LETTERS

Memory CD8 T-cell compartment grows in size with immunological experience

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Memory CD8 T cells, generated by natural pathogen exposure or intentional vaccination, protect the host against specific viral infections[1]. It has long been proposed that the number of memory CD8 T cells in the host is inflexible, and that individual cells are constantly competing for limited space[2,3]. Consequently, vaccines that introduce over-abundant quantities of memory CD8 T cells specific for an agent of interest could have catastrophic consequences for the host by displacing memory CD8 T cells specific for all previous infections[4–6]. To test this paradigm, we developed a vaccination regimen in mice that introduced as many new long-lived memory CD8 T cells specific for a single vaccine antigen as there were memory CD8 T cells in the host before vaccination. Here we show that, in contrast to expectations, the size of the memory CD8 T-cell compartment doubled to accommodate these new cells, a change due solely to the addition of effector memory CD8 T cells. This increase did not affect the number of CD4 T cells, B cells or naive CD8 T cells, and pre-existing memory CD8 T cells specific for a previously encountered infection were largely preserved. Thus, the number of effector memory CD8 T cells in the mammalian host adapts according to immunological experience. Developing vaccines that abundantly introduce new memory CD8 T cells should not necessarily ablate pre-existing immunity to other infections.

Conventional knowledge dictates that the size and composition of the adaptive immune system, including the number of memory CD8 T cells, is invariable. This paradigm serves as the basis for theories regarding the regulation of immune memory and stipulates that new infections and vaccines erode pre-existing immunity. However, because of variability between mice and the fact that single infections result in the addition of relatively small new memory populations, it is actually quite challenging to enumerate changes in the composition and size of different lymphocyte compartments induced by the addition of new memory CD8 T cells. We overcame this difficulty by developing a unique heterologous prime–boost vaccination strategy designed to introduce enormous numbers of memory CD8 T cells specific for a single antigen[7], equal in number to the entire population of memory CD8 T cells specific for all previously encountered infections in unvaccinated mice. This event could reflect what occurs naturally on repeated exposure to serological variants of pathogens that express shared CD8 T-cell epitopes, or more probably, such a response could be intentionally induced to test the potential of memory CD8 T cells to protect against pathogens for which antibody-based vaccines have consistently failed[8]. Most importantly, in this context, it provides a more stringent test of the impact of introducing new memory CD8 T cells on the size of the naive and memory CD8 T-cell compartments, the size of the B and CD4 T-cell compartments, and the number of pre-existing memory CD8 T cells specific for a different pathogen.

To establish a defined and easily detectable population of pre-existing memory CD8 T cells, we transferred naive P14 cells, which are CD8 T cells specific for an immunodominant epitope of lymphocytic choriomeningitis virus (LCMV), into C57Bl/6J mice, and then infected recipient mice with LCMV (Armstrong strain)[9]. This allowed us to track precisely a population of non-cross-reactive monoclonal memory CD8 T cells. After LCMV-specific memory was established, mice were subjected to three heterologous prime–boost immunizations, which resulted in a large addition of memory CD8 T cells specific for the 'N' peptide of vesicular stomatitis virus (Fig. 1a–c)[7]. Control mice received phosphate buffered saline (PBS) instead of three heterologous prime–boost immunizations, which did not result in an N-peptide-specific response (Fig. 1b).

Consistent with previous reports[10,11], new immune challenges caused a substantial reduction in the percentage of CD8 T cells specific for LCMV, suggesting profound attrition of pre-existing memory CD8 T cells (Fig. 1b, c). However, we noted that the proportion of $CD8^+$ lymphocytes in blood unexpectedly increased permanently, from $11.1 \pm 0.8\%$ ($\pm$ s.e.m.) in control mice to $25.4 \pm 1.1\%$ in boosted mice ($P < 0.0001$, measured 94 days after final immunization or PBS inoculation, Fig. 1d). If the size of the CD8 T-cell compartment is malleable, it confounds interpretation based on percentages alone (Fig. 1e).

For this reason, we counted the numbers of CD8 T, CD4 T, and B cells in various tissues between 80 and 160 days after the final immunization. We found that heterologous prime–boost immunizations induced a permanent increase in the number of CD8 T cells in almost all tissues, with no significant affect on the number of CD4 T or B cells. For example, the number of CD8 T cells in the spleen increased by 54.9% relative to control mice (Fig. 2), and similar, or even greater, increases were noted in liver, bone marrow (data not shown) and lung (94.5% increase, Supplementary Fig. 1a). The one exception was lymph node, in which there was no change in the number or composition of lymphocytes (Supplementary Fig. 1b). In addition, no changes were seen among any subset of CD4 T cells in any tissue (Supplementary Figs 1 and 2).

We then determined whether the increase in CD8 T cells reflected changes in the numbers of naive CD8 T cells, memory CD8 T cells or both by staining cells for CD44 and CD62L. We found that heterologous prime–boost immunization induced no change among naive ($CD44^{lo}$) CD8 T cells (Fig. 3). Consistent with these data, prime–boost immunized mice mounted primary CD8 T-cell responses that were equivalent to age-matched controls when challenged with a new infection (Supplementary Fig. 3), suggesting no defect in immunocompetence or reduction in naive CD8 T-cell responsiveness.

The increase in CD8 T cells in heterologous prime–boost immunized mice was entirely due to a large increase in the number of

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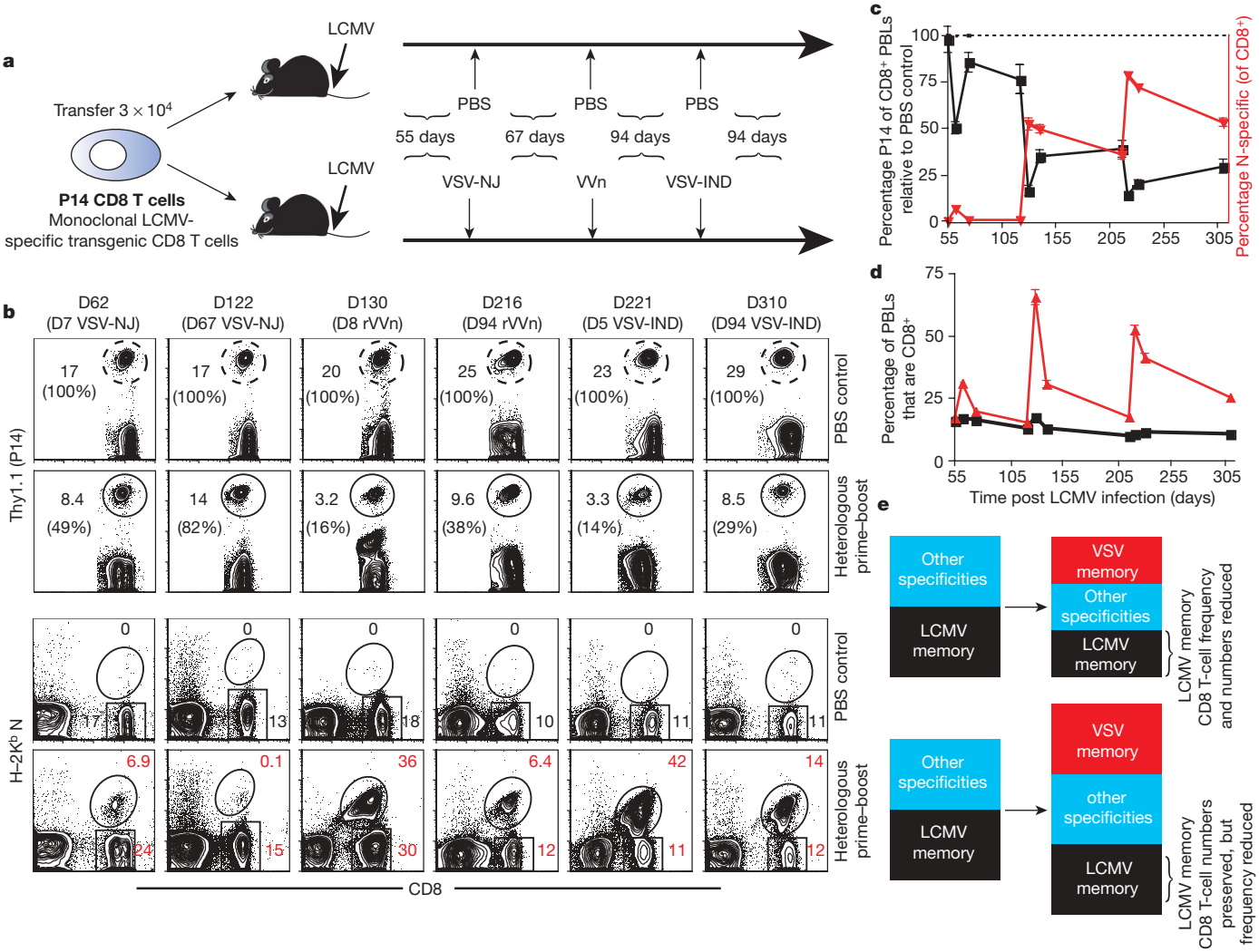

Figure 1 | Heterologous prime–boost vaccination induces a huge addition of memory CD8 T cells and a permanent increase in the proportion of CD8$^+$ lymphocytes. **a**, A monoclonal population of memory CD8 T cells was generated by transferring naive P14 CD8 T cells to naive mice and immunizing with 2×10^5 p.f.u. LCMV. After 55 days, mice were subjected to the heterologous prime–boost regimen (as described in ref. 7 and Methods) or three inoculations of PBS (controls). **b**, **c**, Analysis of virus-specific CD8 T cells in blood. In **b**, representative flow cytometry data indicate the percentage of P14 among CD8 T cells (top two rows). The numbers in parentheses indicate the percentage of P14 CD8 T cells in the heterologous prime–boosted group relative to control mice. The bottom two rows indicate the percentage of N-antigen-specific CD8 T cells (as visualized by H–2K^b N tetramer staining) and the percentage of other CD8 T cells among total lymphocytes on various days (D) after infection. In **c**, black squares indicate the percentage of P14 among CD8 T cells, normalized to control mice. Red triangles indicate the percentage among total CD8 T cells that are specific for N antigen. **d**, Percentage of CD8$^+$ cells among all PBLs from control (black) or boosted (red) mice. Error bars indicate s.e.m. and $n = 16$–18. **e**, Interpretation of memory CD8 T-cell homeostasis depends on whether the size of the compartment is fixed or flexible. The upper panels represent a model of fixed homeostasis in which total cell numbers are constant and introduction of new memory specificities erodes the absolute number of memory cells for other pathogens. In contrast, in the flexible memory compartment model (lower panels), addition of new memory cells is accommodated without attrition of other clones, but the frequency of unrelated pre-existing memory CD8 T cells declines nevertheless.

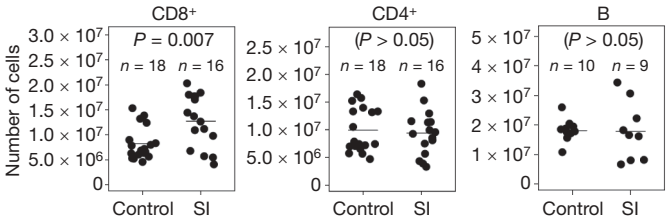

Figure 2 | Total numbers of CD8 T, CD4 T, and B lymphocytes in the spleen of control mice and mice that have been sequentially immunized with VSV-NJ, VVn and VSV-IND. Between 80 and 160 days post final immunization, the numbers of CD8 T, CD4 T, and B cells were counted in the spleen of sequentially immunized mice (SI), and compared to that of control mice. Statistics were determined by unpaired two-tailed *t*-test.

memory (CD44^{hi}) CD8 T cells (Fig. 3). In fact, this increase occurred almost exclusively in the CD62L^{lo} subset of memory CD8 T cells that are often referred to as non-lymphoid effector memory T cells. These data demonstrate that the size of the memory CD8 T-cell compartment is not fixed, and grows with immunological experience.

We then counted the number of new N-peptide-specific memory CD8 T cells, as well as the number of pre-existing memory (P14) CD8 T cells specific for LCMV (Fig. 4). As expected, no N-peptide-specific memory CD8 T cells were detected in control mice. The heterologous prime–boost regimen resulted in so many memory CD8 T cells specific for N, that the population was equivalent in size to the entire CD44^{hi} (memory) CD8 T-cell population in control mice. If the size of the memory CD8 T-cell compartment was fixed, one would predict that almost 100% of pre-existing memory CD8 T cells would be ablated. In contrast, memory P14 CD8 T cells specific for LCMV only

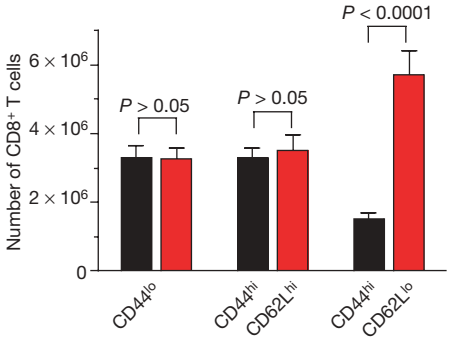

Figure 3 | Increase among CD8 T cells is due solely to an increase in CD62L^{lo} CD44^{hi} memory CD8 T cells. Between 80 and 160 days post final immunization, splenocytes were stained with anti-CD8, -CD44 and -CD62L, and the number of CD8 T cells in each subset were determined ($n = 16$–18). Black bars indicate control mice and red bars indicate heterologous prime–boosted mice. Statistics were determined by unpaired two-tailed *t*-test; error bars are s.e.m.

underwent a modest reduction (from $1.38 \pm 0.17 \times 10^6$ to $9.15 \pm 1.36 \times 10^5$, or a 33.6% reduction, $P = 0.0484$, $n = 16$–18 per group). Modest reductions in memory P14 CD8 T cells were also seen in other tissues (25.6% reduction in lung, $P = 0.485$, and 33.4% reduction in lymph node, $P = 0.390$, $n = 13$–15 per group). These data demonstrate that when very large numbers of new memory CD8 T cells are introduced, the erosion of pre-existing memory CD8 T cells is far less than predicted by a model of fixed homeostasis.

In other experiments, polyclonal endogenous LCMV-specific memory CD8 T cells in C57Bl/6J and/or Balb/c mice did not undergo attrition in response to infections with vaccinia (a virus that elicits a large CD8 T cell response), *Listeria monocytogenes* (an intracellular bacteria) or *Plasmodium yoelii* (a parasite that causes spleens of mice to increase to $\sim 900 \times 10^6$ lymphocytes), as shown in Supplementary Fig. 4. Although these results do not exclude that certain infections could result in memory T-cell loss—as a result of infection-associated pathology, destruction of lymphoid tissue or direct infection of lymphocytes—we found little evidence of attrition in all models tested.

In conclusion, the memory CD8 T-cell compartment is not fixed in size, as previously thought. Instead, the size and proportion of different subsets of the adaptive immune system are flexible and reflect adaptation to the immunological experience of the individual. These results seem to contradict earlier work describing profound attrition of memory CD8 T cells on exposure to new pathogens[10,11]. However, it should be noted that previous studies quantified this decline as a percentage of CD8 T cells. We also observed a substantial

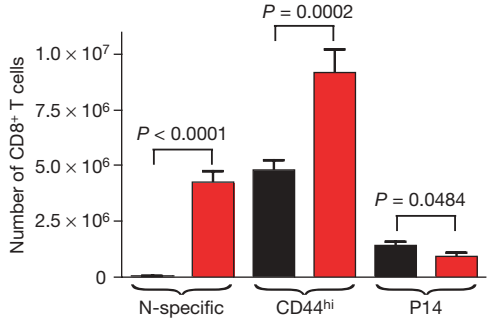

Figure 4 | Addition of very large numbers of memory CD8 T cells induces only modest attrition of pre-existing memory CD8 T cells. Total CD44^{hi}, P14 and N-specific CD8 T cells were counted between 80 and 160 days post final immunization and compared to control mice ($n = 16$–18). Black bars indicate control mice and red bars indicate heterologous prime–boosted mice. Statistics were determined by unpaired two-tailed *t*-test, error bars are s.e.m.

reduction in the frequency of pre-existing memory CD8 T cells specific for LCMV (Fig. 1). However, because the number of CD8 T cells increased in the organism, the erosion of pre-existing memory CD8 T cells was much less than predicted by frequency measurements alone. It should also be noted that previous studies restricted analysis to secondary lymphoid tissue after primary infections that result predominantly in the generation of central memory CD8 T cells. Heterologous prime–boost vaccination preferentially generates effector memory CD8 T cells that are enriched within non-lymphoid tissues (Supplementary Fig. 5 and ref. 7). Indeed, we found no evidence of an increase in total memory CD8 T cells within lymph node, nor an increase in CD62L$^+$ memory CD8 T cells in any tissue (Fig. 3 and Supplementary Fig. 1). Thus, our study does not reject the hypothesis that central memory CD8 T cells, which are enriched in lymph nodes and depend on interleukin (IL)-15 for survival[12], may be fixed in number. However, it does suggest that effector memory CD8 T cells, which express low levels of IL-15R and undergo very little 'homeostatic' division[9,13,14], may not be subject to the same regulation. It should also be noted that the pathological response to certain infections, or situations in which pathogens directly infect memory lymphocytes, could result in the attrition of memory CD8 T cells.

Our data help to explain observed phenomena, and have practical implications. According to the fixed homeostasis model, it is predicted that the number of total memory CD8 T cells remains stable, regardless of exposure to additional pathogens[15]. Two observations cast doubt on this hypothesis. First, effector memory CD8 T cells steadily increase in blood through the first several decades of life[16,17]. Although the number of cells in tissues has not been quantified, and other physiological changes accompany ageing, this supports the hypothesis that the memory CD8 T-cell compartment might enlarge as one is exposed to additional infections. The fixed homeostasis model also predicts that pre-existing CD8 T-cell memory erodes significantly with successive infections. However, the longevity of CD8 T-cell memory after smallpox vaccination was recently shown to be remarkably long-lived ($t_{1/2} = 8$–16 years)[18], which also favours a model of a flexible memory CD8 T-cell compartment.

The fact that the adaptive immune system has the capacity to accommodate increased numbers of memory CD8 T cells outside of lymph nodes could be exploited for vaccination. Most successful vaccines elicit protection by means of antibodies. This approach has not rendered a protective HIV vaccine, prompting many to consider vaccines that elicit effector memory CD8 T cells, for which the recognition mechanism is less vulnerable to viral mutations[19]. However, CD8 T cells only eliminate viruses after co-localizing with cells that are already infected, suggesting that low frequencies of CD8 T cells might not act rapidly enough to prevent chronic infection. So far, clinical vaccine trials using CD8 T cells have failed to protect against HIV[20,21]. However, we show that aggressive heterologous prime–boost vaccination with live replicating vectors has the potential to generate ~100-fold larger memory CD8 T-cell frequencies than what is achieved with current approaches (Fig. 1)[22]. Crucially, we show that this only caused very modest attrition of pre-existing cellular immunity to other pathogens (Fig. 4). With this major caveat removed, perhaps the time has come to examine the potential of massive numbers of memory CD8 T cells to mediate very rapid clearance of viral infections.

METHODS SUMMARY

Mice and infections. C57Bl/6J mice were purchased from The Jackson Laboratory. Thy1.1$^+$ P14 mice bearing the H–2D^b gp33-specific T-cell receptor were fully backcrossed to C57Bl/6J mice and maintained in our animal colony. We generated P14 chimaeras by transferring 3×10^4 naive transgenic Thy1.1$^+$ P14 T cells into naive C57Bl/6J mice. The next day, mice were infected intraperitoneally (i.p.) with 2×10^5 plaque-forming units (p.f.u.) LCMV (Armstrong strain). Fifty-five days later, mice were infected with 5×10^5 p.f.u. vesicular stomatitis virus (New Jersey strain, VSV-NJ) intravenously (i.v.), rested for 67 days, infected with 2×10^6 p.f.u. recombinant vaccinia virus expressing the

VSV nucleoprotein (VVn) i.v., rested for 94 days, and then infected with 1×10^6 p.f.u. VSV (Indiana strain, VSV-IND) i.v. Control mice received PBS i.v. in lieu of VSV and VVn infections. $n = 16$–18 mice per group. All mice were used in accordance with National Institutes of Health and the University of Minnesota or Emory University Institutional Animal Care and Use Committee guidelines.

Isolation of lymphocytes and immunofluorescence. Blood was obtained by disruption of the retro-orbital plexus, and peripheral blood lymphocytes (PBLs) were purified by underlaying Histopaque (Sigma-Aldrich) and performing density centrifugation (800*g* at 20 °C for 20 min). Lymphocytes were isolated from spleen, lung and inguinal lymph nodes as previously described[7]. Single-cell suspensions were surface-stained with anti-CD8, -Thy1.1, -CD44, -CD62L, -CD4, -B220, -CD3 and -IgD (BD Pharmingen). VSV-nucleoprotein-specific CD8 T cells were identified by staining with H–2K^b tetramers constructed with the $N_{52–59}$ peptide, as previously described[23]. Endogenous LCMV-specific CD8 T cells (Supplementary Fig. 4) were identified by staining with H–2D^b $np_{396–404}$, H–2D^b $gp_{33–41}$, H–2D^b $gp_{276–286}$ and H–2L^d $np_{118–126}$ tetramers, constructed as previously described (np and gp refer to LCMV nuleoprotein and glycoprotein, respectively)[24]. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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1. Christensen, J. P., Doherty, P. C., Branum, K. C. & Riberdy, J. M. Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of CD8$^+$ T-cell memory. *J. Virol.* **74,** 11690–11696 (2000).
2. Freitas, A. A. & Rocha, B. Population biology of lymphocytes: the flight for survival. *Annu. Rev. Immunol.* **18,** 83–111 (2000).
3. Goldrath, A. W. Maintaining the status quo: T-cell homeostasis. *Microbes Infect.* **4,** 539–545 (2002).
4. Welsh, R. M., Selin, L. K. & Szomolanyi-Tsuda, E. Immunological memory to viral infections. *Annu. Rev. Immunol.* **22,** 711–743 (2004).
5. Surh, C. D., Boyman, O., Purton, J. F. & Sprent, J. Homeostasis of memory T cells. *Immunol. Rev.* **211,** 154–163 (2006).
6. Sad, S. & Krishnan, L. Maintenance and attrition of T-cell memory. *Crit. Rev. Immunol.* **23,** 129–147 (2003).
7. Masopust, D., Ha, S. J., Vezys, V. & Ahmed, R. Stimulation history dictates memory CD8 T cell phenotype: implications for prime–boost vaccination. *J. Immunol.* **177,** 831–839 (2006).
8. Woodland, D. L. Jump-starting the immune system: prime–boosting comes of age. *Trends Immunol.* **25,** 98–104 (2004).
9. Wherry, E. J. *et al.* Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nature Immunol.* **4,** 225–234 (2003).
10. Selin, L. K., Vergilis, K., Welsh, R. M. & Nahill, S. R. Reduction of otherwise remarkably stable virus-specific cytotoxic T lymphocyte memory by heterologous viral infections. *J. Exp. Med.* **183,** 2489–2499 (1996).
11. Selin, L. K. *et al.* Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. *Immunity* **11,** 733–742 (1999).
12. Schluns, K. S. & Lefrancois, L. Cytokine control of memory T-cell development and survival. *Nature Rev. Immunol.* **3,** 269–279 (2003).
13. Masopust, D., Vezys, V., Wherry, E. J., Barber, D. L. & Ahmed, R. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J. Immunol.* **176,** 2079–2083 (2006).
14. Jabbari, A. & Harty, J. T. Secondary memory CD8$^+$ T cells are more protective but slower to acquire a central-memory phenotype. *J. Exp. Med.* **203,** 919–932 (2006).
15. Antia, R., Ganusov, V. V. & Ahmed, R. The role of models in understanding CD8$^+$ T-cell memory. *Nature Rev. Immunol.* **5,** 101–111 (2005).
16. Jackola, D. R. & Hallgren, H. M. Dynamic phenotypic restructuring of the CD4 and CD8 T-cell subsets with age in healthy humans: a compartmental model analysis. *Mech. Ageing Dev.* **105,** 241–264 (1998).
17. Czesnikiewicz-Guzik, M. *et al.* T cell subset-specific susceptibility to aging. *Clin. Immunol.* **127,** 107–118 (2008).
18. Hammarlund, E. *et al.* Duration of antiviral immunity after smallpox vaccination. *Nature Med.* **9,** 1131–1137 (2003).
19. Pantaleo, G. & Koup, R. A. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nature Med.* **10,** 806–810 (2004).
20. Sekaly, R. P. The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J. Exp. Med.* **205,** 7–12 (2008).
21. Kaiser, J. AIDS research. Review of vaccine failure prompts a return to basics. *Science* **320,** 30–31 (2008).
22. Shiver, J. W. & Emini, E. A. Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. *Annu. Rev. Med.* **55,** 355–372 (2004).
23. Lefrancois, L., Olson, S. & Masopust, D. A critical role for CD40–CD40 ligand interactions in amplification of the mucosal CD8 T cell response. *J. Exp. Med.* **190,** 1275–1284 (1999).
24. Murali-Krishna, K. *et al.* Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8,** 177–187 (1998).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Mice and infections. To test the impact of large numbers of pre-existing memory CD8 T cells on responses to new infections (Supplementary Fig. 3), naive C57Bl/6J mice were subjected to the heterologous prime–boost regimen as follows: mice were infected with 5×10^5 p.f.u. VSV-NJ i.v., rested for 70 days, infected with 2×10^6 p.f.u. VVn i.v., rested for 140 days, and then infected with 1×10^6 p.f.u. VSV-IND i.v. and rested for 146 days. Age-matched control mice were rested without receiving the heterologous prime–boost regimen. Both groups were then challenged with 2×10^5 p.f.u. LCMV Armstrong strain i.p. and analysed 43 days later.

For the experiments described in Supplementary Fig. 4, C57Bl/6J or Balb/c mice were infected with 2×10^5 p.f.u. LCMV Armstrong strain. 45–60 days later, mice were challenged with 2×10^6 p.f.u. vaccinia virus i.p., 2×10^4 colony-forming units *Listeria monocytogenes* i.v. or 1×10^6 red blood cells parasitized with *Plasmodium yoelii*[25] i.p., as indicated.

Quantification of antibody-secreting cells. Using a modification of the enzyme-linked immunospot (ELISPOT) technique, 96-well nitrocellulose plates were coated with LCMV-infected BHK cell lysate for approximately 16 h[26]. After washing off unbound antigen, splenocytes from immune and immune-challenged mice were plated and incubated for 5 h at 37 °C. Mouse immunoglobulin G was detected by means of an HRP-conjugated antibody and visualized with aminoethyl carbazole as the chromagen substrate. Spots were enumerated by means of a stereomicroscope and total numbers of antibody-secreting cells per spleen were calculated by multiplying total spleen cell numbers with numbers of spots per well.

25. Hunter, R. L., Kidd, M. R., Olsen, M. R., Patterson, P. S. & Lal, A. A. Induction of long-lasting immunity to *Plasmodium yoelii* malaria with whole blood-stage antigens and copolymer adjuvants. *J. Immunol.* **154,** 1762–1769 (1995).
26. Slifka, M. K., Matloubian, M. & Ahmed, R. Bone marrow is a major site of long-term antibody production after acute viral infection. *J. Virol.* **69,** 1895–1902 (1995).