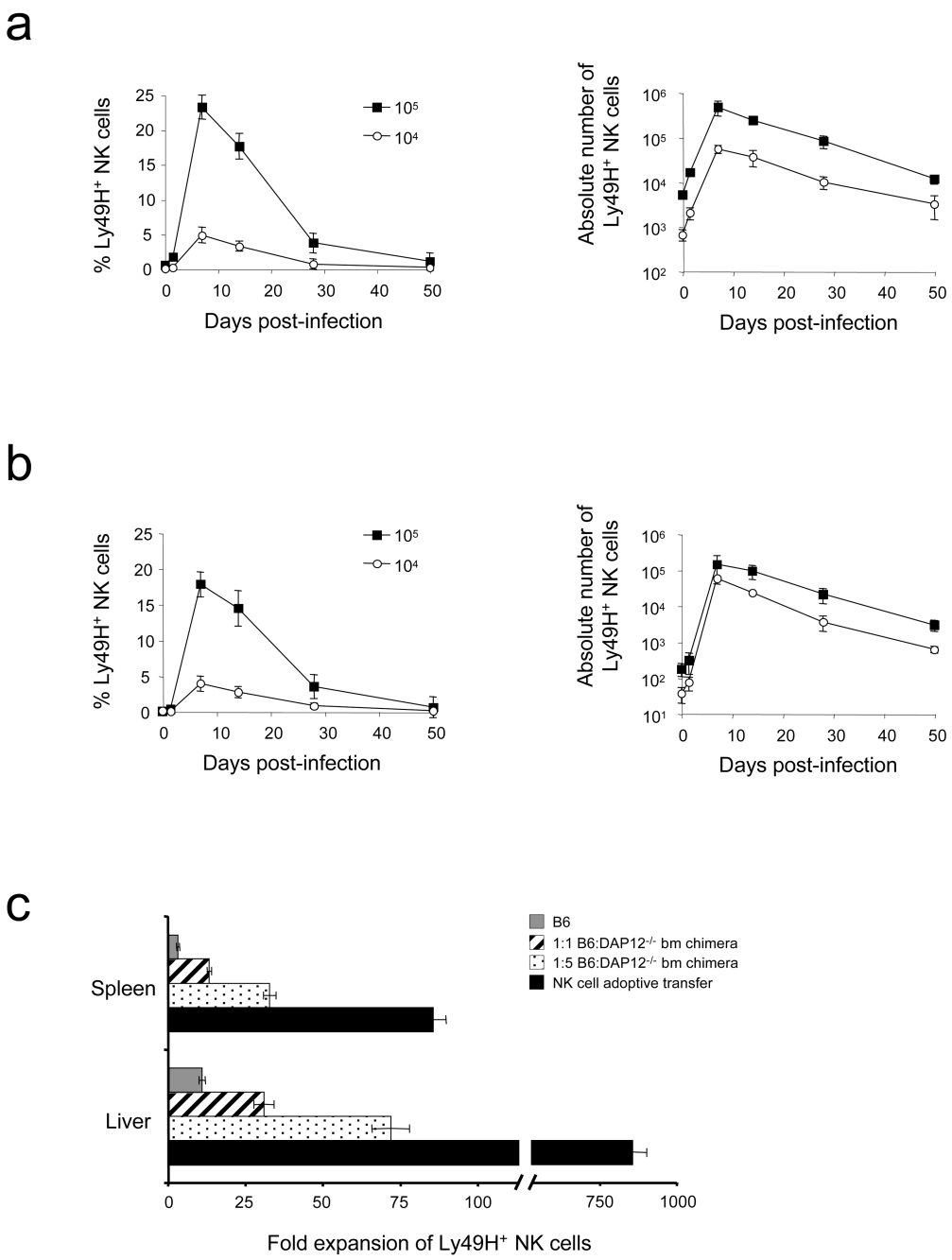


Figure 3. Expansion and contraction of NK cells in lymphoid and non-lymphoid tissues results in "memory" NK cells

a–b, Following adoptive transfer of 10^5 (squares) or 10^4 (circles) wild-type NK cells (CD45.1$^+$) into DAP12-deficient mice and MCMV infection, percentages (left) and absolute numbers (right) of Ly49H$^+$ NK cells in spleen (**a**) and liver (**b**). Error bars display s.e.m. (n = 3–5). Data are representative of 3 experiments. **c**, Fold expansions of Ly49H$^+$ NK cells over 7 days of infection were calculated in B6 mice, in 1:1 and 1:5 wild-type:DAP12-deficient bone marrow chimeric mice, and following adoptive transfer of wild-type NK cells

into DAP12-deficient mice. Error bars display s.e.m. from 3 experiments in each group of mice indicated.

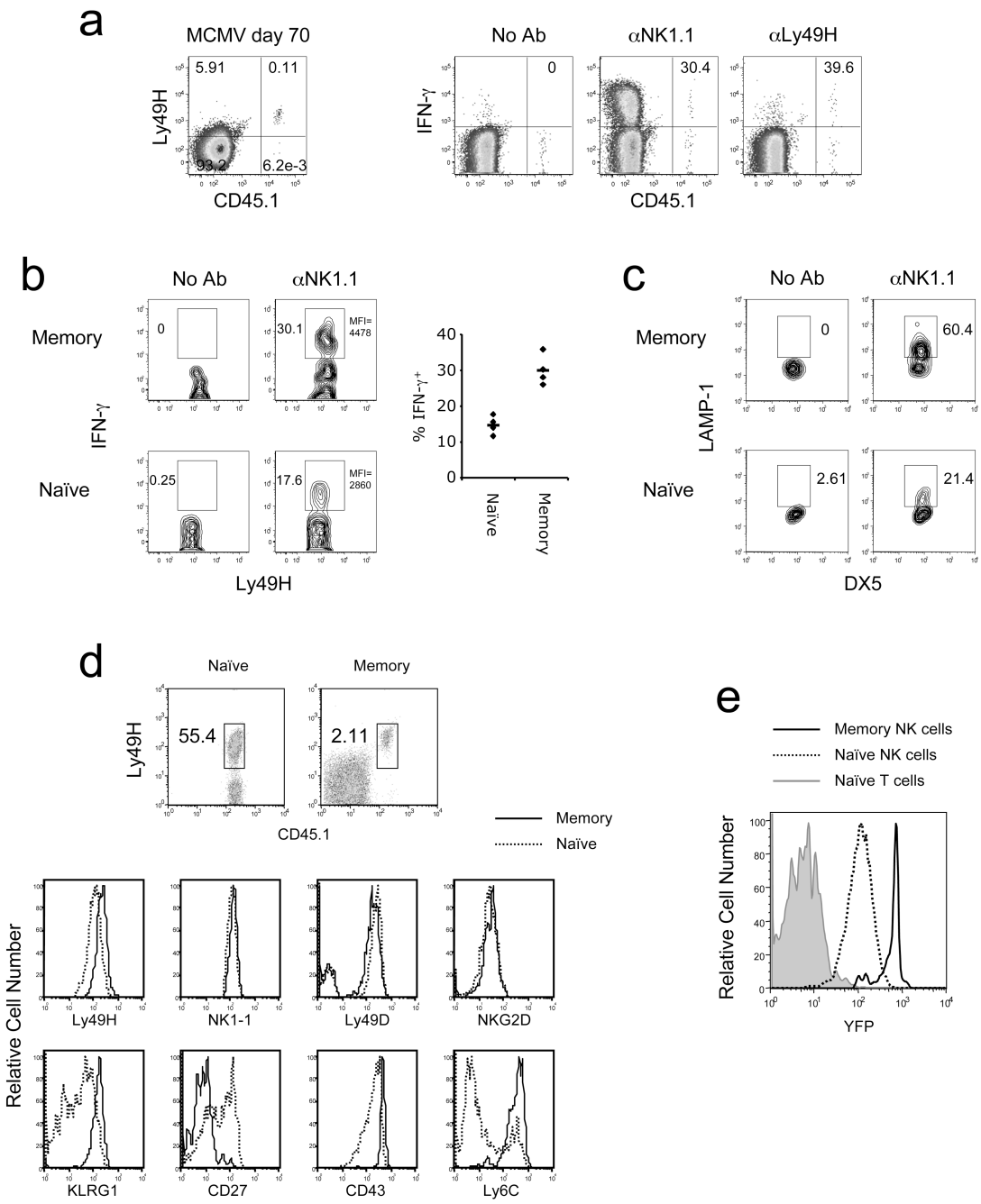
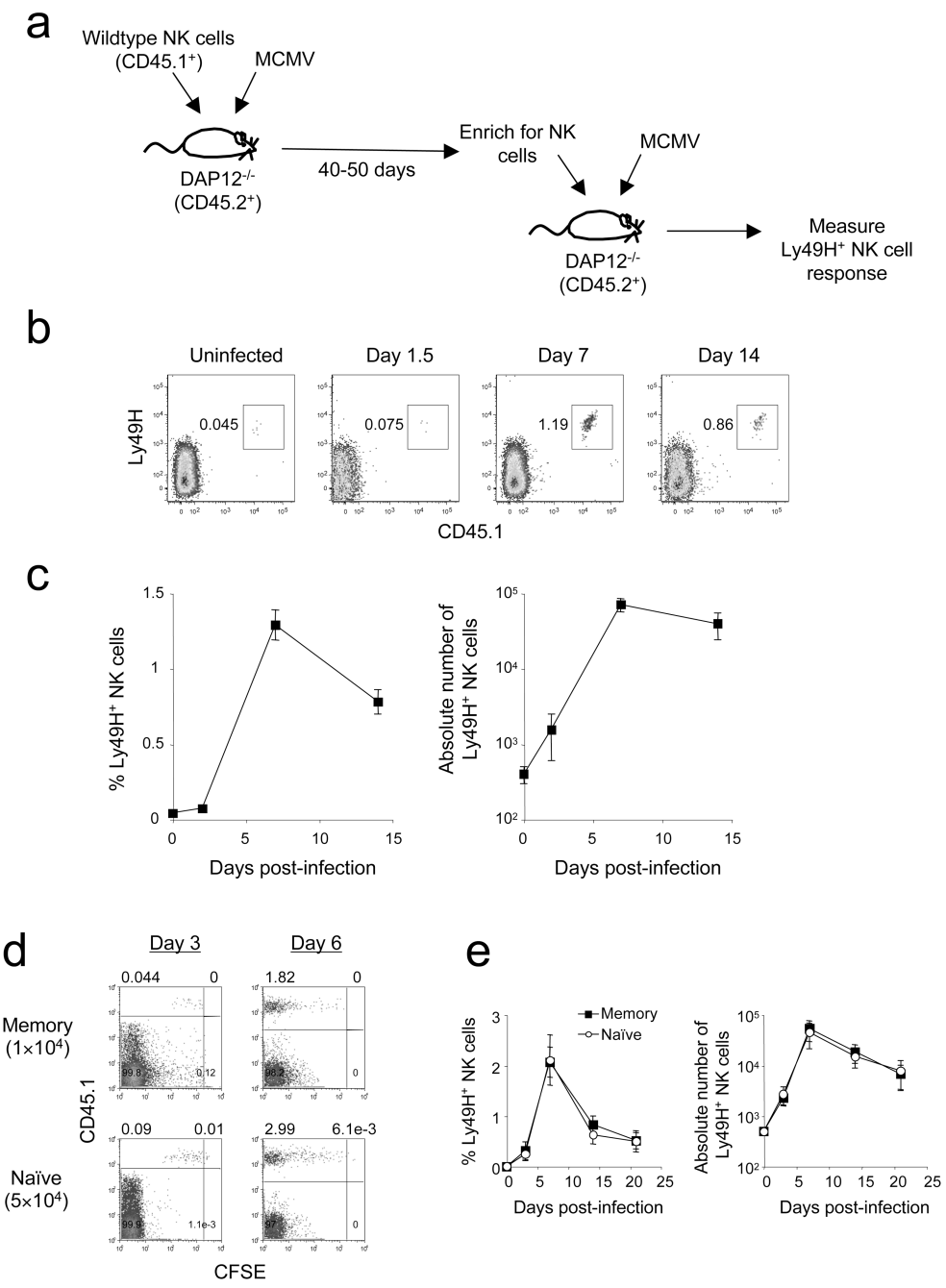


Figure 4. Function and phenotype of "memory" NK cells

a, 10^5 wild-type NK cells (CD45.1$^+$) transferred into DAP12-deficient mice (CD45.2$^+$) were analyzed 70 days after MCMV infection. Left, percentage of CD45.1$^+$ Ly49H$^+$ cells within total NK cell population. Right, percentages of CD45.1$^+$ NK cells producing IFN-γ after stimulation. **b**, "Memory" NK cells compared to naïve NK cells from uninfected mice after anti-NK1.1 stimulation. Percentages of Ly49H$^+$ NK cells (gated on total NK cells) producing IFN-γ. Right, percentages of Ly49H$^+$ NK cells producing IFN-γ (4 mice/group, horizontal bar is mean, p = 0.0009). **c**, LAMP-1 on "memory" NK cells (day 55 PI) versus

naïve NK cells (gated on Ly49H$^+$ NK cells) after anti-NK1.1 stimulation. **d**, Surface markers on "memory" NK cells (day 45 PI) versus naïve NK cells. **e**, NK cells from day 7 MCMV-infected *Yeti* mice were transferred into DAP12-deficient *Rag2*$^{-/-}$ mice. YFP in "memory" Ly49H$^+$ NK cells after 28 days compared to Ly49H$^+$ NK cells and T cells in uninfected *Yeti* mouse.



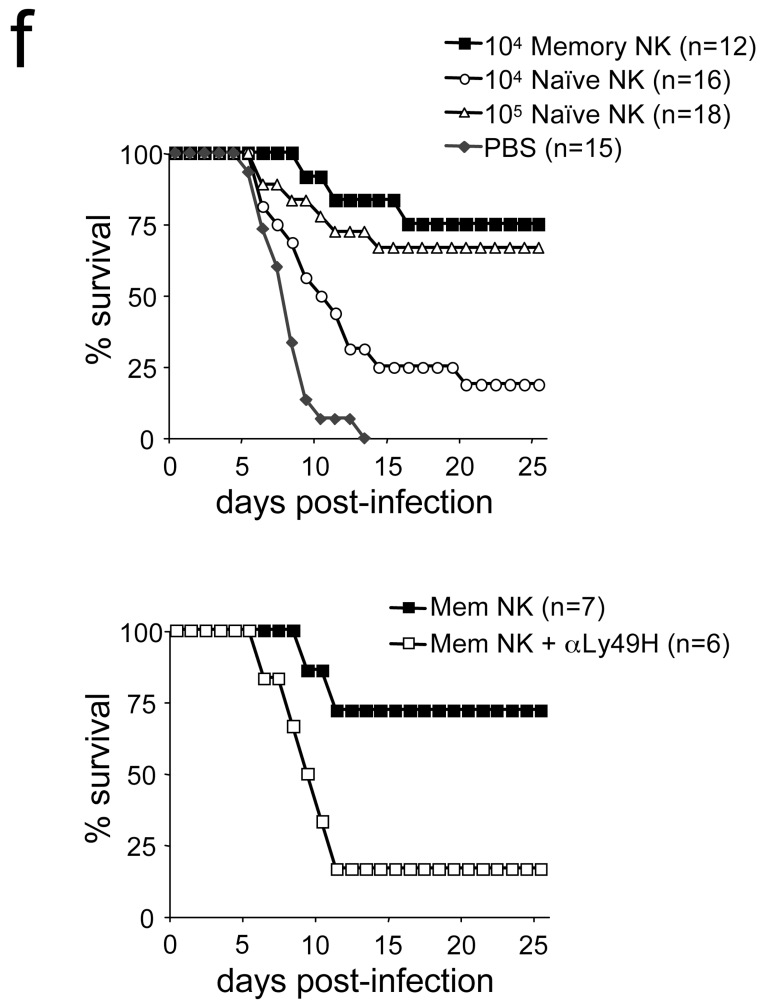


Figure 5. Secondary expansion and protective immunity in "memory" NK cells

a, 10^5 wild-type NK cells (CD45.1$^+$) transferred to DAP12-deficient mice (CD45.2$^+$) were isolated 40–50 days after primary MCMV infection and transferred into DAP12-deficient mice (CD45.2$^+$). Following infection of the second host, Ly49H$^+$ NK cells were analyzed. **b**, Percentages of transferred "memory" Ly49H$^+$ NK cells in the second host. **c**, Percentages (left) and absolute number (right) of Ly49H$^+$ NK cells within the total NK cell population in second host. Error bars display s.e.m. (n = 3–5). Data are representative of 5 experiments. **d**, Analysis of CSFE-labeled "memory" and naïve NK cells transferred into DAP12-deficient recipient mice (day 3 and 6 PI). **e**, Expansion and contraction of "memory" and naïve NK cells (1×10^4 input) shown as a percentages (left) and absolute number (right) of Ly49H$^+$ NK cells within the total NK cell population. Error bars display s.e.m. (n = 3–5). Data are representative of 3 experiments. **f**, Survival of DAP12-deficient neonatal mice receiving 1×10^4 or 1×10^5 naive, or 1×10^4 "memory" NK cells (or PBS as control) followed by MCMV infection, with or without anti-Ly49H blocking. Data were pooled from 3 experiments.