Expansible residence decentralizes immune homeostasis

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In metazoans, specific tasks are relegated to dedicated organs that are established early in development, occupy discrete locations and typically remain fixed in size. The adult immune system arises from a centralized haematopoietic niche that maintains self-renewing potential¹,², and—upon maturation—becomes distributed throughout the body to monitor environmental perturbations, regulate tissue homeostasis and mediate organism-wide defence. Here we examine how immunity is integrated within adult mouse tissues, and address issues of durability, expansibility and contributions to organ cellularity. Focusing on antiviral T cell immunity, we observed durable maintenance of resident memory T cells up to 450 days after infection. Once established, resident T cells did not require the T cell receptor for survival or retention of a poised, effector-like state. Although resident memory indefinitely dominated most mucosal organs, surgical separation of parabiotic mice revealed a tissue-resident provenance for blood-borne effector memory T cells, and circulating memory slowly made substantial contributions to tissue immunity in some organs. After serial immunizations or cohousing with pet-shop mice, we found that in most tissues, tissue pliancy (the capacity of tissues to vary their proportion of immune cells) enables the accretion of tissue-resident memory, without axiomatic erosion of pre-existing antiviral T cell immunity. Extending these findings, we demonstrate that tissue residence and organ pliancy are generalizable aspects that underlie homeostasis of innate and adaptive immunity. The immune system grows commensurate with microbial experience, reaching up to 25% of visceral organ cellularity. Regardless of the location, many populations of white blood cells adopted a tissue-residency program within nonlymphoid organs. Thus, residence—rather than renewal or recirculation—typifies nonlymphoid immune surveillance, and organs serve as pliant storage reservoirs that can accommodate continuous expansion of the cellular immune system throughout life. Although haematopoiesis restores some elements of the immune system, nonlymphoid organs sustain an accrual of durable tissue-autonomous cellular immunity that results in progressive decentralization of organismal immune homeostasis.

Most immune cells function locally³,⁴. For example, T cells require contact with neighbouring cells to sense and eliminate infections or tumours⁵,⁶. To systematically evaluate the longevity of memory T cells in different locations, we transferred CD8⁺ T cells from P14 transgenic mice (hereafter, P14 CD8⁺ T cells) that were also positive for CD45.1 or Thy1.1 into naive female C57Bl/6J mice, followed by infection with the Armstrong strain of lymphocytic choriomeningitis virus (LCMV) (hereafter referred to as P14-immune chimeric mice). Nonlymphoid tissues are surveyed by resident memory T (TRes) cells that are vulnerable to cell death upon tissue digestion, which makes enumeration by this method inadequate⁸,⁹. To mitigate this issue, we visually enumerated P14 CD8⁺ T cells by quantitative immunofluorescent microscopy between 5 and 450 days after infection with LCMV in the tissues of 80–96 mice (Fig. 1a–e). The response to LCMV peaked within 5–9 days, before contracting. A stable population of memory T cells was established in the lymph node by 16 days after infection. We found considerable variation in the durability of nonlymphoid TRes cells, which were stable in salivary gland, decayed modestly in the small intestine (which was best modelled by biphasic decay) and underwent continued attrition in the uterus. We particularly noted attrition of TRes cells, which were stable in salivary gland, decayed modestly in the small intestine (which was best modelled by biphasic decay) and underwent continued attrition in the uterus. We particularly noted attrition of TRes cells, which were stable in salivary gland, decayed modestly in the small intestine (which was best modelled by biphasic decay) and underwent continued attrition in the uterus. We particularly noted attrition of TRes cells, which were stable in salivary gland, decayed modestly in the small intestine (which was best modelled by biphasic decay) and underwent continued attrition in the uterus.
cells within the uterine endometrium, which underwent vacuolation and glandular atrophy in aged mice (Extended Data Fig. 1a–c) that coincided with infertility. Ageing was also associated with the development of prominent peripheral node addressin-expressing tertiary lymphoid organs in salivary glands (excluded from the numbers of T\text{RM} cells in Fig. 1c, Extended Data Fig. 1d, e). These data indicate that the longevity of memory CD8\(^+\) T cells varies by location, but that—in some compartments—these cells can persist indefinitely.

**T\text{RM} cell longevity is TCR-independent**

Naive and central memory T cells recirculate between the blood and lymph nodes. Naive T cells depend on constitutive T cell receptor (TCR) signalling for survival, whereas central memory T cells do not\(^{10}\). Unlike central memory T cells, T\text{RM} cells share many properties with T cells that have undergone recent TCR stimulation\(^{11,12}\). To test whether the persistence of T\text{RM} cells depends on the TCR, we took advantage of UBC–Cre\text{ER}\(^{2}\) × Trac\text{cre}\(^{2}\) mice (hereafter, Trac\text{cre}\(^{2}\) mice) so that we could genetically ablate the TCR by tamoxifen treatment in established memory T cells (Methods, Extended Data Figs. 2, 3). We compared wild-type Thy1.1\(^{+}\) and Trac\text{cre}\(^{2}\) Thy1.1\(^{+}\) cells within the same mice, and confirmed TCR ablation by staining for TCR\(\beta\) and a failure to produce cytokines in response to ex vivo peptide stimulation (Fig. If, g, Extended Data Figs. 2a–e, 3a–c). No significant difference was observed between the longevity of wild-type and TCR-ablated T\text{RM} cells in all of the tissues we examined, nor was expression of CD69 and CD103 reduced in TCR-ablated memory T cells (Fig. Ih–j, Extended Data Figs. 2f–h, 3d). Thus, CD8\(^{+}\) T\text{RM} cells are not maintained by persistent viral antigen, self-peptide–MHC I complexes or cross-reactivity with microbial TCR ligands.
Durability of TRM cell organ surveillance

The defining characteristic of TRM cells is the absence of migration. However, migration experiments are typically short-term, which raises the question of whether TRM cell populations are gradually replenished by central memory T cells or slowly recirculate. To address this issue, we generated mice containing CD45.1+ or Thy1.1+ memory P14 T cells. Here we apply a reductionist approach that was previously used to test whether the circulating memory CD8+ T cell population in lymphoid organs as expansible or rigidly defined to the analysis of host-derived P14 CD8+ T cells (Fig. 1a–e).

Primary infections induce CD62L- KLRG1+ long-lived effector memory T cells that patrol blood without entering lymph nodes. After surgical separation, we observed a gradual disequilibrium in blood in favour of host-derived P14 CD8+ T cells (Fig. 1f–i, Extended Data Fig. 5a, b). These data indicate that T cells that failed to equilibrate during parabiosis (that is, TRM cells) slowly join the blood circulation. Over time, these ex-TRM cells came to comprise between 15 and 30% of all memory P14 T cells in peripheral blood (Extended Data Fig. 5a, b), and were significantly enriched within the KLRG1- effector memory T cell subset (Fig. 1n). Additional profiling revealed that expression of the CD43 glycoform recognized by the IB11 antibody clone further distinguished ex-TRM cells, consistent with expression of this glycoform on tissue-resident memory cells (Fig. 1o, Extended Data Figs. 5c, 6a, b). Thus, although TRM cells wane in some nonlymphoid tissues, they contemporaneously give rise to blood-borne effector memory T cells.

The TRM cell niche is expansible

Cell populations are often numerically fixed in size by cell-extrinsic regulators. Cytokine (for example, IL-15) abundance, metabolite availability and constitutive TCR signalling all function to restrict T cell abundance, but these factors do not axiomatic control T cell survival in tissues. We hypothesized that the resident memory pool may circumvent the numerical constraints imposed on blood-borne T cells. Here we apply a reductionist approach that was previously used to test whether the circulating memory CD8+ T cell population in lymphoid organs as expansible or rigidly defined to the analysis of nonlymphoid tissues dominated by TRM cells (Fig. 1). We established P14 CD8+ T cell memory by infection with LCMV, as in Fig. 1a–e. Sixty days after infection with LCMV, P14-immune chimeric mice were cohoused with mice obtained from pet shops (Fig. 2) to facilitate microbial exposure. Memory P14 CD8+ T cells in nonlymphoid tissues of host origin in most nonlymphoid tissues, which indicates that TRM cells autonomously dominate immunosurveillance for the lifespan of the mouse (Fig. 1l). Organized lymphoid structures—including tertiary lymphoid organs in salivary glands and Peyers patches in the small intestine—were surveyed by both recirculating memory T cells and TRM cells. However, extravascular memory T cells in the lung parenchyma demonstrated near-complete equilibration, regardless of CD69 expression (Fig. 1l, Extended Data Fig. 4a, b). Although TRM cells seed the lung shortly after infection, these data indicate that long-term pulmonary surveillance may depend on circulating memory T cells that enter the tissue, which has ramifications for the durability of T-cell-dependent protection against respiratory infections. Circulating memory T cells also eventually made substantial contributions to the maintenance of memory in liver and kidney (Fig. 1l). Although these observations offer support for a centralized source for nonlymphoid memory in some locations, for most tissues, residence is responsible for long-term surveillance after clearance of primary infections in mice housed under specific-pathogen-free laboratory conditions.
EpCAM demonstrated that TRM cells were numerically preserved (Fig. 2d). In terms of P14 CD8+ T cells using quantitative immunofluorescent microscopy there was a reduction in the relative frequency of pre-existing P14 CD8+ T cells. Concomitant with the expansion of N-specific memory T cells, there is an increase in the in vivo frequency of N-specific memory T cells specific for the immunodominant epitope of MCMV, which indicates that circulating memory T cells were preserved in the face of substantial attrition of P14 CD8+ T cells, which suggests that residence itself is not an artefact of artificial clean mouse husbandry. Small-intestinal TRM cells constituted an exception, and were nearly sixfold less abundant after cohousing. The attrition of intestinal TRM cells may reflect the saturation of anatomical space, heightened damage-associated molecular patterns that induce cellular toxicity or alterations to the microbiome that modulate local survival cues. In two infection models that generate either lung- or skin-resident memory CD8+ T cells, pre-existing memory was preserved after cohousing (Extended Data Fig. 8a–g). Thus, in most tissues, heterologous prime-boost vaccination or physiological exposures to microorganisms did not induce erosion of TRM cells—instead, tissues accommodated more resident cells.

**The immune system is an expandible tissue component**

Homeostasis balances cellular self-renewal with cell death, and maintains organ size in adult organisms. This process preserves organ function while preventing cancer. The expandible nature of the T cell compartment raises questions about whether the immune system in totality of a fixed size or exhibits plasticity in proportion to microbial experience. Cohousing induced a durable increase in nearly all types of leukocytes in blood, as well as persistent enlargement of lymph nodes and spleen (Fig. 3a–c). Both memory CD4+ and CD8+ T cells increased after cohousing, and even the so-called ‘innate’ populations of the immune system demonstrated an ‘adaptive’ ability to durably expand in size (Fig. 3a–c). Extensive analysis using quantitative immunofluorescent microscopy revealed that 5–20% of most nonlymphoid tissues of specific-pathogen-free mice were composed of white blood cells. However, the frequency of immune cells was significantly and durably expanded after cohousing, which indicates...
that the immune system occupies a considerable fraction of visceral and mucosal organs, is flexible in size and is capable of long-lived adaptation in relation to exposure to microorganisms (Fig. 3d, e).

Residency typifies tissue immunity
Memory T cells achieve durable immune surveillance of the entire organism through prolonged residence in most nonlymphoid tissues (Fig. 1). Tissue residency has previously been demonstrated for several types of immune cell, primarily in immunologically naive or single-infection mouse models26,27. We asked whether residence is a common mechanism used by the immune system to achieve broad immunological surveillance, by performing parabiosis surgery of cohoused B6 mice that have diverse microbial experiences (Extended Data Fig. 10a). Leukocytes (distinguished by CD45) equilibrated completely within peripheral blood of parabiotic mice (Extended Data Fig. 10b), but tissue-resident immune populations occupied—and often dominated—most organs (Fig. 4a). Over one month, many CD4+ and CD8+ T cells of the adaptive immune system, as well as macrophages, innate lymphoid cells and natural killer cells of the innate immune system, stably occupied nonlymphoid tissues (Fig. 4b, Extended Data Fig. 10c–f). Consistent with their rapid turnover, granulocytes in tissues relied on continuous replenishment from blood (Fig. 4b, Extended Data Fig. 10g). B cells, which differentiate into the antibody-secreting cells of the immune system, largely equilibrated between parabionts (Fig. 4b, Extended Data Fig. 10h). Overall, residence was a shared feature that was exhibited by many adaptive and innate immune cell types in mice that were exposed to diverse microorganisms.

Conclusion
The immune system has defied classification as a commonly recognized organ system, but is partially captured by the skeletal system (which includes the bone marrow), the cardiovascular system (which includes blood cells) and the lymphatic system (which includes the secondary lymphoid organs)28,29. This framework excludes many immune cells in the body, particularly those that are most responsible for regional immune surveillance and effector functions. Our study generalizes two features of immunity outside of dedicated lymphoid organs: (1) most immune cells stably reside in—rather than recirculate through—tissues and (2) the size of the immune system durably adapts to microbial experience to accommodate additional cells. Although parenchymal cell populations are numerically constrained to maintain organ homeostasis, the adaptive flexibility of the immune system allows leukocytes to comprise a considerable fraction of the body—up to a quarter of visceral organs in microbially experienced mice. Analyses that are limited to blood and lymphoid organs have emphasized the renewable and migratory aspects of the immune system29–33. Our study, which initially focused reductively on T cells, shows that resident T cell immunity can autonomously endure over time, without displacement by subsequent inflammation, infection or competition for a fixed immunological niche. Given that most infections and tumours develop in nonlymphoid organs, these are relevant findings for immunization strategies that seek to harness T cell immunity34,35. In contrast to expectations, we observed that, after a primary infection, most long-lived blood-borne effector memory T cells are ex-TBM cells (Fig. 1); recent reports indicate that reactivated TBM cells can contribute even further to circulating memory populations36–38. The phenomenon of expansible residence that we observed for T cells extended to most populations of immune cells. Although immune responses develop in centralized sites, our data indicate that nonlymphoid organs provide a flexible reservoir for the long-term preservation of adaptive and innate immunity. Given these findings, it may be reasonable to conceptualize the immune system as its own organ system, albeit one that consists of a diverse network of motile sensory cells that are durably integrated throughout the entire body, and that is permissive to considerable plasticity in cell number, composition and distribution.

Online content
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Methods

Mice
Female, SPF C57BL/6j (CD45.2 - B6) and B6. SJL-Ptpcr⁻/⁺Pepcb⁻/⁻BoyJ (CD45.1 - B6) mice at 6 - 8 weeks of age were purchased from The Jackson Laboratory. For cohousing experiments, female, SPF CD45.2⁻ and CD45.1⁻ B6 mice at 6 - 8 weeks of age were purchased from Charles River Laboratories. Female pet shop mice (age not provided by vendor) were purchased from pet shops in the greater Minneapolis - St Paul metropolitan area. Cohousing of SPF mice with sex-matched pet shop mice was performed as previously described, within the University of Minnesota biosafety level 3 facility. The following housing conditions were regulated: ambient temperature (20.0 - 23.3 °C), humidity (30 - 70%) and light/dark cycling (14-h on/10-h off). Tracfl/fl mice were fully backcrossed to UBC-CreER² mice (JAX stock no. 007001) to generate UBC-CreER² Tracfl/fl mice (provided by K. Hogquist). Male and female UBC-CreER² Tracfl/fl mice at 6 - 10 weeks of age were used in tandem with age- and sex-matched Thy1.1⁻ B6 mice. CD45.1⁻ sex-matched mice at 6 - 12 weeks of age were used as recipient mice. P14 CDS⁻ T-cell-transgenic, OT-1 CDS⁻ T-cell-transgenic, Thy1.1⁻ B6 and CD45.1⁻ B6 mouse strains were maintained in-house. All mice were used in accordance with guidelines established by the Institutional Animal Care and Use Committee at the University of Minnesota. The University of Minnesota Institutional Review Board approved all protocols used.

Adoptive transfers and infections
P14-immune chimeric mice were generated by intravenous adoptive transfer of 5 x 10^6 P14 CDS⁻ splenocytes into naive female B6, mice and infection with 2 x 10^5 plaque-forming units (PFU) of the Armstrong strain of LCMV via intraperitoneal injection on the subsequent day. Alternatively, P14-immune chimeric mice were generated by intravenous adoptive transfer of 5 x 10^6 P14 CDS⁻ splenocytes into naive female B6 mice and intranasal infection with 500 PFU PR8-gp33 recombinant influenza virus (provided by R. Langlois) on the subsequent day. OT-1-immune chimeric mice were generated by intravenous transfer of 5 x 10^5 OT-1 CDS⁻ splenocytes into naive female B6 mice and infection with 10^6 PFU VSV–OVA the following day. For experiments using Tracfl/fl mice, Tracfl/fl mice and wild-type Thy1.1⁻ B6 mice were intraperitoneally infected with 2 x 10^5 PFU of LCMV. After 30 days, 10^7 lymphocytes — isolated from secondary lymphoid organs — were intravenously transferred into naive CD45.1⁻ B6 mice. On the subsequent day, CD45.1⁻ recipients were intraperitoneally infected with 10^5 PFU of LCMV. To generate primary polyclonal memory with Tracfl/fl mice, naive Tracfl/fl mice and wild-type Thy1.1⁻ B6 mice were killed, and CD8⁺ T cells were enriched from secondary lymphoid organs via negative selection per the manufacturer's protocol (Stem Cell Technologies). A total of 2 x 10⁶ enriched cells were stimulated per well in flat-bottom 12-well plates with anti-CD3e (clone 145-2C11, 10 µg ml⁻¹) (Bio X Cell) and rB7-1/Fc chimeric protein (0.8 µg ml⁻¹) (R&D Systems) immobilized on the surface in the presence of 5 U ml⁻¹ IL-2 (R&D Systems) with 10 ng ml⁻¹ mouse recombinant IL-12 (R&D Systems) as previously described. After 3 days of in vitro activation, 10⁶ CD8⁺ T cells isolated from Tracfl/fl mice and wild-type Thy1.1⁻ B6 mice were intravenously co-transferred into naive CD45.1⁻ B6 mice. For heterologous prime – boost immunization, three viruses were administered by intravenous injection at 30-day intervals in the following order, as previously described: (1) 10⁵ PFU of VSV, New Jersey serotype; (2) 2 x 10⁶ PFU of recombinant vaccinia expressing the VSV nucleoprotein; and (3) 10⁵ PFU of VSV, Indiana serotype.

Quantitative immunofluorescence microscopy
Collected mouse tissues were fixed in 2% paraformaldehyde for 2 h, followed by overnight cryoprotection in 30% sucrose solution at 4 °C. Sucrose-treated tissue was embedded in optimal cutting temperature (OCT) compound and frozen in a chilled isopentane bath. Alternatively, collected mouse tissues were directly embedded in OCT compound and snap-frozen in a chilled isopentane bath. Studies of the salivary gland focused exclusively on the submandibular gland. Frozen tissue blocks were sectioned at 7 µm in a Leica cryostat. Sections were stained with primary and secondary antibodies, counterstained with DAPI or SYTOX Green to detect nuclei, and immunofluorescence microscopy was performed using a Leica DM6000 B microscope. Monoclonal anti-mouse antibodies, used at a 1:100 dilution unless otherwise noted, were: CD8α (53-6.7), CD8β (53-6.7), CD4 (RM4-5), CD44 (H12F11), CD45 (30-F11), CD41 (A20), Thy-1.1 (OX7) (1:100), B220 (RA3-6B2), EpCAM (G8.8) (1:500) and PNAd (MECA-79), all from BioLegend. Polyclonal goat anti-mouse collagen type IV antibody (1:200) from MilliporeSigma and secondary bovine anti-goat IgG (H+L) antibody (1:300) from Jackson Immunoresearch were used. Images were processed using Fiji software and cell enumeration was performed manually as previously described, or using ImageJ scripts developed in house.

Leukocyte isolation and phenotyping of cells
An intravascular staining method was used to discriminate between cells with the vasculature and those within the parenchyma of tissues. Three minutes before being killed, mice were intravenously injected with each 3 µg of biotinylated- or fluorescein-conjugated CD8α (53-6.7) antibody or 2 µg of fluorescein-conjugated CD45 (30-F11) antibody Antibodies were collected and leukocytes isolated as previously described. Studies of the salivary gland focused exclusively on the submandibular gland. Isolated leukocytes were surface-stained with the following monoclonal anti-mouse monoclonal antibodies at a 1:100 dilution, unless otherwise noted: CD4 (GK1.5), CD3 (53-7.3), CD8α (53-6.7), CD8β (53-5.8), CD11c (N418), CD14 (24G2), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD62L (ME-14), CD64 (55-7.5), CD69 (H1.2F3), CD103 (2E7), Thy1.1 (OX7) (1:250), B220 (RA3-6B2), F4/80 (BM8), Ly6C (HK1.4) (1:400), Ly6G (1A8), Nkp46 (29.1A4), PD-1 (2F.11A2), CX3CR1 (S011F11) and CXCR3 (CXCR3-173), from BioLegend; CD11b (MI70), CD19 (ID3), NK1.1 (PK136), Siglec-F (E50-2440) and TCF-1 (S33-966) (1:50), from BD; CD3e (145-2C11), CD127 (A7R34), and KLRG1 (2F1) and TCF-1 (57-979) from Tonbo Biosciences. Cell viability was determined using Ghost Dye Violet S10 or Ghost Dye Red 780 (Tonbo Biosciences) (1:300). To identify VSV N-specific CD8⁺ T cells, leukocytes were stained with H-2K²/N (MHC class I tetramer) (1:200), conjugated to PE. To identify pp33-specific CD8⁺ T cells, leukocytes were stained with H-2D²/gp33 (MHC class I tetramer) (1:200), conjugated to APC. Staining for intracellular transcription factors and proteins was performed using a transcription factor staining buffer kit (Tonbo Biosciences) with monoclonal anti-mouse antibodies: T-bet (4B10) from BioLegend; Eomes (Dn1Img), FOXP3 (FJK-16s), GATA-3 (TWAJ), Ki67 (53-6.7), CD8β (53-5.8), CD11c (N418), CD43 (1B11), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD62L (ME-14), CD64 (55-7.5), CD69 (H1.2F3), CD103 (2E7), Thy1.1 (OX7) (1:250), B220 (RA3-6B2), F4/80 (BM8), Ly6C (HK1.4) (1:400), Ly6G (1A8), Nkp46 (29.1A4), PD-1 (2F.11A2), CX3CR1 (S011F11) and CXCR3 (CXCR3-173), from BioLegend; CD11b (MI70), CD19 (ID3), NK1.1 (PK136), Siglec-F (E50-2440) and TCF-1 (S33-966) (1:50), from BD; CD3e (145-2C11), CD127 (A7R34), and KLRG1 (2F1) and TCF-1 (57-979) from Tonbo Biosciences. The stained samples were acquired using LSR1 or LSR Fortessa flow cytometers (BD) and analysed with FlowJo software (BD). Neutrophils were distinguished by expression of CD11b and Ly6G. Eosinophils were identified by Siglec-F expression. Innate lymphoid cell, natural killer cell, B cell and monocyte and macrophage populations were distinguished after excluding lineage-positive cells using combinations of CD3, CD5, CD19, B220 and Ly6G, and then using recommended lineage-defining markers as previously described. Monocytes were further subdivided into classical and patrolling populations on the basis of Ly6C expression. Lung macrophages were subdivided into alveolar and interstitial populations on the basis of Siglec-F and CD11b expression.

Parabiosis and separation surgeries
Parabiosis and separation surgeries were performed with age-matched female mice as previously described, with some modifications. For surgeries, anaesthesia to full muscle relaxation was achieved using avertin (250 mg kg⁻¹) by intraperitoneal injection, and surgical site preparation included betadine application to surgical site in a gradually enlarging circular pattern. For parabiosis, corresponding lateral aspects of mice were thoroughly shaved with electric clippers from...
about 1 cm superior to the shoulder and about 1 cm inferior to the hip. Excess hair was wiped off with alcohol preparation pads. Following surgical site preparation, matching skin incisions were made about 0.5 cm superior to the shoulder and about 0.5 cm inferior to the hip. Subcutaneous fascia was bluntly dissected to create around 0.5 cm of free skin. Dorsal and ventral skins of adjacent mice were approximated by interrupted horizontal mattress stitches with 3-0 Prolene suture and overlying surgical wound clips. To separate parabiotic mice, pre-existing suture and wound clips were removed. Excess hair was shaved and wiped off with alcohol preparation pads. After surgical site preparation, a longitudinal incision was made with sharp scissors lateral to the initially conjoined skin. Newly formed fascia was gently detached with a pair of curved forceps. The superior and inferior aspects of the skin of each mouse were then approximated by running stitch with a single 4-0 Vicryl suture. For analgesic treatment, mice received preoperative subcutaneous bupivacaine (2 mg kg⁻¹) and postoperative subcutaneous bupivacaine (2 mg kg⁻¹) and carprofen (5 mg kg⁻¹). Mice were kept on heating pads during and after surgery, and their recovery was monitored continuously.

Tamoxifen administration
Tamoxifen was dissolved in corn oil at 37 °C with shaking overnight to a working concentration of 20 mg ml⁻¹. Working stocks were freshly prepared for each experiment. For CreERT² induction, tamoxifen was administered to mice intraperitoneally at a dose of 75 mg kg⁻¹ every 24 h over 5 consecutive days.

In vitro stimulation assays
Isolated lymphocytes were incubated at 37 °C for 4 h in stimulation media with or without gp33–41 peptide (0.2 μg ml⁻¹) or phorbol myristate acetate and ionomycin (cell stimulation cocktail, ThermoFisher Scientific). Stimulation medium consisted of RPMI1640, 10% FCS, 2 mM l-glutamine, 100 U ml⁻¹ penicillin, 100 mg ml⁻¹ streptomycin, 50 mM 2-mercaptoethanol and brefeldin A (GolgiPlug, BD). Intracellular staining for cytokines was performed using the Cytofix/Cytoperm kit per the manufacturer’s directions (BD) with anti-mouse antibodies: IFNγ (XMGL.2) from BioLegend and TNF (MP6-XT22) from ThermoFisher Scientific.

Quantification and statistical analysis
No statistical methods were used to predetermine sample size. Mice were randomly assigned to experimental groups and investigators were not blinded to allocation during experiments and outcome assessment. Specific statistical tests, sample size (n) and P values can be found in figure legends. Individual data points represent biological replicates. All statistical tests were two-tailed and, generally, non-parametric tests were used to test for significance (Mann–Whitney U test for unpaired samples and Wilcoxon matched-pairs signed-rank test for paired samples). All statistical analysis was done using Prism (GraphPad). For all experiments, a P value <0.05 was considered significant. Mean and s.e.m. are used to represent the centre and dispersion, unless otherwise stated. Nonlinear regression analysis using Prism (GraphPad) was used to model memory T cell population kinetics data, using data points between 30 and 450 days after infection. Model constraints imposed that decay plateau = 0 and a positive rate constant. An exponential decay model was fit to tissue populations if R² > 0. Either one-phase or two-phase exponential decay models were selected after comparison using extra sum-of-squares F test and the Akaike information criterion.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Code availability
ImageJ scripts developed for cell enumeration are available at http://github.com/wijey001/count.

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Author contributions
S.W., L.K.B., M.J.P., J.M.S., O.A.A., R.R., E.M.S. and P.C.R. performed the experiments; S.W., V.V. and D.M. designed the experiments and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Extended Data Fig. 1 | Compartmentalized decay of uterine T cells concomitant with morphological changes in tissue architecture over time.

**a**, Representative immunofluorescence of uterine tissue. **b**, The frequency of P14 memory CD8⁺ T cells in uterine compartments was assessed by quantitative immunofluorescent microscopy at day 60 (n = 6 mice) and day 200 (n = 7 mice) after LCMV infection in one experiment. **c**, Representative immunofluorescence images of mouse uterine tissue at various ages, demonstrating endometrial vacuolations in older mice. **d, e**, Representative immunofluorescence images of mouse salivary gland at various time points demonstrating emergence of salivary gland tertiary lymphoid organs in older mice (d) and expression of peripheral node addressin (PNAd) (e). Morphology representative of n > 12 mice, PNAd staining representative of n = 5 mice (c–e). Scale bar, 100 μm (a), 500 μm (c, 70 weeks in d), 200 μm (10 and 35 weeks in d, e). Statistical significance was determined by two-tailed Mann–Whitney U test (b). *P = 0.0221, **P = 0.0023 (endometrium) or **P = 0.0082 (perimetrium). Data are mean ± s.e.m.
Extended Data Fig. 2 | Selective TCR ablation using Trac^fl/fl mice reveals TCR-independent homeostasis of T_RM cells. 

**a**, Experimental model. Thy1.1^−^CD45.2^+^Trac^fl/fl^ mice and Thy1.1^+^CD45.2^+^wild-type B6 mice were infected with LCMV. After 30 days, 10^7^ lymphocytes—isolated from secondary lymphoid organs—were transferred into naive CD45.1^+^B6 mice, which were subsequently infected with LCMV. Forty days after infection, CD45.1^+^mice were treated with tamoxifen to selectively ablate TCR from transferred Thy1.1^−^CD45.2^+^Trac^fl/fl^ secondary memory T cells. **b, c,** LCMV-specific secondary memory T cells in peripheral blood are shown 40 days after LCMV infection (before tamoxifen treatment) (**b**). Data pooled from 3 independent experiments for a total of n = 8 mice (**c**). **d, e,** Selective TCR ablation of Trac^fl/fl^ secondary memory CD8^+^ T cells, as measured by ex vivo peptide stimulation assay. Sixty days after tamoxifen treatment of CD45.1^+^B6 recipient mice, splenocytes were isolated and stimulated in vitro with gp33-41 peptide. Cytokine production by TCR^−^Trac^fl/fl^ memory CD8^+^ T cells and TCR^+^wild-type memory CD8^+^ T cells from spleen is shown, and reflects n = 6 mice. **f,** Frequency of cells that lack TCRβ expression on Trac^fl/fl^ memory CD8^+^ T cells. Data pooled from 4 independent experiments for a total of n = 8–10 mice (n varies by tissue). **g,** Representative flow cytometry, depicting expression of tissue-resident markers on small-intestine epithelial memory CD8^+^ T cells 60 days after tamoxifen treatment. **h,** Frequency of CD69^+^ memory CD8^+^ T cells in the spleen for wild-type and TCRβ^−^Trac^fl/fl^ populations. Data pooled from four independent experiments, for a total of n = 10 mice. Statistical significance was determined by two-tailed Wilcoxon matched-pairs signed-rank test (**e, h**). *P* = 0.0313. Data are mean ± s.e.m.
Extended Data Fig. 3 | In vitro activation of Tracfl/fl naive T cells generates primary T RM cells that are maintained in the absence of constitutive TCR signalling. 

**a,** Experimental model. Lymphocytes were isolated from secondary lymphoid organs of CD45.2 Tracfl/fl mice and wild-type Thy1.1 B6 mice, and enriched for naive CD8 T cells via magnetic bead enrichment. T cells were activated in vitro for 3 days with anti-CD3ε and rB7-1, and 10^7 cells were co-transferred into naive CD45.1 B6 mice. Thirty days later, recipient mice were treated with tamoxifen. 

**b,** Thirty days after tamoxifen treatment, transferred CD8 T cells were evaluated for CD44 expression, as compared to endogenous CD8 T cells, shown via representative flow cytometry of CD8 T cells isolated from blood. 

**c,** Expression of TCRβ was evaluated for Tracfl/fl and wild-type CD8 T cells, as shown via representative flow cytometry of peripheral blood. 

**d,** The ratio of Tracfl/fl to wild-type CD8 T cells was quantified 30 days after tamoxifen treatment in various tissues, normalized to values from blood, and was not significantly different from 1:1. Data show n = 4 biologically independent mice from 1 experiment. Statistical significance was determined by two-tailed one-sample Wilcoxon test, using 0 as a hypothetical mean. Data are box plots showing median, IQR and extremes.
Extended Data Fig. 4 | CD69 does not unequivocally distinguish long-lived T<sub>EM</sub> cells in the lung. 

**a, b.** Representative flow cytometry (a) and graph (b), demonstrating the degree of disequilibrium among CD69<sup>+</sup> extravascular memory P14 CD8<sup>+</sup> T cells in tissues of separated parabiotic mice (n = 8–10), 260 days after LCMV infection from 1 experiment. Top panels in a are gated on extravascular CD45.1-BV650 memory P14 CD8<sup>+</sup> T cells. Data are mean ± s.e.m.
Extended Data Fig. 5 | Ex-TRM cells comprise a substantial fraction of blood-borne memory. a, b, Longitudinal graphs depicting the frequency of host-derived memory P14 CD8+ T cells (a) or the frequency of ex-TRM cells of P14 CD8+ T cells, as calculated (b) in the peripheral blood of separated parabiotic mice from two independent experiments (n = 17). Data are mean ± s.e.m.; in b, coloured dotted lines reflect s.e.m. c, d, More than 200 days after separation of congenically distinct parabiotic P14-immune chimeric mice (n = 17), host- and donor-derived P14 CD8+ T cells were evaluated for expression of markers of antigen experience, tissue-trafficking and differentiation potential (d). Gating strategy for P14 CD8+ T cells in separated parabiotic mice shown in c is generally representative of the flow cytometry panels in Figs. 1, 2, Extended Data Figs. 2–4, 6.
Extended Data Fig. 6 | The glycoform of CD43 recognized by 1B11 is expressed on CD8⁺ T_{em} cells. a, b, Representative flow cytometry (a) and quantification (b) of CD43–1B11 antibody staining on memory P14 CD8⁺ T cells in nonlymphoid tissues of mice (n = 9) 200 days after infection with LCMV. In a, naive CD8⁺ T cells isolated from peripheral blood (in red) serve as basis for comparison. Data are mean ± s.e.m.
Extended Data Fig. 7 | Pre-existing memory T cells retain functional potency after heterologous prime–boost immunization. a, Sixty days after infection with LCMV, P14-immune chimeric mice were subjected to a heterologous prime–boost regimen. The ex vivo functionality of memory P14 CD8+ T cells in various tissues was compared, and found to be not significantly different ($P > 0.05$) between $n = 4$ or 5 mice ($n$ varies by tissue) receiving heterologous prime–boost and $n = 5$ age-matched control mice, from one of two independent experiments with similar results. Statistical significance was determined by two-tailed Mann–Whitney U test. Data are mean ± s.e.m.
Extended Data Fig. 8 | Lung or skin memory CD8+ T cells are preserved after microbial experience. a–d, P14 CD8+ T cells were transferred into naive mice, which were intranasally infected with PR8–gp33 influenza virus and, 30 days later, mice were cohoused for 45 days with mice obtained from pet shops (a). P14 CD8+ T cells from spleen (b), extravascular lung (c) and bronchoalveolar lavage (BAL) fluid (d) of cohoused mice (n = 8) were enumerated and compared to infection-matched mice housed in SPF conditions (n = 8) from 1 experiment. Statistical significance was determined by two-tailed Mann–Whitney U test. **P = 0.0047 (b); **P = 0.0012 (f). Data are box plots showing median, IQR and extremes.

e–g, OT-1 CD8+ T cells were transferred into naive, which were intravenously infected with VSV–OVA; 30 days later, mice were cohoused for 60 days with mice obtained from pet shops (e). OT-1 CD8+ T cells from spleen (f) and epidermal skin (g) of cohoused mice (n = 6) were enumerated and compared to infection-matched mice housed in SPF conditions (n = 7) from 1 experiment. Statistical significance was determined by two-tailed Mann–Whitney U test. **P = 0.0047 (b); **P = 0.0012 (f). Data are box plots showing median, IQR and extremes.
Extended Data Fig. 9 | Both CD4+ and CD8+ memory T cell populations are expansible. 

a, b, CD45+ cells increase in tissues after cohousing (Fig. 3). Here we examined relative frequencies of memory T cells. C57Bl/6 SPF laboratory mice were cohoused for >60 days with mice obtained from pet shops. Age-matched, conventionally housed SPF mice served as controls. The frequency of CD4+ memory T cells (a) and CD8+ memory T cells (b) as a proportion of CD45+ immune cells is depicted in various tissues in both groups of mice. Memory T cells were defined as CD44+PD1−. mLN, mesenteric lymph node. Data are pooled from 2–4 independent experiments for a total of n = 4–14 mice (n varies by tissue) per group. Data are mean ± s.e.m.
Extended Data Fig. 10 | Tissue residence typifies immune surveillance for many leukocyte populations. a, Model depicting the cohousing of CD45.1+ and CD45.2+ C57Bl/6 SPF laboratory mice for $>60$ days with mice obtained from pet shops, followed by parabiosis of laboratory mice for 28–32 days. b, Between 28 and 32 days after parabiosis, the equilibration of leukocyte populations in peripheral blood was evaluated in $n=8–14$ mice. c–h, Between 28 and 32 days after parabiosis, the tissue disequilibrium of innate lymphoid cells (c, $n=3–12$ mice), natural killer cells (d, $n=5–14$ mice), monocytes and macrophages (e, $n=4–12$ mice), CD44+PD1− memory T cells (f, $n=7–14$ mice), granulocytes (g, $n=4–12$ mice) and B cells (h, $n=2–14$ mice) was evaluated. Data are pooled from four independent experiments and $n$ varies dependent on tissue and population of interest (as not all cell populations were abundantly detected in each tissue or each experiment). AM, alveolar macrophages; IM, interstitial macrophages; mesLN, mesenteric lymph node. Data are mean ± s.e.m.
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- □ □ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- □ □ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- □ □ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

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 8.0.1, Leica Application Suite X 3.4.1.17822 |
| Data analysis         | FlowJo 10, Graphpad Prism 8, Fiji (ImageJ) 1.53c |

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The data that support the findings of this study are available from the corresponding author upon reasonable request. ImageJ scripts developed for cell enumeration are available at http://github.com/wjey001/count.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: Sample size was determined based on previous studies (Beura et al, Nature Immunology 2018; Steinert et al, Cell, 2015). In general, 5 to 10 mice was sufficient to detect differences between groups with power value 0.8 and a 5% significant value.

Data exclusions: No data were excluded.

Replication: All experiments were replicated or verified with 1-3 repeat experiments, with the exception of experiments in Fig. 11, Extended Data Fig. 3 and 8.

Randomization: In experiments with multiple groups, littermates of the same sex were randomly assigned to experimental groups.

Blinding: Blinding was not possible in this study, as technical aspects of all experiments were coordinated by a single investigator.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a Involved in the study
- Antibodies
- Eukaryotic cell lines
- Palaeontology and archaeology
- Animals and other organisms
- Human research participants
- Clinical data
- Dual use research of concern

Methods

n/a Involved in the study
- ChIP
- Flow cytometry
- MRI-based neuroimaging

Antibodies

Antibodies used

- Anti-mouse antibodies are listed below:
  - Antibody, Clone, Company, Catalog #

  Antibodies for immunofluorescence:
  - CD45.1, A20, Biolegend, 110720
  - Thy1.1, OX 7, Biolegend, 566090
  - CD8b, YTS156.7.7, Biolegend, 126608
  - CD9a, 55-6.7, Biolegend, 100708
  - CD45, 30-F11, Biolegend, 103106
  - EPCAM, G8.8, Biolegend, 118222
  - B220, RA3-6B2, Biolegend, 565631
  - PNA, MECA-79, Biolegend, 120808
  - Collagen IV, Polyclonal, Millipore Sigma, AB769
  - Anti-Goat IgG (H+L), Polyclonal, Jackson ImmunoResearch, 805-545-180

  Antibodies for flow cytometry:
  - CD11b, M1/70, BD, 564443
  - CD19, 103, BD, 563557
  - NK1.1, PK136, BD, 564144
  - Siglec-F, E50-2440, BD, 740280
  - B220, RA3-6B2, Biolegend, 103208
  - CD103, 2E7, Biolegend, 173423
  - CD11c, N418, Biolegend, 117311
Validation

All antibodies were validated by and purchased from commercial vendors and were used per manufacturer’s instructions. All antibodies from Biolegend, BD Biosciences,clone Bio-sciences, ThermoFisher Scientific, Millipore Sigma, Jackson ImmunoResearch were subjected to quality control testing.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research.

Laboratory animals
All laboratory mice (Mus musculus) used in these studies were aged 6-12 at initiation of experiment and were female, with the exception of one experiment involving TCRaf/Ii mice that used two male mice. Strains used include C57BL/6, C57BL/6N, B6.SJL-Ptprc<sup>Peptc</sup>/Boy, B6-Thy<sup>1</sup>, C57BL/6, C57BL/10, C57BL/6J, C57BL/10 TCR transgenic mice, Ub CreaT2, TCRaf/Ii.

Wild animals
Female pet shop mice (age not provided by vendor) were purchased from various pet stores in the greater Minneapolis-St. Paul metropolitan area and housed in a BSL-3 facility. No wild animals were used in this study.

Field-collected samples
No field-collected samples were used in this study.

Ethics oversight
All mice were used in accordance with guidelines established by the Institutional Animal Care and Use Committee at the University of Minnesota.

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

Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- 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

For isolation of small intestinal epithelial lymphocytes (SI-IELS), the small intestine was removed, Peyer’s patches were excised, and the intestine was cut longitudinally and then laterally into 0.5-1 cm² pieces. Large intestines were cut similarly. To isolate IELs, intestinal pieces were incubated with 0.154 mg/ml dithioerythritol (DTE) in 10% HBSS/1% PBS bicarbonate for 30 min at 37°C, stirring at 450 rpm. Following IEL isolation, intestinal pieces were further processed to remove lamina propria lymphocytes (LPL), by treatment with 100 U/ml type I collagenase (Worthington) in RPMI 1640, 5% FBS, 2 mM MgCl₂, 2 mM
CaCl2 for 45 min at 37°C, stirring at 450 rpm. The following tissues were cut into pieces and enzymatically digested with 100 U/ml type I collagenase (Worthington) in RPMI 1640, 5% FBS, 2 mM MgCl2, 2 mM CaCl2 at 37°C, stirring at 450 rpm; salivary gland (30 min), fat (30 min), brain (45 min), spinal cord (45 min), skin (1 hr) and lung (1 hr). For isolation of the female reproductive tract, the uterine horns, cervix, and vaginal tissue were resected and cut into small pieces prior to treatment with 0.5 mg/ml type IV collagenase (Sigma) RPMI 1640, 5% FBS, 2 mM MgCl2, 2 mM CaCl2 [treated for 1 hr] at 37°C, stirring at 450 rpm. After enzymatic treatment, the remaining tissue pieces were further mechanically disrupted by a gentleMACS Dissociator. The liver was mechanically dissociated using the back of a syringe over a 70-mm nylon cell strainer (Falcon). From single cell suspensions, lymphocytes were separated using a 44%/67% Percoll density gradient. Spleen, lymph nodes, and thymus were mechanically dissociated using the back of a syringe against a polystyrene Petri dish that had previously been scored. Peripheral blood, spleen, and liver were treated with ACK lysis buffer.

| Instrument | BD LSRII or LSR Fortessa |
|------------|--------------------------|
| Software   | BD FACS Diva was used for data collection and FlowJo 10 was used for data analysis. |
| Cell population abundance | No post-sort fractions were collected in these studies. |
| Gating strategy | FSC-A/SSC-A gating was used to identify singlets of appropriate leukocyte size. Viability dye was used to exclude dead cells. For studies of memory CD8 T cells, CD8a+ T cells were gated on followed by CD44+  memory T cells labeled by CD45.1+, Thy1.1+ or reactive to tetramer of interest (gp33, N). Neutrophils were distinguished by expression of CD11b and Ly6G. Eosinophils were identified by Siglec-F expression. Intra lymphoid, natural killer, B cell, and monocyte/macrophage populations were distinguished after excluding lineage-positive cells using combinations of CD3, CD5, CD19, B220, and Ly6G and then using recommended lineage defining markers as described36,37. Monocytes were further subdivided into classical and patrolling populations on the basis of Ly6C expression. Lung macrophages were subdivided into alveolar and interstitial populations on the basis of Siglec-F and CD11b expression. |

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