

Barrier immune memory is programmed by intestinal epithelial cell presentation of cytosol-delivered bacterial antigens

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Antigen compartmentalization is known to influence CD4 and CD8 T cell recognition but its impact on immune responses to different antigens expressed by the same pathogen is poorly understood. By tracking the clonal CD4 T cell response to a single epitope shuttled between different virulence proteins of *Citrobacter rodentium*, we found that response magnitude and quality depended on antigen localization. Antigens retained within bacteria elicited limited responses, whereas antigens injected into colonocyte cytosol through a type III secretion system generated robust mucosal CD4 T cell responses and promoted formation of epithelial-resident memory T cells. This process required direct presentation by infected epithelial cells. Single-cell transcriptomic analyses revealed that sustained, bidirectional communication between epithelial cells and T cells was essential to elicit both barrier-protective functions and transcriptional programs favoring tissue residency over central memory differentiation. These findings identify nonprofessional antigen-presenting cells as key regulators of CD4 T cell memory fate.

T cell antigen recognition is focused on the surface of cells displaying antigenic peptides bound to major histocompatibility (MHC) molecules: MHC class I for CD8 T cells and MHC class II for CD4 T cells. Whereas MHC class I is ubiquitously expressed by nucleated cells, MHC class II is largely restricted to ‘professional’ antigen-presenting cells (APCs) of hematopoietic origin (dendritic cells (DCs), macrophages and B cells). However, some nonhematopoietic cells, including epithelial cells lining barrier tissues at sites of pathogen entry^{1–7}, can be induced to

express MHC class II and act as ‘nonprofessional’ APCs. Although these cells lack costimulatory molecules and cannot prime naive T cells^{8–10}, they may recruit CD4 T cell help by presenting pathogen-derived antigens, which is akin to the recruitment of cytotoxic CD8 T cells by infected MHC class I⁺ cells to target them for death.

We recently discovered a subset of absorptive intestinal epithelial cells (IECs) termed distal colonocytes (DCCs), so named for their unique distribution to the distal colon, which are selectively targeted

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by the murine enteropathogenic bacterium, *Citrobacter rodentium*¹. *C. rodentium* belongs to a family of closely related Gram-negative bacteria that includes the human pathogens enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC)^{11,12}, which deploy a type III secretion system (T3SS) to colonize the apical surface of host IECs, where they form characteristic attaching and effacing lesions^{11,13,14}. Virulence proteins encoded by the locus of the enterocyte effacement (LEE) pathogenicity island^{15,16} are expressed early in infection to assemble the T3SS molecular syringe, which injects bacterial effectors into the EC, where they localize to the apical membrane or cytosol^{17–20}. Both LEE and non-LEE gene loci (for example, *nle* family) encode T3SS-secreted effectors that contribute to *Cr* pathogenicity^{21,22}. Reflecting the tropism of *C. rodentium* for DCCs, but not absorptive colonic ECs (cECs) that line the proximal colon (proximal colonocytes (PCCs)), DCCs recruit CD4 T cells that produce the epithelium-protective cytokine interleukin-22 (IL-22), contingent on their expression of MHC class II¹. However, which bacterial virulence proteins contribute to the CD4 T cell response, and how their compartmentalization between bacterium and host epithelial cell affects the amplitude and quality of that response, are undefined.

Using isogenic strains of *C. rodentium* engineered to express the same CD4 T cell epitope fused to different *C. rodentium* virulence proteins, we have mapped a clonal CD4 T cell response based on the bacterial ‘carrier’ protein used to deliver cognate antigen, thereby controlling for differences in peptide affinity for MHC class II. We find striking effects on the magnitude and quality of the clonal CD4 T cell response contingent on antigen localization—bacterial, epithelial membrane or epithelial cytosol. Only antigens delivered to the cEC cytosol promoted robust clonal T cell expansion, retention in the infected mucosa and development into tissue-resident memory T (T_{RM}) cells. In the absence of antigenic localization to the cEC cytosol, the clonal response is blunted and memory cell development defaults to a nonresident fate, generating few memory cells in the intestinal epithelium. Thus, cytosolic antigen delivery promotes positioning of CD4 memory cells within the intestinal epithelium, where they are best poised for rapid local response upon reinfection. These results establish a central role for nonprofessional APCs in controlling CD4 T cell memory fate, with implications for mucosal vaccine design.

Results

Injected bacterial antigens induce enhanced mucosal T cell responses

To assess how subcellular localization of bacterial antigens affects CD4 T cell responses, we engineered *C. rodentium* strains that express the lymphocytic choriomeningitis virus (LCMV) gp66–80 peptide recognized by the SMARTA T cell receptor (TCR) fused to different

bacterial proteins, including a hemagglutinin (HA) tag for tracking fusion protein expression²³ (Fig. 1a,b, Extended Data Fig. 1a–c and Supplementary Table 1). Four proteins targeted to different compartments were selected: intimin (*eae*), an LEE-encoded surface adhesin on the outer bacterial membrane; the translocated intimin receptor (*tir*), an LEE-encoded effector that localizes to the apical plasma membrane after injection into cECs, where it binds intimin to establish firm adhesion; *nleA*, a non-LEE-encoded effector that distributes diffusely in the host cell cytosol; and *espZ*, an LEE-encoded effector that concentrates in the cytosol beneath adherent bacteria (Fig. 1b,d). All engineered strains retained virulence comparable to wild-type (WT) *C. rodentium* (DBS100) in susceptible C3H/HeJ mice, indicating that epitope insertion did not compromise bacterial fitness or pathogenicity (Fig. 1c).

Confocal microscopy of colonocytes (CCs) isolated at day 8 after infection confirmed expected subcellular distributions of HA-tagged antigens (Fig. 1d)^{24–29}. Intimin-gp66 decorated the bacterial cell surface, while *tir*-gp66 formed puncta at sites of bacterial attachment to the apical plasma membrane. Both *nleA*-gp66 and *espZ*-gp66 were delivered into the host cell cytoplasm, *nleA* diffusely and *espZ* in intracytoplasmic clusters suggestive of partial association with bacterial pedestals²⁹.

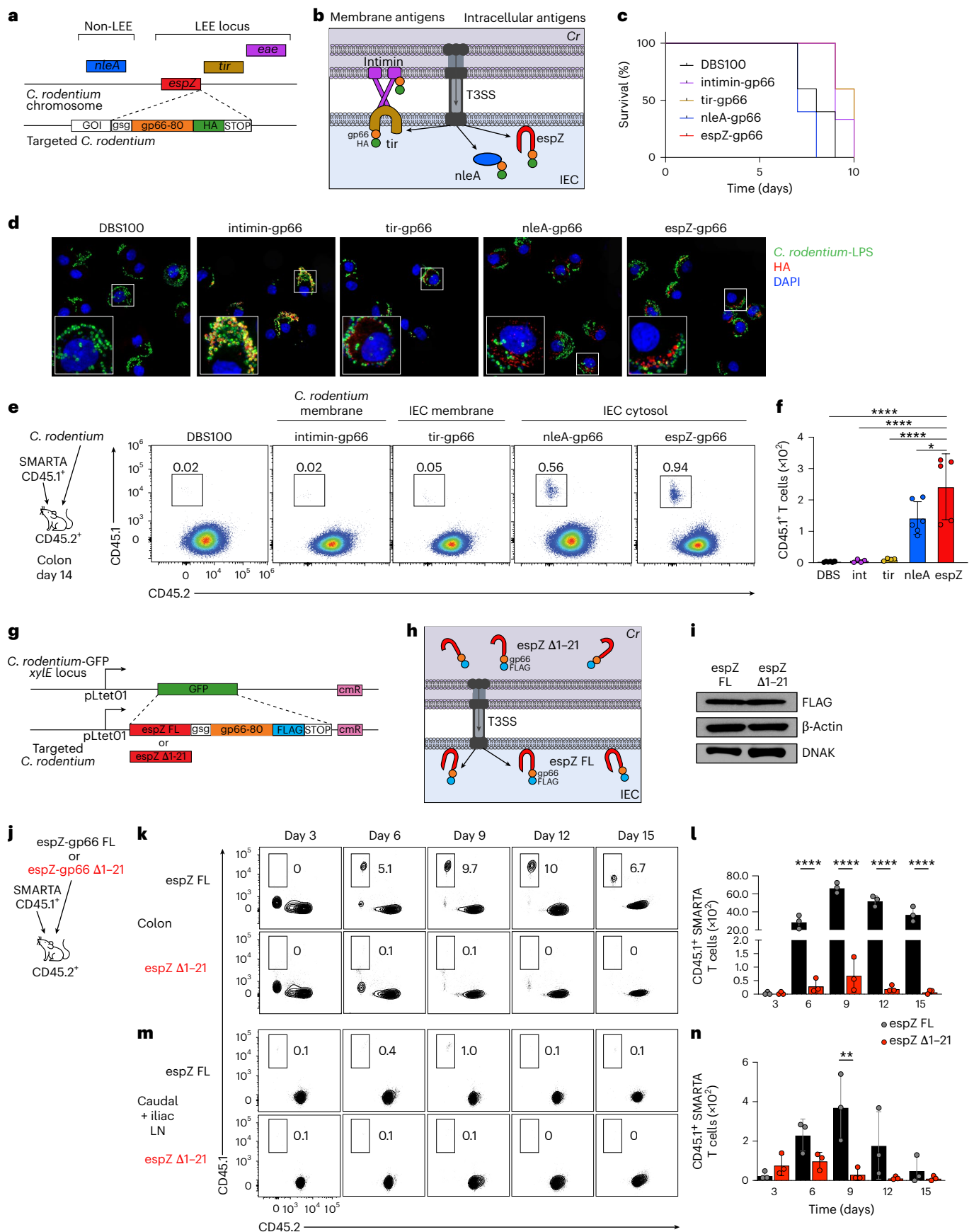
Using a modified adoptive transfer protocol to track antigen-naïve, congenically marked SMARTA T cells, recipient mice were challenged with the different *C. rodentium* strains (Extended Data Fig. 1d–i). SMARTA T cell numbers recovered from the infected mid-distal colon at the peak of the mucosal response (day 14 after infection) were strikingly different (Fig. 1e,f). While membrane-localized antigen, whether bacterial (intimin-gp66) or cEC (*tir*-gp66), failed to induce accumulation of SMARTA cells that differed significantly from control WT *C. rodentium*, both cytosolically delivered antigens (*nleA* and *espZ*) induced robust responses. *nleA*-gp66 and *espZ*-gp66 induced 28-fold and 47-fold greater SMARTA T cell accumulation in the colon, respectively, compared to intimin-gp66.

To determine whether antigen abundance might underlie these differences, we quantified expression of the HA-tagged proteins in vitro and in vivo (Extended Data Fig. 1j–l). Immunoblotting of *C. rodentium* cultures activated to express virulence proteins indicated comparable expression of intimin to *tir* and *nleA* in bacterial cell pellets, but it was absent in culture supernatants, confirming its bacterial membrane localization. *Tir* and *nleA* appeared in both pellets and supernatants, reflecting secretion, with *tir* the most abundantly secreted protein in vitro. *EspZ* was not detected in vitro, which is consistent with previous reports that this effector is poorly induced in vitro³⁰.

In vivo expression was quantified in *C. rodentium*-DCC conjugates from infected mice, using the four epitope-tagged variants engineered into a green fluorescent protein (GFP)-expressing *C. rodentium* background (*C. rodentium*-GFP) to enable flow cytometry sorting^{1,31}.

Fig. 1 | Subcellular localization of *C. rodentium* antigens determines the magnitude of colonic CD4⁺ T cell responses. Candidate *C. rodentium* proteins were tagged with LCMV gp66–80 epitope and used for adoptive transfer experiments with CD45.1⁺ SMARTA T cells. **a**, Schematic of the gsg-gp66–80-HA construct inserted into *C. rodentium* LEE genes (LEE locus; *eae* (intimin), *tir* and *espZ*) and non-LEE gene, *nleA*. **b**, Schematic of subcellular localizations of the *C. rodentium* effector proteins intimin, *tir*, *nleA* and *espZ* relative to IEC-bound *C. rodentium* cell. **c**, Survival of C3H/HeJ-susceptible mice infected with DBS100 (WT), intimin-gp66, *tir*-gp66, *nleA*-gp66 and *espZ*-gp66. *n* = 5 mice per group. Data are from one experiment representative of two independent experiments. log-rank (Mantel–Cox) test. **d**, D8-infected EpCAM⁺ GFP⁺ IECs were sorted from mid-distal colons of C57BL/6 mice infected with *C. rodentium*-GFP (WT), intimin-gp66 *C. rodentium*-GFP, *tir*-HA *C. rodentium*-GFP, *nleA*-gp66 *C. rodentium*-GFP and *espZ*-gp66 *C. rodentium*-GFP, were analyzed using immunofluorescence microscopy. **e,f**, Transferred CD45.1⁺ SMARTA T cells were recovered from the mid-distal colons of C57BL/6 mice infected with DBS100, intimin-gp66, *tir*-gp66, *nleA*-gp66 or *espZ*-gp66 on day 14 of infection, stained for CD45.1 and CD45.2 (**e**) and quantified (**f**) using flow cytometry. Data are shown as the mean ± s.d. with

individual mice shown; *n* = 6 (DBS100), 4 (intimin-gp66), 4 (*tir*-gp66), 6 (*nleA*-gp66) and 5 (*espZ*-gp66) mice. Data are from one experiment representative of two independent experiments. One-way analysis of variance (ANOVA) with Tukey's post hoc test; **P* < 0.05, *****P* < 0.0001. **g**, Schematic of the *espZ* FL-gp66-FLAG and *espZ* Δ1–21-gp66-FLAG constructs used to replace the GFP coding sequence in xyle pseudogene in the *C. rodentium*-GFP parent strain. **h**, Schematic of subcellular locations of full-length *espZ*-gp66 translocated into the host IEC or *espZ* Δ1–21-gp66 protein, which remains associated with *C. rodentium* bacteria. **i**, Total protein lysate from sorted mid-distal Ly6G⁺ DCCs from mice infected with *espZ* FL or *espZ* Δ1–21 were analyzed using immunoblotting for FLAG-tagged antigen, with β-actin and DnaK as loading controls. **j**, Schematic of adoptively transferred naïve CD45.1⁺ SMARTA T cells into C57BL/6 mice infected with either *espZ* FL or *espZ* Δ1–21. **k–n**, On days 3–15 after infection, cells recovered from colon (**k,l**) or caudal/iliac LNs (**m,n**) were stained for CD45.1 and CD45.2 (**l** and **n**) and quantified (**l** and **n**) using flow cytometry. Data are shown as the mean ± s.d. with individual mice shown; *n* = 3 mice per group per time point. Data are from one experiment representative of two independent experiments. One-way ANOVA with Tukey's post hoc test; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.



Intimin and espZ were detected at significantly lower levels than tir or nleA, which were the most abundant proteins in *C. rodentium*-DCC conjugates of those assessed (Extended Data Fig. 1l). Notably, both nleA and espZ induced similar antigen-specific responses despite the lower abundance of espZ. Conversely, tir was most abundant yet induced a minimal response, indicating that compartmentalization, not abundance, was the more critical determinant of immunogenicity. However, because these four carrier proteins differ in structure, stability and turnover, we sought a comparison in which only subcellular localization varied.

Therefore, we isolated translocation as the sole variable, comparing full-length espZ with a truncated variant lacking its T3SS secretion signal—the same protein and epitope, differing only in access to the host epithelium cytosol. gp66 and a FLAG peptide were fused to the carboxy terminus of either full-length espZ (espZ FL) or a truncated espZ variant lacking the N-terminal 21 amino acids (espZ Δ 1–21), each targeted to the *xylE* pseudogene locus of *C. rodentium*^{31,32} (Fig. 1g,h and Extended Data Fig. 1a–c), which drives high-level expression independently of the endogenous *espZ* locus, thereby avoiding perturbation of *C. rodentium* pathogenicity (Extended Data Fig. 1a). Immunoblotting of isolated *C. rodentium*-bound cECs established that full-length espZ-gp66 and the Δ 1–21 mutant were comparably abundant in *C. rodentium*-DCC conjugates, excluding epitope abundance as a confounder (Fig. 1i).

Recipients challenged with the two espZ *C. rodentium* variants showed a marked bifurcation of the antigen-specific response: espZ FL induced a greater than 100-fold increase in mucosal SMARTA T cells over espZ Δ 1–21 at the peak of the response (day 9) (Fig. 1j–l). This did not reflect differential mucosal retention alone, as lymph nodes (LNs) draining the distal colon (caudal and iliac) showed comparable differences over the time course (Fig. 1m,n). Thus, with protein, epitope and abundance held constant, cytosolic access alone accounted for the greatest difference in the clonal CD4 T cell response to antigen.

T cell immunity to *C. rodentium* is initiated in the cecum before spreading to the distal colon

C. rodentium cultured in vitro first colonizes the cecum following infection, where expression of the pathogenicity genes required to assemble the T3SS begins^{17,33,34}, before spreading to the distal colon^{32,35}. The immunological consequences of this early colonization are incompletely understood, as models to track clonal T cell responses to *C. rodentium* antigens have been lacking. Therefore, we asked where antigen-specific T cell responses were initiated, and whether the muted response to noncytosolic antigens reflected altered geography of priming, in the cecum rather than distal colon.

Using fluorescence in situ hybridization (FISH) with probes for total Eubacteria and *C. rodentium*, we examined bacterial localization early in cecal colonization in tissue processed to preserve mucus architecture (Fig. 2a–c). Remarkably, *C. rodentium* exhibited highly focal

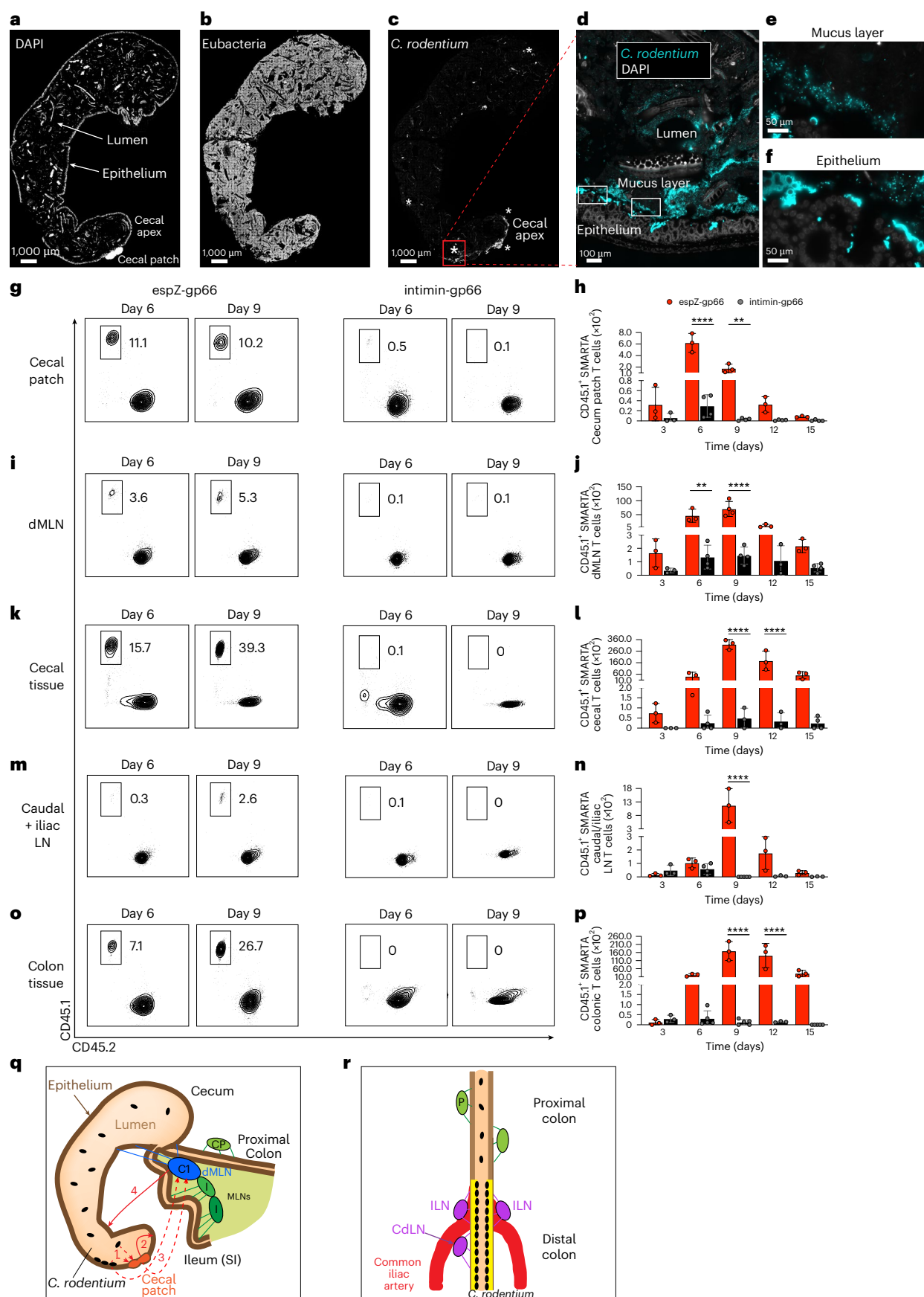
epithelial colonization at the cecal apex, close to, but not directly associated with, the cecal patch, a gut-associated lymphoid tissue (GALT) structure analogous to Peyer's patches³⁶ (Fig. 2c–f). *C. rodentium* was also abundant in the cecal mucus layer and free in the lumen. The basis for this restricted colonization is unknown, but its proximity to the cecal patch raised the possibility that this lymphoid structure serves as an early site of antigen sampling and T cell priming³⁷. Therefore, we tracked SMARTA T cell dynamics after infection with espZ-gp66 or intimin-gp66 across *C. rodentium*-colonized regions: the cecum and its draining distal mesenteric LN (dMLN); and the distal colon and its draining caudal and iliac LNs (Fig. 2g–r).

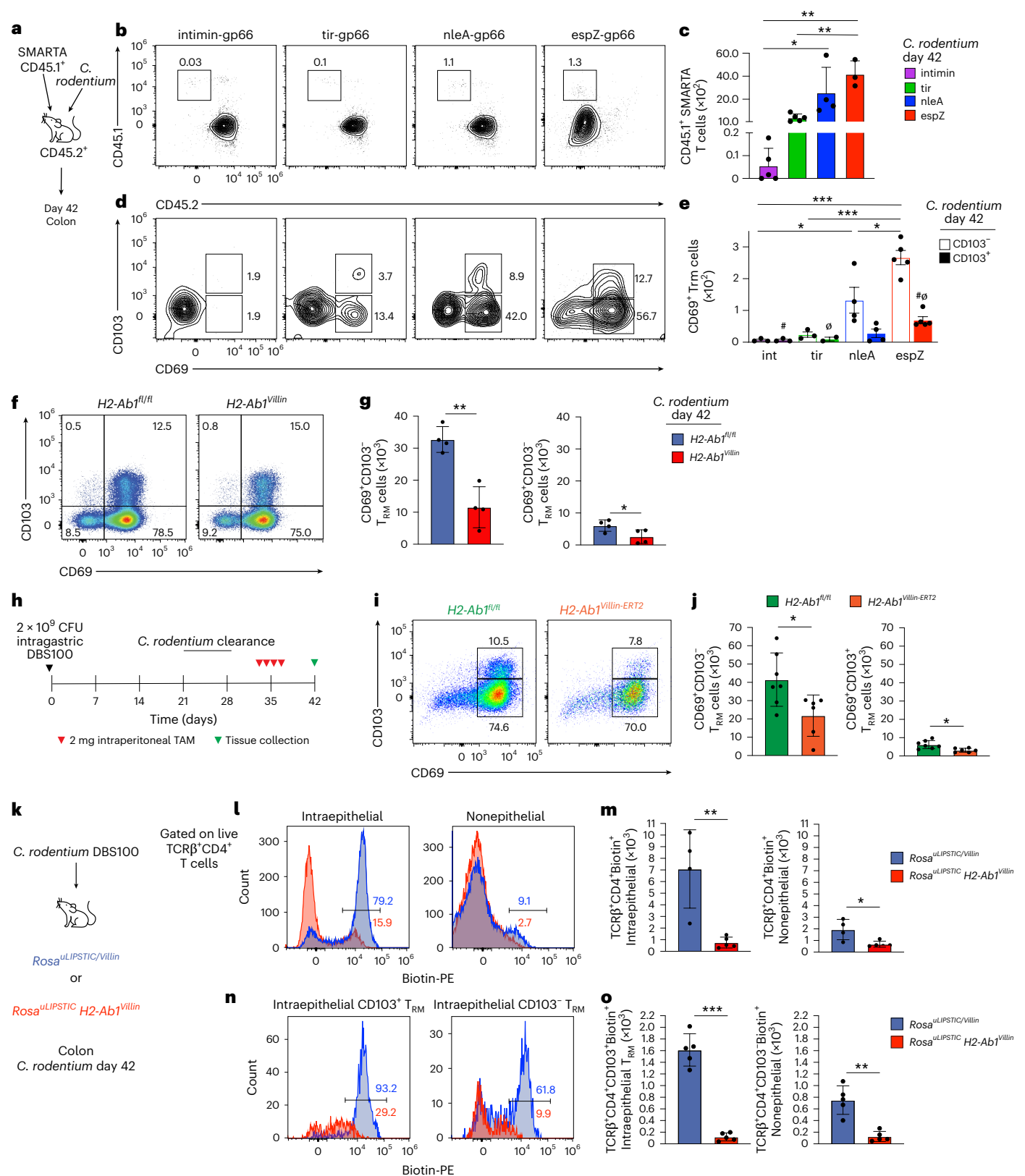
In response to espZ-gp66, SMARTA cell numbers were greatest in the cecal patch at day 6 after infection before rapidly declining by day 9 (Fig. 2g–j), whereas the cecum-draining dMLN (C1) response emerged on day 6, peaked at day 9 and contracted by day 12 (Fig. 2i,j). T cell infiltration into the cecal mucosa was delayed until day 9 and persisted through day 12 before declining (Fig. 2k,l). Although the cecal patch response was modestly accelerated, its peak amplitude was approximately tenfold lower than dMLN and approximately 50-fold lower than the cecal mucosa, suggesting that priming in the cecal patch, presumably via antigen translocation across the patch epithelium rather than direct colonization, is minor relative to that in the dMLN. The clonal response in the colon mucosa (Fig. 2m–p) largely tracked that in the cecum, whereas the dMLN response preceded that of distal colon-draining LNs and exceeded it by more than fivefold. These kinetics indicate that the cecum-draining LN is the dominant site of SMARTA cell priming, which is consistent with initial detection of translocated *C. rodentium* virulence proteins there.

The response to intimin-gp66 was considerably weaker at all sites and time points and, although measurable in draining LNs, was essentially undetectable in cecal and distal colonic mucosae (Figs. 1e,f and 2g–p), excluding differing sites of induction as an explanation. Accordingly, in a similar survey of response to either espZ FL or Δ 1–21 espZ (Extended Data Fig. 2), the magnitude of the clonal response to injected antigen dwarfed that of the same antigen sequestered in the bacterial cytosol, at each time point and tissue site, mirroring findings that compared espZ-gp66 and intimin-gp66. Notably, the kinetics in response to espZ FL were not appreciably accelerated in the cecum despite constitutive expression of espZ from the *xylE* gene locus, which is consistent with rapid expression of native espZ on arrival of *C. rodentium* in the cecum. Also, most SMARTA cells were ultimately recovered from infected mucosae, which is consistent with their retention thereby epithelial antigen recognition. Thus, the superior response to injected antigen is consistent across all cecal and colonic sites of *C. rodentium* colonization, initiated in the cecum-draining LN and tracking with antigen spread to the distal colon (Fig. 2q,r). This establishes that subcellular antigen localization, rather than antigen quantity or regional differences in antigen delivery dynamics, as the major determinant of the magnitude and distribution of the clonal T cell response.

Fig. 2 | Early *C. rodentium* colonization in the cecum initiates CD4⁺ T cell priming in GALT. a–f, Cecae were collected from 48-h DBS100-infected C57BL/6 mice and fixed in ice-cold methacarn solution to preserve mucus layer structure. Cecal sections were stained with 4',6-diamidino-2-phenylindole (DAPI) (a) and FISH probes for total Eubacteria (b) and *C. rodentium* (c–f); *C. rodentium* (cyan) localization near IECs (DAPI, grey) in d, with higher magnification of *C. rodentium* in the loose mucus layer (e) and abutting the epithelium (f). g–p, Adoptively transferred naive CD45.1⁺ SMARTA T cells in C57BL/6 mice infected with espZ-gp66 or intimin-gp66 were analyzed using flow cytometry and quantified from the cecal patch (GALT, g (flow plots), h (quantification)), dMLN-C1 (i (flow plots), j (quantification)), cecal tissue (k (flow plots), l (quantification)), caudal and iliac LNs (m (flow plots), n (quantification)), and colon tissue (o (flow plots), p (quantification)). Representative flow cytometry plots are shown for days 6 and 9 (g,i,k,m,o), with quantification across days 3–15 (h,j,l,n,p). Data are shown as the mean $\pm$ s.d. with individual mice shown. h,j,l,n,p, $n = 3$ espZ-gp66

and 3 intimin-gp66 (day 3), 3 and 4 (day 6), 4 and 4 (day 9), 3 and 4 (day 12) and 3 and 4 (day 15) mice; the same mice were sampled for all five tissues. Data are from one experiment representative of two independent experiments. One-way ANOVA with Tukey's post hoc test; ** $P < 0.01$, **** $P < 0.0001$. q,r, Together, q and r illustrate the spatiotemporal sequence of T cell priming initiated in cecum-draining lymphoid tissue before spreading to the distal colon and its dLNs. q, Schematic of the cecum and proximal colon depicting the proposed sequence of early antigen acquisition and T cell priming after *C. rodentium* colonization of the cecal apex. The numbered arrows indicate the proposed sequence of events, with the dashed lines indicating the routes of antigen and the solid lines indicating the movement of primed effector CD4 T cells to the infected cecal mucosa. r, Schematic of the distal colon and associated lymphoid drainage depicting *C. rodentium* attachment to DCCs and subsequent antigen drainage into the caudal and iliac LNs, which drain the distal colon via lymphatics running alongside the common iliac artery. SI, small intestine.





Epithelial cell presentation of injected bacterial antigens promotes CD4 T_{RM} cell development

Evidence for a role of nonprofessional APCs in memory T cell development has been limited^{38–41}. Given the superior ability of injected antigens to drive mucosal CD4 T cell responses, and our previous finding that IEC antigen presentation recruits CD4 T cell help¹, we asked whether T cell recognition of IEC-presented antigen is central to mucosal memory development.

Before the *C. rodentium* challenge, few colonic CD4 T cells displayed a resident memory phenotype (CD69⁺CD103⁻ and CD69⁺CD103⁺) (Extended Data Fig. 3) but both populations were strongly induced during active infection and persisted into the memory phase (days 43 and 82). Both IL-17A-expressing and interferon- γ (IFN γ)-expressing cells comprised the memory cell pool (Extended Data Fig. 3e–h), with predominance of IFN γ competence at each time point, reflecting greater numbers of type

Fig. 3 | Epithelial cell presentation of injected *C. rodentium* antigens drives CD4⁺ T_{RM} cell formation and maintenance. **a**, Naive CD45.1⁺ SMARTA T cells were adoptively transferred into C57BL/6 mice infected with either intimin-gp66, tir-gp66, nleA-gp66 or espZ-gp66 *C. rodentium* strains. **b,c**, Cells from the colons of mice were collected on day 42 after infection, stained for CD45.1 and CD45.2 (**b**) and quantified (**c**) using flow cytometry. Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 5$ (intimin-gp66), 5 (tir-gp66), 4 (nleA-gp66) and 3 (espZ-gp66) mice. Data are from one experiment representative of two independent experiments. One-way ANOVA with Tukey's post hoc test; $^*P < 0.05$, $^{**}P < 0.01$. The espZ-gp66 panels in **b** and **d** were acquired on a different flow cytometer than intimin-, tir- and nleA-gp66 panels, resulting in a shift in the CD45.2⁺ endogenous population along the x axis; this does not affect the gating of CD45.1⁺ SMARTA T cells or the interpretation of the results. **d,e**, Colonic cells from day 42-infected mice were gated on CD45.1⁺ SMARTA T cells and stained for CD4, TCR beta chain (TCR β), CD69 and CD103 (**d**) and quantified (**e**) using flow cytometry to quantitate the numbers of CD69⁺CD103⁺ and CD69⁺CD103⁺ T_{RM} cells. Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 3$ (intimin-gp66), 3 (tir-gp66), 4 (nleA-gp66) and 5 (espZ-gp66) mice. Data are from a single experiment, independent of that shown in **b,c**; two independent experiments were performed in total. One-way ANOVA with Tukey's post hoc test; $^*P < 0.05$, $^{***}P < 0.001$ (CD103⁺ across antigens); $\#P < 0.05$ and $\phi P < 0.05$ (CD103⁺ across antigens). **f,g**, Cells from the colons of DBS100-infected *H2-Ab1^{fl/fl}* (blue) and *H2-Ab1^{Villin}* (red) mice were collected on day 42, stained for CD4, TCR β , CD69 and CD103 (**f**), and quantified (**g**) using flow cytometry (**f**). Quantification of CD69⁺CD103⁺ and CD69⁺CD103⁺ T_{RM} cell numbers from the colons of DBS100-infected *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice at day 42 (**g**).

Data are shown as the mean $\pm$ s.d. with individual mice shown; **g** left: $n = 4$ *H2-Ab1^{fl/fl}* and 4 *H2-Ab1^{Villin}*; **g** right: $n = 5$ *H2-Ab1^{fl/fl}* and 4 *H2-Ab1^{Villin}* mice. Data are from one experiment representative of two independent experiments. Unpaired two-sided Student's *t*-test; $^*P < 0.05$, $^{**}P < 0.01$. **h**, Schematic of TAM-induced deletion of *H2-Ab1* after *C. rodentium* clearance in *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin-ERT2}* mice receiving TAM on days 33–36 after infection. **i,j**, Cells from the colons of DBS100-infected *H2-Ab1^{fl/fl}* (green) and *H2-Ab1^{Villin-ERT2}* (orange) mice were stained as in **d** (**i**) and quantified (**j**) using flow cytometry on day 42 after infection to quantitate the number of CD69⁺CD103⁺ and CD69⁺CD103⁺ T_{RM} cells after TAM-induced deletion of *H2-Ab1*. Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 7$ *H2-Ab1^{fl/fl}* and $n = 6$ *H2-Ab1^{Villin-ERT2}* mice. Data are from one experiment representative of two independent experiments. Unpaired two-sided Student's *t*-test; $^*P < 0.05$. **k**, Schematic of LIPSTIC experiments using *Rosa^{ulipstic/Villin}* (blue) and *Rosa^{ulipstic}* *H2-Ab1^{Villin}* (red) mice at day 42 after infection, after biotin-LPETG administration. **l,m**, Cells isolated from intraepithelial (left) or nonepithelial (right) compartments of colon from *Rosa^{ulipstic/Villin}* (blue) and *Rosa^{ulipstic}* *H2-Ab1^{Villin}* (red) mice at day 42 after infection were stained as in **d** (**l**); anti-biotin antibody and the numbers of biotin⁺ CD4⁺ cells were quantified (**m**) in the intraepithelial and nonepithelial compartments (**l,m**), as well as the number of biotin⁺ CD69⁺CD103⁺ and CD69⁺CD103⁺ T_{RM} cells in the intraepithelial compartment (**n**, flow plots; **o**, quantification). Data are shown as the mean $\pm$ s.d. with individual mice shown; **m,n**: $n = 4$ *Rosa^{ulipstic/Villin}* and $n = 5$ *Rosa^{ulipstic}* *H2-Ab1^{Villin}*; **o** (both panels): $n = 5$ mice per genotype. Data are from one experiment representative of two independent experiments. Unpaired two-sided Student's *t*-test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

1 helper T (T_H1) cells than type 17 helper T (T_H17) cells during the effector response (day 14 and see below).

Assessment of total CD69⁺CD103⁺ and CD69⁺CD103⁺ SMARTA cell numbers generated in response to different *C. rodentium* strains revealed striking differences in both number and phenotype (Fig. 3a–e). At day 42 after infection, more than 3 weeks after bacterial clearance, few CD69⁺ SMARTA cells (T_{RM} phenotype) were generated in response to intimin-gp66. Despite the modest mucosal T cell response induced by tir-gp66 (Fig. 3a–f), there was significant induction of both CD69⁺CD103⁺ and CD69⁺CD103⁺ T_{RM} cells, albeit substantially lower than the numbers of SMARTA T_{RM} cells responding to nleA-gp66 and espZ-gp66 (22-fold and 30-fold greater, respectively, than intimin-gp66), which represented the majority of the total recovered SMARTA cells (Fig. 3d,e). Interestingly, although injected antigen promoted the population of other intestinal segments by CD69⁺ T_{RM} cells, CD103⁺ T_{RM} cells were restricted to the cecum and distal colon, the sites of *C. rodentium* colonization (Supplementary Data Fig. 1). Rare CD69⁺CD103⁺ and no CD69⁺CD103⁺ T_{RM} cells were found in nonintestinal tissue (salivary gland). Thus, T_{RM} cell development was strongly biased toward injected

antigens, especially cytosolic ones, and induction of CD103⁺ T_{RM} cells was largely restricted to sites of *C. rodentium* colonization.

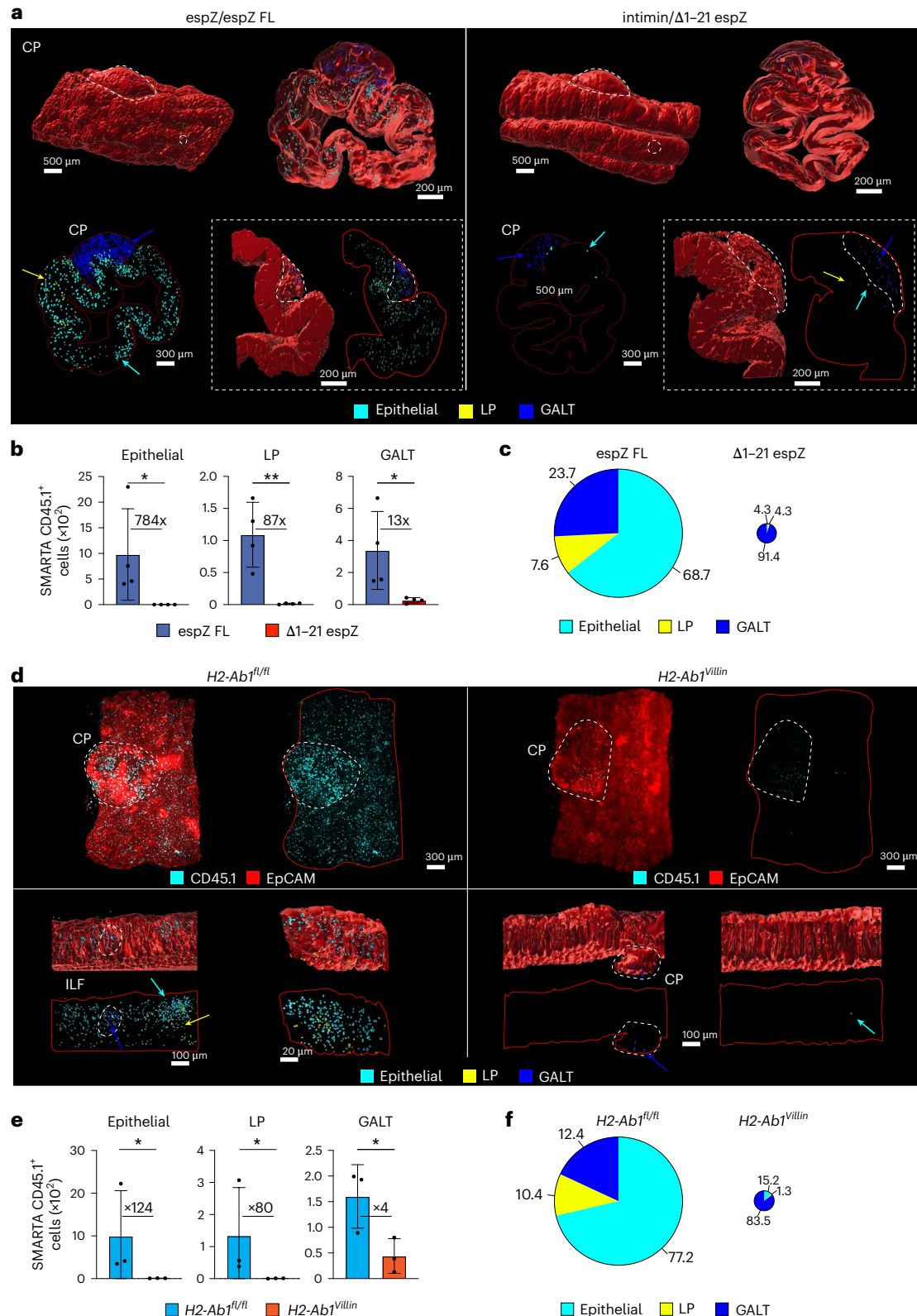
These findings suggested that epithelial antigen presentation is critical for T_{RM} cell programming. We found previously that upregulation of epithelial MHC class II was important for controlling *C. rodentium* infection, while its loss impaired the antigen-specific T cell response¹. To determine whether epithelial MHC class II is required for T_{RM} induction, we compared control *H2-Ab1^{fl/fl}* mice with *H2-Ab1^{Villin}* mice, in which MHC class II deficiency is targeted to IECs. Compared to control mice, *H2-Ab1^{Villin}* mice showed significantly reduced CD69⁺CD103⁺ and CD69⁺CD103⁺ CD4 T_{RM} populations at day 42 after infection (Fig. 3f,g), demonstrating that epithelial MHC class II is necessary for optimal CD4 T_{RM} development. To determine whether ongoing MHC class II expression maintains T_{RM} cells after bacterial clearance, we used tamoxifen (TAM)-inducible deletion of epithelial MHC class II (*H2-Ab1^{Villin-ERT2}* mice) (Fig. 3h). Memory-phase deletion of MHC class II significantly reduced both CD69⁺CD103⁺ and CD69⁺CD103⁺ T_{RM} subsets (Fig. 3i,j), indicating that continued epithelial MHC class II expression contributes to T_{RM} cell maintenance after pathogen clearance.

Fig. 4 | Differential antigen localization promotes distinct tissue patterns of CD4 T cell memory. **a**, Representative LSMF surface renderings of distal colon tissue from WT CD45.2⁺ mice infected with *C. rodentium* expressing espZ-gp66 (left) or espZ FL (left, dashed inset) or intimin-gp66 (right) or Δ 1–21-infected mice (right, dashed inset) that received SMARTA CD45.1⁺ T cells, collected at day 42 after infection. Top: longitudinal and transverse views of the intact colonic tube (300 and 200- μ m optical sections). Bottom: CD45.1⁺ SMARTA T cells pseudocolored according to compartment: epithelial (cyan), LP (yellow) and GALT (blue). CP boundaries are indicated by dashed white outlines. Compartment assignments were made using the criteria described in Extended Data Fig. 4. **b**, Quantification of SMARTA CD45.1⁺ in the epithelial (left), LP (middle) and GALT (right) compartments from espZ FL-infected and espZ Δ 1–21-infected mice. Data are shown as the mean $\pm$ s.d. with individual mice shown; epithelial: $n = 4$ espZ FL and $n = 4$ espZ Δ 1–21; LP: $n = 4$ and 5; GALT: $n = 4$ and 5 mice. Data are from one experiment representative of two independent experiments. Unpaired two-sided Student's *t*-test; $^*P < 0.05$, $^{**}P < 0.01$. The fold differences shown above each compartment indicate the ratio of mean CD45.1⁺ cell number in espZ FL-infected versus espZ Δ 1–21-infected mice and are provided for descriptive comparison only; statistical comparisons are as indicated. **c**, Pie charts showing the relative distribution of SMARTA CD45.1⁺ T cells across epithelial, LP and GALT compartments in espZ FL-infected and espZ

Δ 1–21-infected mice (~ 1.6 mm³ field of view (FOV)). The pie chart area was scaled proportionally to total CD45.1⁺ cell number per FOV. **d**, Representative LSMF images (top: 300- μ m optical sections) and three-dimensional surface renderings (bottom: 100 and 20- μ m optical sections) of distal colon tissue from *H2-Ab1^{fl/fl}* or *H2-Ab1^{Villin}* mice that received SMARTA CD45.1⁺ T cells and were infected with *C. rodentium* espZ FL, collected at day 42 after infection. CD45.1⁺ (cyan) and EpCAM (red) cells are shown in the raw images; surface renderings show CD45.1⁺ cells pseudocolored according to compartment as in **a**. CP and isolated ILF boundaries are indicated by the dashed white outlines. **e**, Quantification of SMARTA CD45.1⁺ T cell numbers across compartments from *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice. Data are shown as the mean $\pm$ s.d. with individual mice shown; epithelial (left): $n = 3$ *H2-Ab1^{fl/fl}* and 3 *H2-Ab1^{Villin}*; LP (middle): $n = 3$ and 3; GALT (right): $n = 4$ and 3 mice. Data are from one experiment representative of two independent experiments. Unpaired two-sided Student's *t*-test; $^*P < 0.05$. The fold differences shown above each compartment indicate the ratio of mean CD45.1⁺ cell numbers in *H2-Ab1^{fl/fl}* versus *H2-Ab1^{Villin}* mice and are provided for descriptive comparison only; statistical comparisons are as indicated. **f**, Pie charts showing the relative distribution of SMARTA CD45.1⁺ T cells across epithelial, LP and GALT compartments in *H2-Ab1^{fl/fl}* (left) and *H2-Ab1^{Villin}* mice (right) (~ 1.6 mm³ FOV). The pie chart area was scaled proportionally to total CD45.1⁺ cell number per FOV.

To extend these results, MHC-class-II-dependent interactions between cECs and CD4 T cells were assessed using the Labeling Immune Partnerships by SorTagging Intercellular Contacts (LIPSTIC) transgenic system^{42,43} (Fig. 3k). In *Rosa^{uLIPSTIC/Villin}* mice, where ECs can transfer biotin to closely interacting partners, 79% of recovered intraepithelial CD4 T_{RM} cells were biotin⁺ at day 42 after infection (Fig. 3l,m). Loss of epithelial MHC-class-II-dependent interactions in *Rosa^{uLIPSTIC}/H2-Ab1^{Villin}* mice markedly reduced biotin labeling of intraepithelial CD4 T_{RM} cells

to 16% (approximate fivefold reduction). In contrast, nonepithelial CD4 T cells showed only modest MHC-class-II-dependent labeling, indicating infrequent interactions with IECs relative to intraepithelial CD4 T cells. Although CC-T_{RM} cell interactions were less frequent for CD103[−] than CD103⁺ CD4 T_{RM} cells, despite their greater abundance within the colonic epithelium, they remained substantial (~62% biotin⁺), indicating that the majority of both CD103[−] and CD103⁺ T_{RM} subsets sustain ongoing interactions with cECs that are



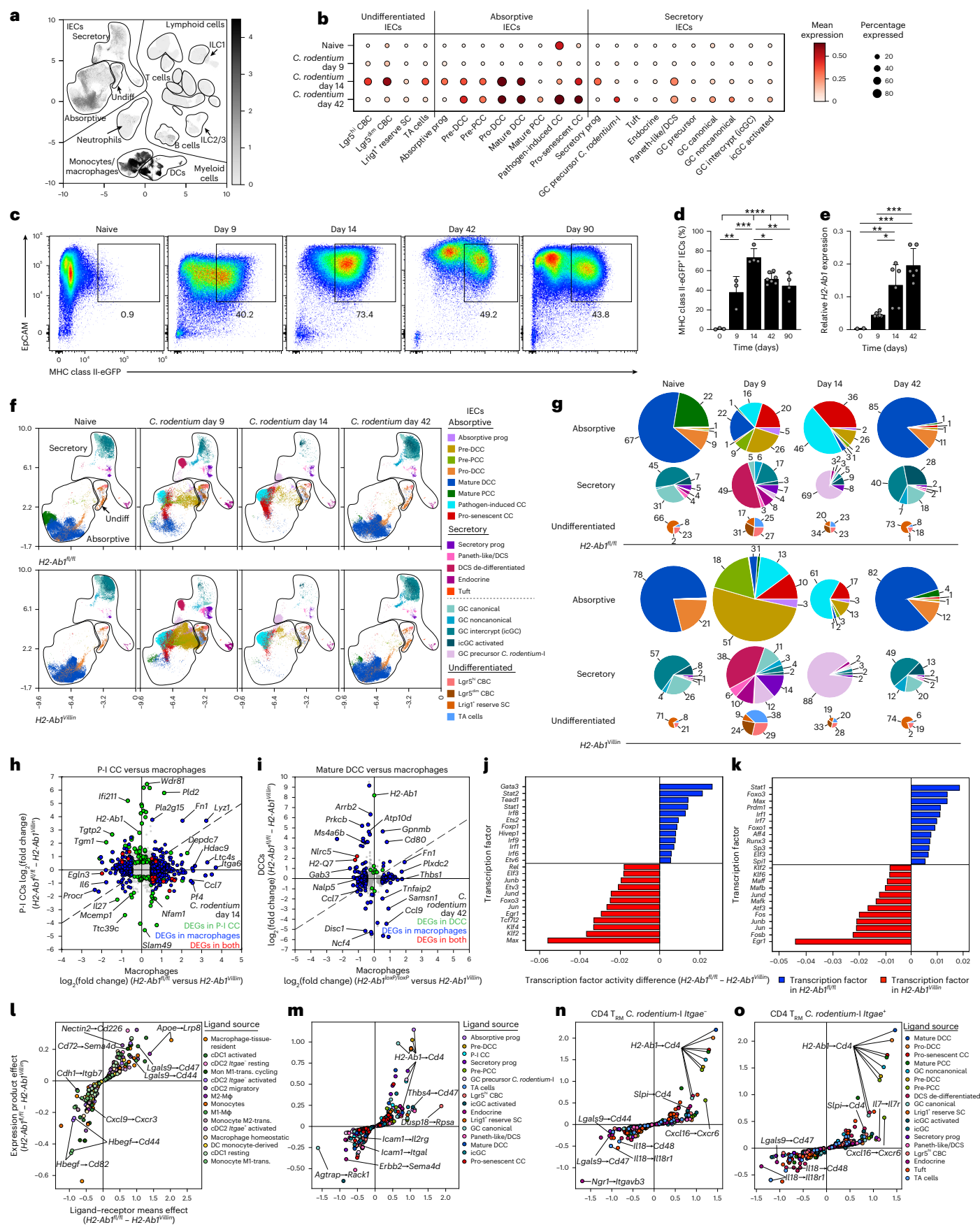


Fig. 5 | Epithelial MHC class II expression sustains IEC-T cell communication during and after *C. rodentium* infection. scRNA-seq was performed on EpCAM⁺ and CD45⁺ cells isolated from intraepithelial and nonepithelial compartments from mid-distal colons of *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice that were uninfected (naive) or *C. rodentium*-infected for 9, 14 or 42 days ($n = 2$). **a**, Uniform manifold approximation and projection (UMAP) analysis of *H2-Ab1* expression in key labeled clusters (see Extended Data Fig. 7 for detailed demarcation of clusters). **b**, Dot plot of *H2-Ab1* expression in epithelial cell clusters in naive and day 9, 14 or 42 *C. rodentium*-infected mice. **c, d**, EpCAM⁺ IECs isolated from naive or day 9, 14, 42 or 90 *C. rodentium*-infected MHC class II-enhanced GFP (eGFP) mice were analyzed using flow cytometry (**c**) and quantified by MHCII⁺ frequency (**d**). Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 3$ (naive), 3 (day 9), 4 (day 14), 7 (day 42) and 4 (day 90) mice. Data are from one experiment representative of two independent experiments. One-way ANOVA with Tukey's post hoc test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. **e**, *H2-Ab1* gene expression in EpCAM⁺ IECs sorted from naive or day 9, 14 or 42 of *C. rodentium*-infected C57BL/6 mice determined using quantitative PCR with reverse transcription (RT-qPCR). Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 2$ (naive), 6 (day 9), 6 (day 14) and 6 (day 42) mice. Data are from one

critically dependent on MHC class II (Fig. 3n,o). Thus, CC presentation of injected bacterial antigens promotes the development and maintenance of both major subsets of mucosal CD4 T_{RM} cells via sustained, MHC-class-II-dependent interactions.

Differential antigen localization promotes distinct patterns of CD4 T cell memory

The foregoing results established a bias favoring effector and memory CD4 T cells responding to cEC-presented injected antigens but did not define their microanatomical disposition. Therefore, we used light-sheet fluorescence microscopy (LSFM) to quantify the spatial distribution of transferred SMARTA T cells as a function of antigen localization, whether CC-associated (espZ-gp66 and espZ FL) or bacterial-associated (intimin-gp66 and espZ $\Delta 1-21$), and epithelial MHC class II expression during the memory phase. Assignment of cells to the epithelium, lamina propria (LP) or GALT, colonic patches (CPs) and isolated lymphoid follicles (ILFs) was enabled using intravital immunostaining of transferred SMARTA cells (CD45.1⁺), IECs (EpCAM⁺) B cells (CD19) (Extended Data Fig. 4) and vasculature (CD31) (Extended Data Fig. 5).

In WT mice infected with *C. rodentium* variants expressing either espZ FL or espZ-gp66, SMARTA cells robustly populated the distal colonic mucosa at day 42 after infection (Fig. 4a–c, Supplementary Data Fig. 2 and Supplementary Videos 1–3). Among quantified SMARTA cells, the greatest proportion and density of cells were associated with the epithelium (espZ FL: 68.7% of 1,427 cells total; tissue density 612 cells per mm³), followed by GALT (23.7% of cells; 211 cells per mm³) and LP (7.6% of cells; 68 cells per mm³) in that order. Thus, the epithelial SMARTA cell density was approximately threefold and ninefold higher

experiment representative of two independent experiments. One-way ANOVA with Tukey's post hoc test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **f**, UMAP of IEC clusters from naive and day 9, 14 or 42 *C. rodentium*-infected *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice, with cluster colors corresponding to the key shown. The comparison of **f** and **g** best illustrates genotype-dependent shifts in IEC subset composition during active infection. **g**, Pie charts depicting the percentages of absorptive, secretory and undifferentiated IEC clusters at the indicated time points and genotypes. Pie chart areas are normalized to the largest cell pool (day 9 *H2-Ab1^{Villin}* mice). Notable shifts in absorptive lineage subset composition, including pro-senescent CCs, between *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice at day 14 are most readily appreciated by comparing **f** and **g** in conjunction with the text. **h, i**, Two-way scatter plot of differentially expressed genes (DEGs) from P-I CCs versus DEGs from pooled *H2-Ab1⁺* macrophage clusters from day 14 (**h**) and DEGs from mature DCCs versus DEGs from pooled *H2-Ab1⁺* macrophage clusters from day 42 (**i**) scRNA-seq data. **j, k**, pyScenic transcription factor regulon analysis performed in P-I CCs at day 14 (**j**) and mature DCCs at day 42 (**k**). **l–o**, Ligand–receptor analysis performed via LIANA on myeloid-CD4 T_{H1} effector interactions (day 14, **l**), IEC-CD4 T_{H1} effector interactions (day 14, **m**), IEC-CD4 CD103⁺ T_{RM} interactions (day 42, **n**) and IEC-CD4 T_{RM} CD103⁺ interactions (day 42, **o**). Undiff, undifferentiated.

than that of GALT and LP, respectively. The relative dearth of SMARTA cells in the LP was notable, as this compartment contained the greatest number and density of cells during the effector response (day 14; see below), suggesting that signals sustaining T cells found there are limited relative to the intraepithelial niche. A similar distribution was observed as late as 240 days after espZ FL infection (Extended Data Fig. 6 and Supplementary Video 4), indicating durable persistence of antigen-specific memory cells months after *C. rodentium* clearance.

In stark contrast, intimin-gp66 or espZ $\Delta 1-21$ *C. rodentium* variants generated only rare SMARTA T cells in the epithelium or LP of WT mice, with only ~4–5% of CD45.1⁺ cells quantified in the intimin-gp66 and espZ $\Delta 1-21$ challenged mice combined at day 42 (Fig. 4a–c, Supplementary Data Fig. 2 and Supplementary Videos 1 and 5). Even fewer SMARTA cells were identified in the LP (1–2%). Instead, most SMARTA T cells (91–95%) localized to the GALT. Thus, the disposition of antigen-specific T cells responding to bacterially associated antigen was markedly diminished and strongly favored GALT during the memory phase.

Consistent with a critical role for epithelial antigen presentation in driving the amplitude and developmental trajectory of *C. rodentium*-reactive CD4 T cells, the number of SMARTA cells in the mucosa of *H2-Ab1^{Villin}* mice challenged with espZ FL was reduced more than 20-fold relative to control *H2-Ab1^{fl/fl}* mice (53 versus 1,317 mean total quantified cells, respectively) (Fig. 4d–f), a reduction remarkably similar to that of WT mice infected with either espZ $\Delta 1-21$ or intimin-gp66. Moreover, few SMARTA cells were identified in the epithelium or LP of *H2-Ab1^{Villin}* mice, with the majority localized to the GALT (84%). Thus, without epithelial antigen presentation, *C. rodentium*-specific memory T cells were markedly reduced and intraepithelial T_{RM} development was minimal. These imaging

Fig. 6 | EC antigen presentation directs T_{RM} cell transcriptional programming.

a, UMAP analysis of CD4 effector and memory populations from indicated *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice. **b, c**, Pie charts of the percentages of CD4 effector (**b**) and memory (**c**) clusters from naive and days 9, 14 and 42-infected *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice. **d, e**, Colonic intraepithelial cells from day 42-infected *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice were stained for CD4, TCR β , CD44 and CD62L, and analyzed using flow cytometry (**d**) to quantify the number of CD44⁺CD62L⁺ T_{CM} cells (**e**). Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 6$ mice per genotype. Data are from one experiment representative of two independent experiments. Unpaired two-sided Student's *t*-test; * $P < 0.05$. **f, g**, Colonic intraepithelial cells from day 42-infected *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice were stained for CD4, TCR β , CD69 and CD103, and then analyzed using flow cytometry (**f**) to quantify the number of CD69⁺CD103⁺ and CD69⁺CD103⁺ T_{RM} cells (**g**). Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 6$ mice per genotype. Data are from one experiment representative of two independent experiments. Unpaired two-sided Student's *t*-test; * $P < 0.05$, ** $P < 0.01$. **h**, Ratio of T_{CM} cells to CD69⁺CD103⁺ and CD69⁺CD103⁺ T_{RM} cells from the colonic intraepithelial

compartment in day 42-infected *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice. Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 6$ mice per genotype. Data are from one experiment representative of two independent experiments. Unpaired two-sided Student's *t*-test; * $P < 0.05$, ** $P < 0.01$. **i**, Two-way scatter plot of DEGs from CD103⁺ T_{RM} clusters versus DEGs from CD103⁺ T_{RM} clusters from day 42 scRNA-seq data. **j–n**, Two-way scatter plots of DEGs from T_{CM} versus DEGs from CD103⁺ T_{RM} clusters (**j**), DEGs from T_{EM} versus DEGs from CD103⁺ T_{RM} clusters (**k**) and DEGs from T_{CM} versus DEGs from T_{EM} clusters (**l**) from day 42 scRNA-seq data. pyScenic transcription factor regulon analysis performed in CD103⁺ T_{RM} (**m**) and CD103⁺ T_{RM} (**n**) from day 42 scRNA-seq data. **o**, Summary schematic depicting normal effector CD4 T cell development during acute *C. rodentium* infection and subsequent differentiation into memory T cell subsets (T_{CM}, T_{EM} and T_{RM}) in *H2-Ab1^{fl/fl}* mice. In *H2-Ab1^{Villin}* mice lacking epithelial MHC class II, T_{EM} and T_{RM} populations are reduced with a reciprocal increase in T_{CM} cells. Additionally, T_{RM} cells that develop in *H2-Ab1^{Villin}* mice display an altered transcriptional program compared with those from *H2-Ab1^{fl/fl}* mice.

memory T (T_{CM}) cells and secondary lymphoid tissue-resident cells stationed there.

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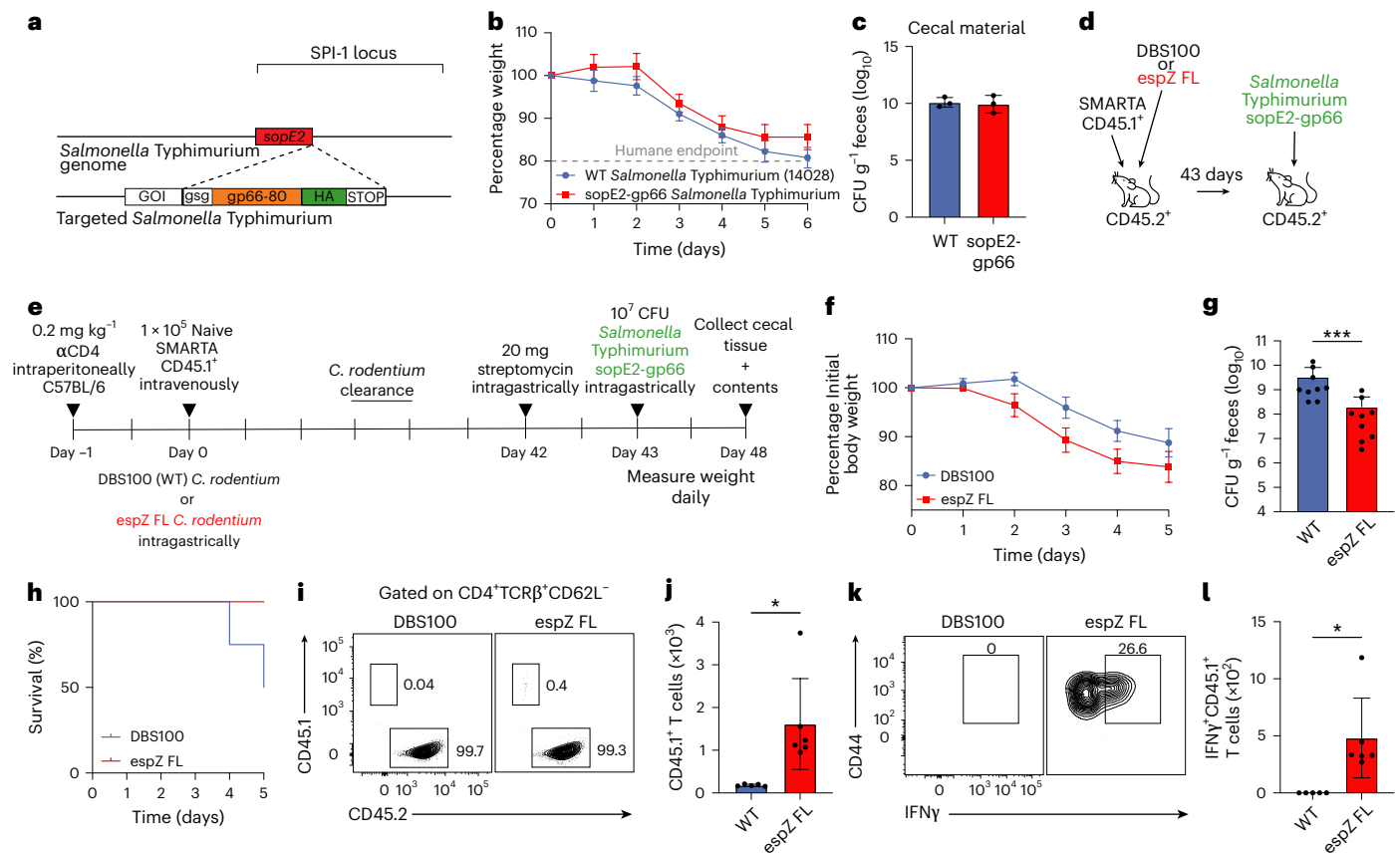


Fig. 7 | Functional recall response of *C. rodentium*-primed T_{RM} cells.

a, Schematic of the allelic exchange strategy used to generate *sopE2*-gp66 *Salmonella Typhimurium*. **b**, Body weight of C57BL/6 mice after oral gavage with WT *Salmonella Typhimurium* (strain no. 14028s) or *sopE2*-gp66 *Salmonella Typhimurium* (4×10^7 colony-forming units (CFU); 20 mg streptomycin sulfate pretreatment). Data are shown as the mean $\pm$ s.e.m.; $n = 3$ mice per group. **c**, Fecal CFU g^{-1} from cecal contents of mice in **b** at the day of collection. Data are shown as the mean $\pm$ s.d. with individual mice shown; $n = 3$ mice per group. **d**, Cartoon schematic of the heterologous challenge experimental design. **e**, Experimental timeline. C57BL/6 mice received anti-CD4 (GK1.5; 0.2 mg kg^{-1}) intraperitoneally on day -1, followed by adoptive transfer of 10^5 naive CD45.1⁺ SMARTA T cells (intravenously) and oral infection with 2×10^9 CFU DBS100 (WT) or *espZ* FL *C. rodentium* on day 0. After *C. rodentium* clearance, mice received streptomycin sulfate (20 mg, oral gavage) on day 42 and were challenged with 4×10^7 CFU *sopE2*-gp66 *Salmonella Typhimurium* (oral gavage) on day 43. Cecal tissue and contents were collected on day 48. **f**, Body weight of mice in **e** after *Salmonella*

Typhimurium challenge. Data are shown as the mean $\pm$ s.e.m.; $n = 9$ DBS100 (WT) and $n = 9$ *espZ* FL mice, pooled from two independent experiments (the same animals quantified in **g**). **g**, Fecal CFU g^{-1} from cecal contents of mice in **e** on day 5 after *Salmonella Typhimurium* challenge. *** $P < 0.001$. **h**, Survival of mice in **e** after *sopE2*-gp66 *Salmonella Typhimurium* challenge. **i**, Representative flow cytometry plots of CD45.1 versus CD45.2 expression gated on CD4⁺TCR β ⁺CD62L⁺ cecal lymphocytes from mice in **e** on day 5 after *Salmonella Typhimurium* challenge. **j**, Quantification of CD45.1⁺ SMARTA T cells recovered from the cecal tissue of mice in **e**. **k**, Representative flow cytometry plots of IFN γ versus CD44 expression among CD45.1⁺ cells from **i** after ex vivo restimulation with phorbol 12-myristate 13-acetate and ionomycin. **l**, Quantification of IFN γ ⁺ CD45.1⁺ SMARTA T cells from the cecal tissue of mice in **e**. **g**, **j**, **l**, Data are the mean $\pm$ s.d. with individual mice shown. **g**, $n = 9$ DBS100 (WT) and $n = 9$ *espZ* FL mice, pooled from two independent experiments. **j**, $n = 5$ DBS100 (WT) and $n = 6$ *espZ* FL mice from a single experiment. Unpaired two-sided Student's *t*-test; * $P < 0.05$. Survival in **h** was compared using a log-rank (Mantel-Cox) test; * $P < 0.05$.

DCCs and responding T cells. Single-cell RNA sequencing (scRNA-seq) analysis was performed on sorted EpCAM⁺ epithelial cells and CD45⁺ immune cells from the intraepithelial and nonepithelial compartments of distal colonic mucosa of naive and infected *H2-Ab1*^{fl/fl} and *H2-Ab1*^{villin} mice (Extended Data Fig. 7 and Supplementary Table 2).

Across all clusters, professional mucosal APCs (for example, DCs and macrophages) maintained high *H2-Ab1* (MHC class II) expression at all time points, whereas specific epithelial subpopulations upregulated MHC class II in response to infection (Fig. 5a and Extended Data Fig. 8). Expression of *H2-Ab1* peaked at day 14 of infection in DCCs, particularly pro-DCCs, mature DCCs and pro-senescent CCs (Fig. 5b), and persisted through the end of the observation period (day 90), well beyond the clearance of *C. rodentium*. Persistent surface MHC class II expression was confirmed using flow cytometry and independent transcriptomic analysis (Fig. 5c–e).

Comparison of EC developmental trajectories between *H2-Ab1*^{fl/fl} and *H2-Ab1*^{villin} mice highlighted altered CC dynamics during active infection in the absence of epithelial MHC class II, principally among

cells of the absorptive lineage (Fig. 5f,g). No differences were observed between genotypes at homeostasis, reflecting absent CC MHC class II at steady state. During active infection, *H2-Ab1*^{villin} mice failed to show the normal increase in shedding of pro-senescent CCs, which is consistent with a critical role for T cell-derived IL-22 in programming these cells to counter *C. rodentium* effector proteins that promote DCC retention¹ (Fig. 5g). This was evident as decreased relative and absolute numbers of pro-senescent CCs and increased total DCCs in *H2-Ab1*^{villin} mice at the peak of bacterial load (day 9). Notably, the total number of DCCs in *H2-Ab1*^{villin} mice contracted at day 14, which is consistent with impaired recruitment of T cell-derived IFN γ signaling, a key driver of absorptive lineage proliferation during infection⁴⁴ (see below). Secretory subsets were less affected, probably reflecting the absence of antigen loading by injected *C. rodentium* proteins, although goblet cell (GC) precursor *C. rodentium*-I cells selectively increased in *H2-Ab1*^{villin} mice, perhaps reflecting reduced IFN γ -dependent GC contraction.

Differential gene expression analysis confirmed that *H2-Ab1* deletion was specific to ECs, with no significant effect on *H2-Ab1*

expression by myeloid cells (Fig. 5h,i). Although macrophage numbers and subset composition shifted during infection contingent on MHC-class-II-dependent DCC–CD4 T cell interactions (Extended Data Fig. 9), *H2-Ab1* expression by *H2-Ab1^{fl/fl}* and *H2-Ab1^{villin}* macrophage or DC populations was not significantly altered (Extended Data Fig. 8), indicating retained antigen-presenting function. Thus, macrophage and DC subsets from both genotypes showed comparable upregulation of genes downstream of CD40 signaling (Fig. 5h,i), which is consistent with retained T cell–APC interactions.

Transcription factor regulon analysis using pySCENIC⁴⁵ revealed that IFN γ -responsive transcription factors, including Stat1, Stat2, Irf1, Irf7 and Irf8, were the most significantly enriched in infected *H2-Ab1^{fl/fl}* IECs (for example, pathogen-induced (P-I) CCs at day 14 and mature DCCs at day 42) compared to MHC-class-II-deficient IECs (Fig. 5j,k). This implicated T_H1 responses to IEC antigen presentation as a major driver of DCC programming during and after infection. The signals maintaining this program after clearance are unclear, but sustained IFN γ signatures suggest that ongoing EC–T cell crosstalk dampens innate programs that otherwise fail to extinguish. A Prdm1 regulon induced downstream of IFN signaling restrains pro-inflammatory signals^{46–48}, cooperating with Foxo1 and Foxo3 against NOD-family signaling via AP-1 and NF- κ B⁴⁹. Enrichment of AP-1 regulons in CCs of *H2-Ab1^{villin}* mice (for example, Fos, Jun, Junb, Jund)^{50–52} may thus reflect lost IFN γ feedback restraint. Klf2 and Klf4, which likewise repress AP-1 activity in IECs⁵³, were also enriched in *H2-Ab1^{villin}* mice, suggesting that this feedback does not require direct T cell input.

LIANA⁵⁴ analysis of intercellular communication showed no differential H2-Ab1–Cd4 interactions between myeloid cells and effector T cells at day 14 (Fig. 5h,i,l). Supporting the differential gene expression analysis above (Fig. 5h,i), no differential H2-Ab1–Cd4 interactions were evident between different myeloid cells and effector T cells at day 14 (Fig. 5l). In stark contrast, H2-Ab1–Cd4 interactions dominated EC–T cell interactions, particularly those involving numerically favored T_H1 cells (Fig. 5m). This persisted at day 42, with strong *H2-Ab1*-dependent interactions between ECs and both CD103⁺ and CD103⁺ CD4 T_{RM} cells (Fig. 5n,o). Additional interactions in *H2-Ab1^{fl/fl}* mice included Cxcl16–Cxcr6, which supports T cell tissue residency in colonic epithelium^{55–57}, and Il-7–Il-7r, which supports T cell survival^{58,59}. *Il7* and *Il15* transcripts also shifted significantly across IEC subsets over the course of *C. rodentium* infection (Extended Data Fig. 10), suggesting changing epithelial sources of survival signals during the transition to memory. In *H2-Ab1^{villin}* mice, we also found Il-18–Cd48 and Il-18–Il-18r1 IEC–T cell signatures persisting into the memory phase, which is consistent with the regulon data described above (Fig. 5j,k).

Absence of epithelial MHC class II decreased total effector CD4 T cell numbers during active infection (day 14) but minimally affected the relative composition of effector or memory subsets, which is consistent with our previous findings¹ (Fig. 6a,b and Supplementary Table 2). In contrast, there were significant shifts in the composition of memory cell subsets despite only modest changes in total memory cell numbers. In agreement with LSFM data showing near-complete absence of SMARTA T_{RM} cells at day 42 (Fig. 4d–f), deployment of both CD103⁺ and CD103⁺ epithelium-associated T_{RM} cells was highly dependent on CC antigen presentation (Fig. 6a,c). Thus, *H2-Ab1^{villin}* mice exhibited a shift favoring transcriptionally defined T_{CM} cells, with decreases in T_{RM} and effector memory (T_{EM}) populations (Fig. 6c), and a T_{CM}-to-T_{RM} ratio significantly skewed toward T_{CM} in *H2-Ab1^{villin}* mice (Fig. 6d–h). Together with the differential programming of T_{RM} and T_{CM} cells contingent on injected versus noninjected *C. rodentium* antigens (Fig. 4a–c and Supplementary Data Fig. 2), these results indicate that T cell recognition of DCC-expressed antigen is required for optimal T_{RM} development, whereas its absence favors a default, central memory-like transcriptional fate and disposition to GALT rather than intraepithelial or LP compartments⁶⁰.

Differential gene expression analysis revealed distinct memory cell transcriptional programs contingent on epithelial antigen recognition, most prominently in T_{RM} cells (Fig. 6i–l). For example, CD103⁺ T_{RM} cells developing in *H2-Ab1^{fl/fl}* mice expressed higher tissue residency transcripts, including *Itgae* (CD103), *Jun*, *Ifng*, *Ccl5* and *Gzma*. Among the most differentially expressed MHC-class-II-dependent genes was *Hdac11*, the sole class IV histone deacetylase, which represses effector gene expression in memory T cells⁶⁰ and may contribute to CD103⁺ and CD103⁺ T_{RM} cell quiescence. Notably, several transcription factor transcripts enriched in T_{RM} cells (*Hic1*, *Ahr*, *Nr4a1*) were expressed independently of IEC antigen presentation, indicating regulation by other microenvironmental cues. In *H2-Ab1^{villin}* mice, T_{RM} cells that developed showed altered transcriptional identity, with increased *Klf3*, *Klf4*, *Socs3* and *Ccr2*—markers of central memory and migratory programs^{61–63}—and loss of effector-associated genes. Although relative T_{CM} numbers diverged between *H2-Ab1^{fl/fl}* and *H2-Ab1^{villin}* mice, few genes were differentially expressed, which is consistent with similar ‘default’ programming in the absence of antigen recognition on IECs.

Transcription factor regulon analysis confirmed these findings. Thus, both CD103⁺ and CD103⁺ T_{RM} cells developing in *H2-Ab1^{fl/fl}* mice exhibited higher activity of Fos, Tbx21, Jun, Runx2, Runx3 and Prdm1, factors associated with more terminal development, tissue residency and sustained effector competency^{64–66} (Fig. 6m,n). In contrast, T_{RM} cells that developed in *H2-Ab1^{villin}* mice showed increased activity of Mxd1, Tcf7, Bcl6 and Bach2, which are associated with central memory plasticity and suppression of tissue residency. Klf2, Tcf7/Lef1 and Bach2 regulate the expression of *Klf4*, *Socs3* and *Ccr2*, DEG signatures noted above. The Klf7 regulon was prominent in both CD103⁺ T_{RM} cells from *H2-Ab1^{fl/fl}* mice and CD103⁺ T_{RM} cells from *H2-Ab1^{villin}* mice, indicating that its role in memory cell survival and prevention of exhaustion is uncoupled from cEC antigen presentation. Together, these data reveal that MHC-class-II-dependent antigen presentation by DCCs shapes the transcriptional landscape of developing memory T cells, actively promoting tissue-resident programming over default central memory programming (Fig. 6o).

Memory T cells induced by injected antigen confer heterologous protection against bacterial rechallenge

To test whether memory CD4 T cells induced by injected antigen confer mucosal protection in a recall response, we used a heterologous rechallenge strategy. Because humoral immunity after primary *C. rodentium* infection complicates homologous rechallenge^{33,67}, we used *Salmonella* Typhimurium engineered to deliver gp66–80 coupled to the injected effector protein, sopE2 (sopE2-gp66 *Salmonella* Typhimurium) (Fig. 7), which mediates *Salmonella* invasion of IECs after its injection via the SPI-1 T3SS^{68,69}. C-terminal fusion of gp66–80 to sopE2 preserved this function while co-delivering the SMARTA epitope into the IEC cytosol. SopE2-gp66 *Salmonella* Typhimurium exhibited equivalent virulence to WT *Salmonella* Typhimurium (strain no. 14028s) (Fig. 7b,c) and similar colonization of the cecum—the principal site of overlap between *C. rodentium* and *Salmonella* Typhimurium colonization in streptomycin-pretreated mice⁷⁰. CD45.2⁺ WT mice receiving CD45.1⁺ SMARTA T cells were infected with DBS100 (WT) or espZ FL *C. rodentium* (Fig. 7d,e), challenged on day 43 with sopE2-gp66 *Salmonella* Typhimurium after streptomycin pretreatment, and killed on day 48. Although there was no significant difference in weight loss between groups (Fig. 7f), espZ-FL-infected mice exhibited significantly lower cecal bacterial burdens and complete survival through day 5 after challenge, compared to 50% loss of DBS100-infected controls (Fig. 7g,h). Significantly more SMARTA T cells (more than tenfold) were recovered from the ceca of espZ-FL-primed mice; they produced significantly more IFN γ upon ex vivo restimulation (Fig. 7i–l). Thus, the more numerous T_{RM} cells programmed by cytosolic antigen conferred significantly enhanced protection in a recall challenge by an unrelated pathogen delivering the same epitope.

Discussion

In this study, we find that not all bacterial antigens are immunologically equal. At least for bacteria that use a T3SS to colonize host barrier epithelia, there is a hierarchy in the induction and mucosal recruitment of CD4 T cells contingent on the distribution of bacterial antigens, strongly favoring those delivered to the cytosol of the host epithelium, independent of epitope binding affinity for MHC class II or heterogeneity of TCR avidity for the MHC–peptide complexes generated. Antigen localization eclipsed antigen load as the principal determinant of the magnitude and developmental trajectory of the T cell response; injected effectors were the dominant antigen class programming T_{RM} development, thereby focusing T_{RM} responses on epithelium-associated antigens. Coupled with our recent report that epithelial antigen presentation recruits CD4 T cell help for barrier defense during active infection¹, these findings consolidate the importance of nonprofessional APCs in guiding the adaptive response during and after pathogenic challenge.

Both the route by which antigens enter the host and their physical form have long been appreciated as determinants of immunogenicity^{71,72}. Particulate antigens are well suited to uptake by phagocytic APCs, which direct extracellular material to endolysosomal MHC class II loading compartments⁷³, whereas soluble antigens are poorly captured by these cells but can be efficiently delivered to the MHC class II loading compartments of B cells, particularly if they harbor repeated epitopes bound by surface immunoglobulin⁷⁴. In contrast to these professional APCs, epithelial cells lack efficient mechanisms for the uptake of pathogen-associated antigens and therefore rely on the bacterium itself to deliver antigens for presentation. Unlike invasive bacteria such as *Salmonella enterica* serovars or *Listeria monocytogenes*, which have mechanisms for direct bacterial cell entry into IECs, attaching and effacing pathogens such as *C. rodentium* lack such mechanisms; only T3SS-delivered antigens enter host IECs. Thus, bacterial ‘invasion’ is relative: although *C. rodentium* does not penetrate cECs as an intact cell, injection of effector molecules constitutes a barrier breach detected by T cells primed against these antigens.

The quantity and quality of the clonal CD4 T cell response were strongly affected by antigen disposition: bacterium-associated versus epithelium-associated and, within the epithelial cell, membrane-associated versus cytosol-associated. The superior immunogenicity of injected *C. rodentium* antigens indicates that other routes of intestinal antigen uptake, that is, capture by transepithelial dendrites of CX3CR1 macrophages⁷⁵, transport across M cells⁷⁶, GC-associated passages⁷⁷ or neonatal Fc receptor⁷⁸-mediated transport, contribute less. The relatively weak response to bacterially associated antigen was unexpected, as antigens of this class, such as intimin, are targets of an IgG antibody response important in thwarting *C. rodentium* pathogenicity and clearing infection³³. For intimin-gp66 this might reflect its low dose; however, naturally expressed espZ-gp66 was present at similar levels despite inducing a far more potent response. Dose alone cannot explain the muted response to espZ Δ 1–21, which was expressed comparably to FL yet induced more than 100-fold fewer mucosal SMARTA T cells. This suggests that, for attaching and effacing pathogens at least, DCs more efficiently take up EC-associated antigen, whether via efferocytosis of debris from damaged or dying IECs or via a cellular intermediate, as in GC-associated passage-mediated uptake⁷⁹. The basis for preferential responses to cytosolic espZ and nleA over membrane-associated tir remains unclear but may reflect differential display of scavenger receptor targets by damaged basolateral versus apical membrane domains or relative inaccessibility of tir due to its tethering to intimin and the colonocyte cytoskeleton.

Because injected *C. rodentium* antigens bypass the classical endolysosomal MHC class II loading pathway, our results favor macroautophagy as the loading mechanism^{80–82}; this is supported by the absence of KFERQ-like motifs in espZ and nleA (excluding chaperone-mediated autophagy) and the greater immunogenicity

of cytosolic (espZ, nleA) versus membrane-associated (tir) antigens, which is consistent with preferential entry of cytosolic over integral membrane proteins into the autophagy pathway. This will require further study.

Mechanisms controlling the development of different types of T cell memory are an area of active investigation^{83,84}. Our findings highlight an important role for CD4 T cell recognition of MHC class II–peptide complexes on infected ECs in programming tissue residency. Further, they establish a central role for translocated bacterial proteins in CD4 T_{RM} programming, the striking bifurcation of T_{RM} versus non- T_{RM} memory specification driven by antigen localized to the host epithelial cell versus bacterial cell. This makes biological sense: linking antigen recognition on IECs to local tissue residency deploys T_{RM} cells to sites where they will encounter antigen anew upon reinfection. Preferential positioning of T_{RM} cells in the epithelium is further supported by our scRNA-seq data, which show that the T cell-sustaining cytokines IL-7 and IL-15 are differentially produced by CC populations, IL-15 by mature IECs near the luminal surface and IL-7 by immature cECs deeper in crypts. This aligns with recent spatial transcriptomics describing two CD8 T_{RM} subsets arrayed along the crypt–villus axis of the small intestine near spatially segregated epithelial IL-7 or IL-15 producers⁸⁵. Notably, relatively few memory T cells populated the LP; the major sites of accumulation were the epithelium and GALT. The mix of memory cells localized to the GALT consisted largely of circulating T_{CM} cells, although a component of T_{RM} cells stationed there was also apparent. Delineation of the cell types directed to residency in the GALT warrants direct interrogation.

Our scRNA-seq data expose transcriptional networks underpinning bidirectional cEC–T cell signal exchange—altered cEC programming downstream of T cell help and reciprocal programming of T cells engaging cEC-presented antigen. Dominant signaling into cECs was downstream of IFN γ , reflecting the predominance of T_{H1} cells at the peak of the effector response. IFN γ not only programs barrier defense functions in the epithelium⁵ but also activates the antigen processing and presentation machinery required to recruit antigen-dependent help for the epithelium, whether mediated by IFN γ itself or IL-22, the other major epithelium-protective cytokine that relies on cEC antigen presentation to prevent lethality by *C. rodentium*^{1,35,86,87}. The progressive shift in T_{H17}/T_{H22} – T_{H1} balance resulting from transdifferentiation of T_{H17} precursors may be linked to T_{RM} specification as T_{RM} transcriptional programming aligned more closely with the T_{H1} program, many features of which are shared with CD8 T_{RM} cells. Thus, primed T cells entering the mucosa within the more plastic T_{H17} program are progressively constrained as they transition to T_{H1} and T_{RM} cells, with accompanying loss of stem-like characteristics^{88,89}.

Integration of the scRNA-seq data analysis with other findings in our study further elucidate the T cell response to cEC-displayed antigens. A methodological point also emerged: because standard mucosal isolation cannot cleanly separate the epithelium, LP and GALT phenotypes assigned to the epithelial and nonepithelial pools, using dissociation-based methods is necessarily inaccurate and highlights the need for integration with imaging-based localization and spatial transcriptomics. Thus, while there were substantial shifts in relative numbers of T_{RM} and non- T_{RM} memory cells contingent on epithelial antigen presentation, particularly for the tracked clonal population, a requirement for T_{RM} specification of endogenous, polyclonal CD4 T cells was not absolute. However, T_{RM} cells developing in MHC-class-II-deficient mice retained some T_{CM} transcriptional signatures, suggesting that signals promoting full T_{RM} commitment (for example, TGF β ^{59,90}) are deficient without sustained engagement on antigen-bearing cECs. Thus, rather than delivering a unique T_{RM} -inducing signal, cEC-presented antigen sustains engagement within the epithelial microenvironment long enough to complete programming that extinguishes a plastic, migratory memory lifestyle and commits cells to survival on local IL-15 or IL-7. Consistent with this model, the balance of CD103⁺ and CD103⁺

T_{RM} cells and their transcriptional signatures were largely unaffected by loss of EC antigen presentation; universal LIPSTIC labeling showed that most T_{RM} cells of both subsets actively engaged ECs during the memory phase, albeit less so for CD103⁺ cells. Whether this reflects differences in dwell time, positioning along the crypt–surface axis or other factors warrants further study. Finally, our findings extend the characterization of transcriptional networks programming tissue residency in CD4 T cells, revealing considerable similarity to those of better-characterized CD8 T_{RM} cells while exposing new players whose functional characterization, along with the basis for subset-specific differences in epithelial engagement, await further study.

In sum, MHC-class-II-dependent interactions between cECs and CD4 T cells are bidirectional and preferentially mediated by peptides derived from bacterial proteins injected into the host epithelial cytosol. Such presentation not only amplifies the magnitude of the T cell response, increasing effector cell accumulation in the involved mucosa during active bacterial infection, but also directly influences memory T cell fate decisions, promoting the development and maintenance of T_{RM} populations that provide frontline defense at the intestinal barrier. The persistence of epithelial MHC class II expression and continued epithelial–T cell interactions after pathogen clearance suggests that ECs serve as both initiators and long-term maintainers of barrier immunity. These results support a model in which the antigen-presenting EC may not provide indispensable signals for memory subset development; rather, sustained epithelial interactions increase T cell exposure to a microenvironment enriched for T_{RM}-promoting signals such as TGFβ. When these interactions are absent, memory differentiation is biased away from epithelial tissue residency and toward the GALT, as either circulating or secondary lymphoid T_{RM} cells. These findings have important implications for mucosal vaccine design, suggesting that targeting antigens to the cytosol of ECs may enhance protective mucosal immunity. They may also inform strategies for modulating pathological mucosal memory in chronic inflammatory disease.

Online content

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Methods

Mice

C57BL/6 (WT; stock no. 000664), C3H/HeJ (stock no. 000659), *H2-Ab1* floxed (stock no. 013181)⁹¹, SMARTA-1 CD45.1 (stock no. 030450)⁹², *Villin-cre* (stock no. 021504)⁹³ and *Villin:creERT2* (stock no. 020282)⁹⁴ mice were purchased from the Jackson Laboratory. *Rosa26^{ulIPSTC}* (ref. 43) mice were a gift from G. D. Victora. MHCII-EGFP⁹⁵ mice were a gift from H. L. Ploegh. *H2-Ab1^{fl/fl}* and *H2-Ab1^{villin}* mice were screened using PCR and flow cytometry to exclude mice with spontaneous, Cre-independent germline deletion of MHC class II, as reported previously¹. All mice underwent rigorous screening to exclude segmented filamentous bacteria, *Helicobacter* spp. and other potential pathogens. All mouse strains were bred and maintained at the University of Alabama (UAB) in accordance with Institutional Animal Care and Use Committee guidelines.

C. rodentium strains and infections

C. rodentium strain DBS100 (ATCC) was used for the scRNA-seq experiments. For the flow cytometry analysis and cytospin, we used a strain of *C. rodentium* expressing GFP⁹⁶ (derived from DBS100; kindly provided by B. A. Vallance). A fresh, single colony was grown in 10 ml lysogeny broth at 37 °C with rotation at 225 rpm for 12–14 h. Next day, 1 ml of overnight culture was added to 250 ml lysogeny broth (Thermo Fisher Scientific), incubated at 37 °C with rotation (225 rpm) for 3–3.5 h until OD₆₀₀ reached 1.0 on the spectrophotometer (Spectronic 200, Thermo Fisher Scientific). Bacteria were pelleted at 25 °C, 3,000 rpm for 15 min and then resuspended in 5 ml sterile 1× PBS. Mice were inoculated with 2×10^9 CFU in a total volume of 100 µl of PBS using gastric gavage.

Generation of epitope-tagged *C. rodentium* strains

We previously reported the detailed methods for this process¹. Briefly, DNA constructs were synthesized by GeneArt (Thermo Fisher Scientific) and then cloned into the pCAL52 (ref. 97) shuttle vector (kindly provided by S. E. Winter). Transformed *EcNR1Δasd E. coli*⁹⁸ (kindly provided by M. J. Gray) containing the cloned pCAL52 plasmid was grown overnight in lysogeny broth with 25 mg ml⁻¹ chloramphenicol and 1 mg ml⁻¹ 2,6-diaminopimelic acid (DAP) (Sigma-Aldrich) and *C. rodentium* (DBS100 or *C. rodentium*-GFP) was grown overnight in lysogeny broth. Puddle mating was performed on a lysogeny broth agar plate containing 1 mg ml⁻¹ DAP and incubated at 30 °C overnight to promote conjugation. The puddle mating was then plated on lysogeny broth agar plates containing 25 mg ml⁻¹ chloramphenicol (without DAP) and incubated at 37 °C overnight. Single-crossover merodiploid clones were confirmed using PCR and then selected in 25% sucrose lysogeny broth for 3–4 days with daily plating on lysogeny broth agar containing 40 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma-Aldrich) for blue-white detection of clones that had successfully undergone a double-crossover event to remove the *phoN*-containing pCAL52 backbone. White colonies were confirmed as either WT revertants or homozygous insertion mutants using PCR, with the following forward and reverse primers:

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eae_F 5'-GATGGCGTATTCTGAGGCAG-3'
eae_R 5'-CGGGTGCCGGGTTTATATGAT-3'
tir_F 5'-GAAGAGCCTATTATGATGAAGTCGC-3'
tir_R 5'-GTTCTCTGAACATTCATCACTACC-3'
nleA_F 5'-AGTGTGTCGTGGTCTTGCAA-3'
nleA_R 5'-TATGGTGGGCAGGCGCGACA-3'
espZ_F 5'-CGGGAATTGCAGCAATGTGT-3'
espZ_R 5'-GGTTGGGGCTAACGGAGTAT-3'
xylE_F 5'-AGGGTTACACGATCAACC-3'
xylE_R 5'-TCTTACGTGCCGATCAACGT-3'
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Immunoblot detection of epitope-tagged bacterial proteins

Sorted *C. rodentium*-GFP-bound EpCAM⁺ IECs, *C. rodentium* pellets from culture in DMEM, or supernatant proteins from *C. rodentium*

culture in DMEM concentrated with trichloroacetic acid precipitation (overnight at 4 °C) were resuspended in 2× Laemmli buffer (4% SDS, 20% glycerol, 0.005% bromophenol blue, 0.125 M Tris-HCl, pH 6.8 and 10% β-mercaptoethanol). Lysates were transferred to 1.5-ml Eppendorf tubes and briefly sonicated. Total proteins were loaded onto 8–15% SDS–polyacrylamide gel electrophoresis gel for electrophoresis separation. After electrophoresis, proteins on the gel were transferred onto 0.45-µm polyvinylidene fluoride membrane (cat. no. IPVH00005, Merck Millipore) in a sponge sandwich. Membranes were blocked with 5% nonfat milk (cat. no. 170-6404, Bio-Rad Laboratories) and probed with primary antibodies overnight on a shaker at 4 °C. Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The membranes were then incubated with Immobilon Crescendo Western HRP substrate (cat. no. WBLUR0500, Merck Millipore) for 2–5 min before imaging with CL-X Posure X-Ray film (Thermo Fisher Scientific). β-Actin was used as a loading control for IECs; GFP or DnaK were used as loading controls for *C. rodentium*. The following antibodies were used: mouse anti-hemagglutinin (HA) (clone 6E2, Cell Signaling Technology), rat anti-HA (clone 3F10, Sigma), mouse anti-FLAG (clone M2, Sigma-Aldrich), mouse anti-beta-actin HRP (cat. no. sc-47778, Santa Cruz Biotechnology), anti-GFP (rabbit polyclonal, Thermo Fisher Scientific), mouse anti-DnaK (clone 8E2/2, Enzo Life Sciences), anti-rabbit HRP (goat polyclonal, Thermo Fisher Scientific), anti-rat HRP (goat polyclonal, Thermo Fisher Scientific) and anti-mouse HRP (goat polyclonal, Thermo Fisher Scientific).

IEC and intraepithelial lymphocyte isolation

Intestinal tissue was flushed with 1× PBS, cut into 4-cm mid-distal colon, opened longitudinally and then cut into strips of 1-cm-length. Tissue pieces were incubated for 20 min at 37 °C with 1 mM dithiothreitol (Sigma-Aldrich), followed by 2 mM EDTA (Invitrogen) in H5H medium (1× HBSS (Thermo Fisher Scientific), 5% FCS, 20 mM HEPES (Thermo Fisher Scientific), and 2.5 mM 2-β-mercaptoethanol (Gibco)). Tissue pieces were vortexed briefly after each 20-min incubation, followed by washing with H5H before centrifugation at 1,750 rpm for 10 min at 4 °C. IECs were then purified on a 40%/75% Percoll (Cytiva) gradient by centrifugation for 20 min at 25 °C and 2,000 rpm with no brake. For analysis of *C. rodentium*-GFP attached to IECs, tissue pieces from 4 cm of mid-distal colon were incubated for 20 min at 37 °C with 1 mM EDTA in H5H medium, followed by gentle mixing and washing with H5H.

LP lymphocyte isolation

Colons were flushed with 1× PBS, opened longitudinally then cut into small pieces and placed in H5H medium. Tissue was then minced in 1.5-ml microcentrifuge tubes for 2–3 min before being transferred into glass scintillation vials with 10 ml of complete R10 medium (1× Roswell Park Memorial Institute 1640 (Corning), 10% FCS, 1× penicillin/streptomycin (Corning), 1× nonessential amino acid solution (Corning), 1 mM sodium pyruvate (Corning), 2 mM L-glutamine (Corning) and 2.5 mM 2-β-mercaptoethanol (Gibco)) with collagenase IV (100 U ml⁻¹, Sigma-Aldrich) and DNase I (20 mg ml⁻¹, Sigma-Aldrich). Tissue was digested at 37 °C for 40 min with stirring followed by filtering over a 70-µm filter and washing with complete R10 medium. Cells were then centrifuged at 1,750 rpm at 4 °C for 10 min and then purified on a 40%/75% Percoll gradient as described above. Where indicated, cells were stimulated with phorbol-12-myristate-13-acetate (50 ng ml⁻¹, Sigma-Aldrich) and ionomycin (750 ng ml⁻¹, Sigma-Aldrich) at 37 °C for 3–4 h in the presence of GolgiPlug (BD Biosciences).

Cytospin and immunofluorescence staining of bacteria-bound IECs

Sorted ECs ($2-5 \times 10^5$ cells per ml) were cytospun onto glass slides at 1,500 rpm for 2 min at room temperature. Cells were then dried for 10 min, fixed with 4% room temperature paraformaldehyde (PFA)

(Electron Microscopy Sciences) for 10 min and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min before staining. The following antibodies and reagents were used: anti-GFP (cat. no. A-11122, Thermo Fisher Scientific), anti-goat IgG (Thermo Fisher Scientific), anti-HA-tag (polyclonal, Novus Biologicals), anti-rabbit IgG (Thermo Fisher Scientific) and ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific).

Flow cytometry and cell sorting

Colon cells were stained with Fc Block (anti-CD16/CD32, clone 2.4G2) followed by staining with fluorescently labeled antibodies in IEC buffer (1× PBS with 5% FCS and 2 mM EDTA to reduce cell clumping) for IECs and intraepithelial lymphocytes (IELs) or 2% FCS in 1× PBS for lamina propria lymphocyte (LPL) cells on ice in 1.5-ml microcentrifuge tubes or in a 96-well plate. For intracellular staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences). Samples were acquired on an Attune NxT flow cytometer (Thermo Fisher Scientific) or FACSymphony A3 (BD Biosciences) and analyzed with the FlowJo software. Cells were sorted on a FACSymphony S6 (BD Biosciences). The following antibodies/reagents were used: anti-CD45 (clone 30-F11, BioLegend), anti-EpCAM (clone G8.8, Thermo Fisher Scientific), anti-MHCII (clone M5/114.15.2, Thermo Fisher Scientific), LIVE/DEAD Fixable Near-IR dead cell dye (Thermo Fisher Scientific), anti-CD4 (clone RM4-5, BioLegend), anti-TCRβ (clone H57-597, Thermo Fisher Scientific), anti-CD44 (clone IM7, BioLegend), anti-CD45.1 (clone A20, Thermo Fisher Scientific), anti-CD45.2 (clone 104, BD Biosciences), anti-CD69 (clone H1.2F3, Thermo Fisher Scientific), anti-CD103 (clone 2E7, Thermo Fisher Scientific), anti-CD19 (clone 1D3/CD19, BioLegend), anti-CD11b (clone M1/70, BioLegend), anti-CD11c (clone N418, BioLegend), anti-CD62L (clone MEL-14, BioLegend), anti-PD-1 (clone 29F.1A12, BioLegend), anti-CXCR5 (clone L138D7, BioLegend), anti-IL-17A (clone TC11-18H10, BD Biosciences), anti-IFNγ (clone XMGL2, Thermo Fisher Scientific) and anti-IL-22 (clone 1H8PSWR, Thermo Fisher Scientific). T_{RM} cells are defined as CD4⁺TCRβ⁺CD69⁺CD62L⁺CD103⁺ (CD103⁺T_{RM}) or CD4⁺TCRβ⁺CD69⁺CD62L⁺CD103⁺ (CD103⁺T_{RM}). T_{CM} cells are defined as CD4⁺TCRβ⁺CD44⁺CD69⁺CD62L⁺, distinguishing them from naive T cells which are CD44⁺CD62L⁺.

Quantitative PCR with reverse transcription

cDNA synthesis was performed with the iScript Reverse Transcription Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. cDNA amplification was performed with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) in a Bio-Rad CFX qPCR instrument. The following primer sequences were used:

Gapdh_F 5'-TCCATGACAACCTTTGGCATTG-3'
 Gapdh_R 5'-CAGTCTGGGTGGCAGTGA-3'
 IL7_F 5'-CAGTATCACAAAGGCACACAAAC-3'
 IL7_R 5'-CTCTCAGTAGTCTCTTTAGGAAACAT-3'
 IL15_F 5'-ATCCATCTCGTGCTACTTGTG-3'
 IL15_R 5'-CTATCCAGTTGGCCTCTGTTT-3'

Adoptive T cell transfer

Approximately 20–24 h before adoptive transfer of CD45.1⁺ SMARTA cells, mice were administered 0.2 mg kg⁻¹ anti-CD4 (clone GK1.5, Bio X Cell) in 1× PBS intraperitoneally. Spleens and LNs from CD45.1⁺ SMARTA donors were collected and dissociated in complete R10 medium before centrifugation at 1,500 rpm for 5 min at 4 °C. Red blood cells were removed from the cell suspension using ammonium-chloride-potassium (Quality Biological) lysis for 2 min before quenching with complete R10 medium. Naive CD4⁺ T cells from pooled spleens and LNs were purified using the naive CD4⁺ mouse T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Purified T cells were transferred by intravenous retro-orbital injection (1 × 10⁵ total cells) into recipients, followed by *C. rodentium* infection on the same day.

Generation of sopE2-gp66 *Salmonella* Typhimurium using allelic exchange

The DNA sequence containing a Gly-Ser-Gly linker, LCMV glycoprotein residues 66–80 (DIYKGVYQKFSVEFD) and HA-tag flanked by upstream and downstream regions of ~500 bp homologous to *Salmonella* Typhimurium sopE2 containing BamHI and SphI sites was synthesized by GeneArt (Thermo Fisher Scientific) and then cloned into the pRDH10 shuttle vector containing a CmR and sacB selection (a gift from S. Winter) using T4 ligation. All manipulations were performed in *Salmonella* Typhimurium IR715 ΔphoN::Kan (NaI^r, Kan^r; a gift from A. Baumler), a nalidixic acid-resistant derivative of strain 14028s in which phoN has been replaced with a kanamycin resistance cassette. Ligation products were transformed into One Shot PIR2 *E. coli* (Thermo Fisher Scientific) for propagation of the π-dependent R6Kγ origin of replication in pRDH10. Successfully ligated pRDH10 clones were sequence-verified and grown overnight in lysogeny broth at 37 °C. The pRDH10 sopE2-gp66 plasmid was purified from PIR2 *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN). Purified plasmid was transformed into the EcNR1Δasd *E. coli* strain (a gift from M. Gray) that is auxotrophic for DAP and selected for by chloramphenicol resistance on lysogeny broth agar plates containing 25 μg ml⁻¹ chloramphenicol and 1 mg ml⁻¹ DAP for mating. Transformed EcNR1Δasd *E. coli* containing the pRDH10 sopE2-gp66 plasmid was grown overnight in lysogeny broth with 25 μg ml⁻¹ chloramphenicol and 1 mg ml⁻¹ DAP; *Salmonella* Typhimurium IR715 ΔphoN::Kan was grown overnight in lysogeny broth. Puddle mating was performed by mixing 1.3 ml of overnight EcNR1Δasd *E. coli* pRDH10 sopE2-gp66 culture and 200 μl of *Salmonella* Typhimurium IR715 ΔphoN::Kan and centrifugation followed by resuspension in 50 μl lysogeny broth. This mixture was then spotted onto a lysogeny broth agar plate containing 1 mg ml⁻¹ DAP and incubated at 30 °C overnight to promote conjugation. Puddle mating was then collected into 1 ml of lysogeny broth and 2 × 10⁻⁶ dilutions were plated on lysogeny broth agar plates containing 25 μg ml⁻¹ chloramphenicol (without DAP) and incubated at 37 °C overnight. Single-crossover merodiploid clones were confirmed using PCR and then resolved by culture in 25% sucrose lysogeny broth for 3–4 days with daily plating on lysogeny broth agar with and without 25 μg ml⁻¹ chloramphenicol to determine the ratio of merodiploids to total colonies. When merodiploids represented less than 50% of total colonies, 50–100 μl of sucrose culture was plated on lysogeny broth agar; colonies were confirmed as either WT revertants or sopE2-gp66 insertion mutants using PCR, with the following forward and reverse primers: sopE2_F 5'-TGCCAACGCCACAACAATTT-3' and sopE2_R 5'-CTGTCTGGTTGTGCAGGTATTT-3'.

Streptomycin pretreatment model of *Salmonella* Typhimurium colitis

Intestinal *Salmonella* Typhimurium infection was performed using the streptomycin pretreatment colitis model as described previously⁷⁰. Mice received a single oral gavage of streptomycin sulfate (20 mg in 100 μl sterile water) 24 h before bacterial challenge. *Salmonella* Typhimurium IR715 ΔphoN::Kan or sopE2-gp66 STm was grown in 10 ml lysogeny broth at 37 °C with rotation at 225 rpm for 12–14 h. The following day, 1 ml of overnight culture was added to 250 ml lysogeny broth and incubated at 37 °C with rotation (225 rpm) for 3–3.5 h until OD₆₀₀ reached 1.0. Then, 50 ml of bacteria were pelleted at 25 °C, 3,000 rpm for 15 min and resuspended to a final concentration of 10⁸ CFU ml⁻¹ in 1× PBS. Mice were inoculated with 4 × 10⁷ CFU in a total volume of 100 μl using oral gavage. The inoculum dose was confirmed by back-calculation from serial dilution plating on lysogeny broth agar with 50 μg ml⁻¹ kanamycin sulfate. Mice were weighed daily and monitored for signs of disease; mice reaching humane endpoint criteria (≥20% body weight loss) were euthanized before the scheduled collection time point.

Quantification of cecal *Salmonella* Typhimurium bacterial burden

Cecal contents were collected by opening the cecum longitudinally and scraping contents into pre-weighed 1.5-ml microcentrifuge tubes using sterile disposable plastic forceps (130 mm, pointed/angled, polystyrene, sterile, Bürkle). Tubes were weighed to determine sample mass. Contents were resuspended in 1 ml sterile 1× PBS and homogenized using vortexing. Homogenates were serially diluted tenfold in 1× PBS and 10 µl of each dilution was spotted onto lysogeny broth agar plates containing 50 µg ml⁻¹ kanamycin sulfate. Plates were incubated overnight at 37 °C and colonies were enumerated the following day. CFU per gram of cecal content was calculated as: CFU/g = (colony count / 0.01 ml) × dilution factor / sample weight (g). Data are presented as log₁₀ CFU g⁻¹.

Light-sheet microscopy and three-dimensional image analysis

Mice received 20 µg anti-EpCAM-AF594 (clone G8.8, BioLegend) 24 h, and 15 µg anti-CD45.1-AF647 (clone A20, BioLegend) together with 15 µg anti-CD19-APC/Fire750 (clone 6D5, BioLegend) 3 h before euthanasia and tissue collection. One mouse (day 14, espZ FL-gp66) received anti-CD45.1-AF647, anti-CD31-AF594 (10 µg, 3 h before euthanasia, clone MEC13.3, BioLegend) and anti-EpCAM-APC/Fire750 (20 µg, clone G8.8, BioLegend) instead of the standard staining panel. Mid-distal colons were collected into ice-cold 4% PFA (Electron Microscopy Sciences) and stored at 4 °C overnight before subsequent washes in ice-cold 1× PBS. Tissue was then dehydrated in increasing concentrations of ethanol before clearing in 100% ethyl cinnamate (Sigma-Aldrich). Tissue was stored in 100% ethyl cinnamate at room temperature until imaging. LSFM of optically cleared colon samples was performed on an Ultra-Microscope Blaze (Miltenyi Biotec) in multicolor acquisition mode as shown previously⁹⁹. The microscope system uses the ImSpector software (LaVision BioTec). Samples were positioned on a steel holder within the imaging chamber filled with 100% ethyl cinnamate. Tissue orientation was stabilized using ultraviolet-curable optical adhesive (Norland Products). Imaging was performed using a ×12 MI Plan objective (0.53 detection numerical aperture) with either ×1.0 or ×0.6 zoom, bidirectional light-sheet illumination and fluorophore-dependent exposure times ranging from 100 to 250 ms. The light-sheet thickness was set to 3.9 µm (sheet numerical aperture = 0.186); the sheet width was adjusted to 60–70%, corresponding to an effective illumination field width of ~12–14 mm. The intestinal tube was imaged over a z-range of approximately 1,300–1,700 µm per sample with a z-step size of 2 µm between images. Epithelium, stained with anti-EpCAM-AF594, was excited with a 595/20 nm optically pumped semiconductor laser (50 mW) and detected with a 630/30-nm band-pass filter. CD45.1⁺ SMARTA cells were detected using anti-CD45.1-AF647, excited with a 630/30-nm optically pumped semiconductor laser and detected with a 680/30-nm band-pass filter. CD19-APC/Fire750 was excited using a 740/40-nm laser and detected with an 824/55-nm band-pass filter. Tissue autofluorescence was acquired using 520/40-nm excitation and 585/40-nm emission and served as an internal control for fluorescent signal specificity. Three-dimensional rendering and quantitative image analysis were performed in IMARIS v.10.1.0 (Oxford Instruments). Raw LSFM datasets were converted into IMARIS format using the IMARIS file converter. Quantification of total CD45.1⁺ SMARTA cell counts and subsequent classification into epithelial, LP and GALT compartments was conducted using the IMARIS Surface module. Analyses were performed on datasets acquired under matched imaging and reconstruction parameters to ensure comparability of spatial distributions across samples, while acknowledging intrinsic biological variability in tissue architecture and lymphoid structure distribution along the colon. For the EpCAM and CD45.1 fluorescence channels, iterative rounds of machine-learning-assisted threshold refinement (IMARIS machine-learning classifier) were performed to ensure high-accuracy surface segmentation while excluding nonspecific autofluorescent

structures. First, an epithelial surface was generated based on EpCAM fluorescence intensity to define anatomical boundaries for the relative distribution of CD45.1⁺ SMARTA cells in the colon. Epithelial CD45.1⁺ cells were defined as surfaces with a distance to the EpCAM surface less than 0 µm, whereas LP CD45.1⁺ cells were defined by a distance greater than 0 µm. GALT-associated CD45.1⁺ cells were assigned based on the distinctive morphology of CPs and ILFs, with the CD19 signal serving as a supportive marker to confirm B cell-enriched lymphoid structures. A minimum volume filter of 65.5 µm³ (equivalent to an approximate spherical diameter of 5 µm) was applied to CD45.1⁺ surface objects to exclude subcellular fragments, noncellular debris or segmentation artifacts, and to retain events within the lower bound of reported diameters for intact lymphocytes. Throughout the entire rendering workflow, potential nonspecific or autofluorescent structures were carefully inspected in all anatomical planes and removed manually when necessary.

Within the CD31⁺ dataset (*n* = 1), the spatial relationship of CD45.1⁺ SMARTA cells to the colonic vascular endothelium at day 14 was exemplarily assessed. Cells with a distance less than 0 µm to the CD31⁺ endothelium were classified as intravascular or endothelial-adherent. Cells located more than 0 µm and 2 µm or less from CD31⁺ structures were classified as belonging to a proximal perivascular compartment, reflecting proximity to the endothelial niche, including basement membrane and pericyte-associated regions. Cells located more than 2 µm from the CD31⁺ structures were classified as nonvascular-associated.

Single-molecule FISH staining of infected and uninfected cecal sections

Samples were prepared and imaged using published single-molecule FISH (smFISH) protocols¹⁰⁰, with minor modifications. Briefly, cecal tissues were fixed in methacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid; v/v/v) at 4 °C for 48 h to preserve both tissue morphology and RNA integrity. Fixed specimens were subsequently washed with 100% methanol (cat. no. MX0480, Sigma-Aldrich) for 35 min at 4 °C for a total of two times. Next, the tissue was washed in 100% ethanol (cat. no. 04-355-223, Thermo Fisher Scientific) for 30 min at 4 °C for a total of two times to eliminate residual fixative. The tissues were then stored in fresh 100% ethanol at 4 °C for 48–96 h before paraffin embedding, which was performed by the Rodent Histopathology Core at the Dana-Farber/Harvard Cancer Center. During the embedding process, tissues were incubated in xylene (cat. no. 89370-088, VWR) for 1.5 h at room temperature, repeated twice, followed by infiltration with molten paraffin wax (cat. no. 3801340, Leica Biosystems) for 4–5 h at 60 °C. Samples were subsequently embedded in paraffin blocks (cat. no. 3801320, Leica Biosystems). Embedded tissues were sectioned at a thickness of 4 µm and mounted on poly-L-lysine-treated, silanized coverslips to ensure flat adhesion during the downstream staining procedures. Sections were baked at 60 °C for 20 min, then washed four times with xylene (cat. no. 534056, Sigma-Aldrich) for 2.5 min each to remove paraffin. Residual xylene was cleared by two washes in 100% ethanol for 3 min, followed by rehydration through sequential 95% and 70% ethanol washes for 1 min each. Rehydrated sections were post-fixed in 4% (v/v) PFA (cat. no. 15714, Electron Microscopy Sciences) in 1× PBS for 10 min to crosslink RNA, then washed three times in 1× PBS to remove the PFA. Samples were equilibrated in a 30% formamide solution (deionized formamide in 2× SSC, cat. no. AM9344, Thermo Fisher Scientific) before staining. The probe sequences used in the smFISH experiments were designed to hybridize to 16S rRNAs in bacteria and included a 20-nt readout sequence (underlined) appended to each target sequence to allow specific binding of fluorescently labeled oligonucleotides for detection during fluorescence microscopy imaging. The applied *C. rodentium*-specific probe (5'-CCAGATGGGATTAGCTTGTTGGTGAGGTA^{AACTCCACTACTACTCACTCT}-3'), eubacterial probe (5'-AATCTCCTCCAACACTTCTAACGAGATCTCTACGGGAGGCAGCAGTAGGGA-3') and corresponding

fluorescently labeled probes (*C. rodentium*: 5'-Alexa750-S-S-AG AGTGAGTAGTAGTGGAGT-3'; Eubacteria: 5'-Cy5-S-S-TAGAA GTGTTGGAGGAGATT-3') were synthesized and experimentally validated to ensure binding and specificity as described previously^{101,102}. After staining, sections were embedded in a 4% (v/v) polyacrylamide gel prepared from a 19:1 bis-acrylamide mixture (cat. no. 1610144, Bio-Rad Laboratories). The gel solution contained 0.05 M Tris-HCl, pH 7.4 (cat. no. 15568-025, Thermo Fisher Scientific), 0.3 M NaCl (cat. no. AM9759, Thermo Fisher Scientific), 0.03% (w/v) ammonium persulfate (cat. no. 215589, Sigma-Aldrich) and 0.15% (v/v) *N,N,N',N'*-tetramethylethane-1,2-diamine (cat. no. T7024, Sigma-Aldrich). Embedded sections were cleared overnight at 37 °C using a digestion buffer composed of 2% (v/v) SDS (cat. no. AM9822, Thermo Fisher Scientific), 8 U ml⁻¹ proteinase K (cat. no. P8107S, New England Biolabs) and 0.25% (v/v) Triton X-100 (cat. no. T8787, Sigma-Aldrich) in 2× SSC. Cleared samples were washed with 2× SSC for 30 min at room temperature a total of five times and stored at 4 °C in 2× SSC supplemented with murine RNase inhibitor (1:400 dilution, cat. no. M0314L, New England Biolabs) until imaging.

Microscopy imaging and data analysis

Stained samples were imaged on a custom-built Nikon Ti-2 epifluorescence microscope equipped with a Celesta laser light engine (cat. no. 90-10521, Lumencor), a ×60 CFI PlanApo oil objective, a ×10 CFI PlanApo air objective and two Hamamatsu ORCA Flash 4.0 complementary metal-oxide-semiconductor cameras connected via a TwinCam color splitter (Cairn), as described previously¹⁰⁰. Samples were mounted in a Biopetechs FCS2 closed-flow chamber (1-mm gasket, cat. no. 1907-1422-1000, Biopetechs) connected to a custom automated fluidics system consisting of a peristaltic pump and Hamilton modular valve positioner valves. The flow system was programmatically controlled through an established computational pipeline (<https://github.com/ZhuangLab/storm-control>), allowing automated buffer exchanges and multiple smFISH imaging rounds to be performed sequentially. During imaging, mounted samples were stained for at least 15 min with fluorescently labeled oligonucleotide sequences that hybridized to the 20-nt readout regions appended to each rRNA probe. Staining was performed either at the bench or directly on the microscope by configuring the fluidics system to slowly flow a hybridization solution containing the fluorescently labeled oligonucleotides over the sample, ensuring stable probe–target binding. To minimize photobleaching and enhance fluorescence signal intensity, samples were suspended in an oxygen-scavenging imaging buffer containing 4 μM Trolox-quinone, 0.5 mg ml⁻¹ Trolox (cat. no. AB120747, Abcam), 1:500 recombinant protocatechuate 3,4-dioxygenase (cat. no. 46852004, OYC Americas) and 5 mM protocatechuic acid (cat. no. 37580, Sigma-Aldrich) in 2× SSC. During the first round of imaging, samples were bench-top-stained with fluorescent probes targeting *C. rodentium* rRNAs and DAPI for 15–20 min. The samples were rinsed briefly with 2× SSC to remove unbound dyes, mounted on the microscope and suspended in imaging buffer before being illuminated at 750 nm, 635 nm and 405 nm to visualize the *C. rodentium* rRNA signals, background autofluorescence and DAPI-stained host structures, respectively. To obtain a tissue-wide image of bacteria localization within host boundaries, multiple fields of view were photographed from a given sample using automated stage scanning. After the first imaging round, fluorescent signals were removed by incubating the sample in a cleavage buffer containing 50 mM Tris(2-carboxyethyl)phosphine (TCEP25, GoldBio) in 2× SSC. Samples were then washed with fresh 2× SSC to eliminate residual cleavage buffer and cleaved fluorescent molecules. Subsequently, a new set of fluorescently labeled probes was introduced, allowing binding of fluorescently labeled probes to their intended targets, and visualization of eubacterial signals marking the distribution of bacterial populations within the tissue. The produced collection of images from both rounds of microscopy imaging were subsequently stitched together to form a composite mosaic image and displayed using Fiji and Python scripts.

Single-cell isolation and library preparation

IECs, IELs and LPLs were isolated from the mid-distal colon of *H2-Ab1^{fl/m}* and *H2-Ab1^{fl/lin}* mice at four time points: naive (day 0), day 9, day 14 and day 42 after *C. rodentium* infection. For each condition, cells were pooled from three or four mice. After cell isolations as described above, single-cell suspensions were stained and sorted directly into 5 μl PBS supplemented with 5% FCS and 0.1 mM EDTA to obtain live EpCAM⁺ IECs, live CD45⁺ IELs derived from the epithelial fraction and live CD45⁺ LPLs from the LP. B cells were depleted using anti-CD19 antibody during fluorescence-activated cell sorting to enrich for rare T cell populations. For each sample, post-sort viability was confirmed to be 90% or greater using trypan blue exclusion; 10,000 live cells were loaded onto the 10X Chromium Controller. scRNA-seq libraries were prepared using the Chromium Single Cell 3' Gene Expression Kit (10X Genomics) at the FCSC Core Facility (UAB) according to the manufacturer's protocol. Libraries from all 52 samples (Supplemental Table 2) were pooled and sequenced on two Illumina NovaSeq 6000 S4 flow cells using 2 × 100 bp paired-end reads.

Read alignment and gene quantification

Raw sequencing data were processed using Cell Ranger v.9.0.1 (10X Genomics). Raw base call files were converted to FASTQ format using cellranger mkfastq with default parameters. Alignment, barcode processing and unique molecular identifier counting were performed using the cellranger count pipeline with default settings, aligning reads to the mouse reference genome GRCm39. Gene expression matrices were generated based on valid barcodes identified by Cell Ranger.

Quality control, normalization and cell type annotation

All analyses were performed within the scverse ecosystem¹⁰³. Initial pre-processing and quality control were performed using Scanpy (v.1.9.8)¹⁰⁴. Ambient RNA contamination was mitigated using scAR¹⁰⁵, and doublets were identified and removed using Solo¹⁰⁶ implemented via the scvi-tools package (v.1.1.2)¹⁰⁷ with default parameters. Cells in which detected genes were less than 250 or more than 5,500 (±2 s.d. from the mean) were removed; genes expressed in fewer than three cells were also excluded. An initial Leiden (v.0.10.2)¹⁰⁸ clustering step separated epithelial and immune compartments, enabling compartment-specific mitochondrial cutoffs of less than 70% for epithelial cells and less than 25% for immune cells, each derived from ±2 s.d. around the mean. Counts were then normalized to 10,000 per cell using scanpy.pp.normalize_total and log-transformed with scanpy.pp.log1p. Further, sc.tl.score_genes_cell_cycle was used to compute S-phase and G2/M-phase scores for each cell and infer the corresponding cell-cycle phase.

Batch correction and latent embedding were then performed using scVI¹⁰⁹, incorporating batch information (file_id), continuous covariates (n_genes, total counts, mitochondrial and ribosomal percentages) and relevant categorical covariates (genotype, time point, replicate, cell-layer and cell-cycle phase). After integration, neighborhood graphs were computed from the scVI latent space, followed by Leiden clustering (resolution = 0.2) and UMAP visualization¹¹⁰. Major lineages (T cells, epithelial cells, myeloid cells, B cells, neutrophils and fibroblasts) were annotated based on canonical marker expression. To resolve finer cell states, each lineage was iteratively subset and reclustered (resolution 0.3–1.0) to obtain the lowest resolution that yielded biologically distinct, marker-defined subclusters. The subclusters were annotated using (1) literature-derived marker genes, (2) the top 250 DEGs per subcluster determined using sc.tl.rank_genes_groups and (3) the anatomical origin of the cells (IEC, IEL or LPL) (Supplemental Table 2). In total, 559,621 high-quality cells were retained for downstream analysis. Finally, scANVI¹¹¹ was run to improve biological conservation across genotypes, time points and cell fractions.

Pseudobulk differential expression testing

Differential expression analysis was performed using a pseudobulk approach with pyDESeq2 (v.0.4.12)^{112,113}. Cells were grouped according

to cell state, time point and genotype, and raw unique molecular identifier counts were aggregated to form pseudobulk replicates. DESeq2 normalization, dispersion estimation and negative binomial generalized linear modeling were performed using the standard workflow; statistical significance was assessed using the Wald test and *P* values were adjusted using the Benjamini–Hochberg false discovery rate method. Genes with $P_{\text{adj}} < 0.05$ were considered significantly differentially expressed.

Gene regulatory network inference with pySCENIC

Transcription factor regulatory networks were inferred from scRNA-seq data using pySCENIC (v.0.12.1)^{45,114} in a lineage-specific workflow. Analyses were performed separately for each major lineage (epithelial, T, myeloid, neutrophil, innate, B). Within each lineage, genes detected in ten or more cells with three or more counts were retained; 8,000 highly variable genes were combined with all detected transcription factors. To build robust regulatory networks, ~500 cells per cluster were sampled in a genotype × time-point-balanced manner across three independent seeds. First, transcription factor–target coexpression modules were identified using GRNBoost2 (ref. 115), a gradient-boosting method that detects genes whose expression patterns covary with each transcription factor across single cells. This step yields candidate transcription factor–target regulatory edges supported by consistent coexpression relationships. Edges reproducibly detected in at least two of the three seeds were retained. Second, motif enrichment pruning was performed using RcisTarget, which evaluates whether candidate target genes contain enriched transcription factor-binding motifs using the mm10 (v10nr) motif databases. Only transcription factor–target pairs with motif support were preserved; regulons containing eight or more targets were retained. Finally, regulon activity was quantified using AUCell, which computes the area under the recovery curve for each regulon’s gene set within individual cells, producing cell-specific regulon activity scores. This score reflects how highly a regulon’s target genes are expressed relative to all other genes in that cell.

Regulon activity differences between the two genotypes were assessed within each cluster by computing, for every regulon, the genotype-specific median AUCell activity and the resulting activity difference ($\Delta\text{median} = H2\text{-Ab}1^{\text{fl/fl}} - H2\text{-Ab}1^{\text{Vill}^{\text{fl}}}$). For visualization, we generated transcription factor rewiring bar plots, showing the top regulons enriched per cluster in both genotypes based on $|\Delta\text{median}|$, with positive values indicating higher activity in $H2\text{-Ab}1^{\text{fl/fl}}$ and negative values indicating higher activity in $H2\text{-Ab}1^{\text{Vill}^{\text{fl}}}$.

Ligand–receptor interaction analysis

Cell–cell communication was inferred using LIANA+ (v.1.5.1)^{54,116}, using the rank_aggregate method to score ligand–receptor interactions. A unified ligand–receptor reference was constructed by concatenating multiple curated resources (Consensus, MouseConsensus, Baccin2019, CellChatDB, CellPhoneDB, ConnectomeDB2020, Embrace, Ramilowski2015, ICELLNET and LRdb) and removing duplicates. LIANA+ was run per tissue layer (epithelial or LP) and time point, using annotated cell states as sender and receiver identities, with communication scores computed per sample, where each sample was defined by tissue layer × time point × genotype × replicate. LIANA’s rank_aggregate.by_sample method was used to compute interaction scores per sample, retaining only ligand–receptor pairs with sufficient biological support—at least five cells per sender–receiver group and expression in 5% or more of cells expressing the ligand or the receptor.

Differential ligand–receptor activity between $H2\text{-Ab}1^{\text{fl/fl}}$ and $H2\text{-Ab}1^{\text{Vill}^{\text{fl}}}$ was assessed by aggregating per-sample LIANA+ scores across replicates to compute genotype-specific medians for three metrics: (1) mag_score (–magnitude_rank, where higher values indicate stronger interactions); (2) lr_means (mean ligand and receptor expression within sender and receiver); and (3) expr_prod (ligand × receptor

expression product). Only interactions with two or more biological replicates per genotype were retained. Differential effects for the three parameters above were calculated as $(H2\text{-Ab}1^{\text{fl/fl}} - H2\text{-Ab}1^{\text{Vill}^{\text{fl}}})$. The resulting effect-size measures (mag_score_effect, lr_means_effect and expr_prod_effect) were used to identify sender–receiver interactions enriched in either genotype; only interactions with absolute magnitude of differential effect ($|\text{mag_score_effect}|$) of 0.15 or greater were retained. For each receiver subset, the top ten ligand–receptor interactions per sender, ranked using $|\text{mag_score_effect}|$ were selected; differential communication patterns were visualized using scatterplots of lr_means_effect versus expr_prod_effect.

Statistics and reproducibility

Statistical analyses were performed in Prism v.10.3.1 (464) (GraphPad Software), and flow cytometry data were analyzed using FlowJo v.10.9.0. Two groups were compared using unpaired two-tailed Student’s *t*-test; more than two groups were compared using a one-way ANOVA, or a two-way ANOVA where genotype and time point were compared (Extended Data Fig. 9), each followed by a Tukey’s post hoc test with correction for multiple comparisons. Survival after *Salmonella* Typhimurium challenge was compared using a log-rank (Mantel–Cox) test. Data are presented as the mean ± s.d., except for body weight curves (mean ± s.e.m.). For all experiments, the unit of study was the individual mouse; *n* denotes the number of mice per group and is stated exactly in each figure legend; all replicates are biological replicates. For LSF, reported cell numbers and densities represent totals per approximately 1.6 mm³ field of view from one mid-distal colon segment per mouse. All experiments were performed at least twice as independent experiments with similar results; the micrographs and immunoblots shown in Fig. 2a–f and Extended Data Figs. 1j–n and 6a,b are representative of two independent experiments.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Single-cell RNA-sequencing data generated in this study have been deposited in the NCBI Gene Expression Omnibus under accession number GSE314593. Reads were aligned to the mouse reference genome GRCm39. Source data are provided with this paper. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Code availability

No custom software or algorithms were generated in this study. All analyses were performed using previously published software packages with the versions and parameters specified in Methods. Analysis scripts implementing these workflows are available from the corresponding author upon request.

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Author contributions

C.G.W., C.L.Z. and C.T.W. conceived the project and wrote the manuscript. C.G.W., C.L.Z., M.P.A. and C.T.W. performed the experiments or interpreted the results. L.W.D. and J.R.S. assisted with the development of the epitope-tagged *C. rodentium* strains. L.K. and M.G. performed the LSFM and analysis. N.T.-A., Y.W. and J.M. performed the smFISH and analysis. H.S. generated the immunoblot data. A.R.M. assisted with perfusion and collection of tissue specimens for LSFM. S.N.H. and Y.N.-K. conducted the in vitro fertilization procedures to maintain all mouse lines with the same C57BL/6 microbiota. R.D.H. assisted with the scRNA-seq experiments. M.P.A. and V.H.O. performed the bioinformatics analyses of the scRNA-seq data. B.F.F. provided technical expertise and contributed to data interpretation. L.K. and L.W.D. contributed equally as second authors.

Competing interests

The authors declare no competing interests.

Additional information

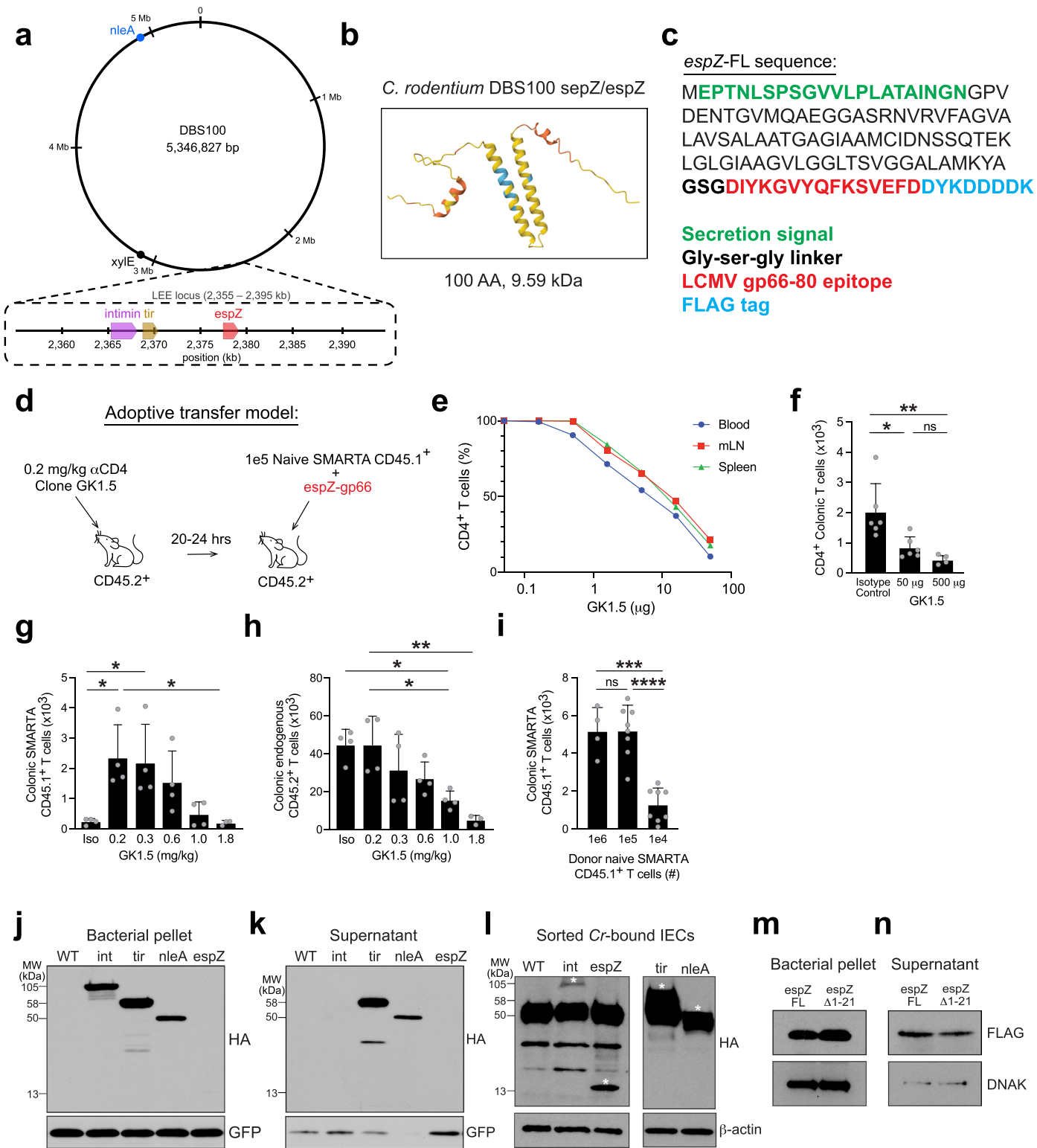
Extended data is available for this paper at <https://doi.org/10.1038/s41590-026-02656-7>.

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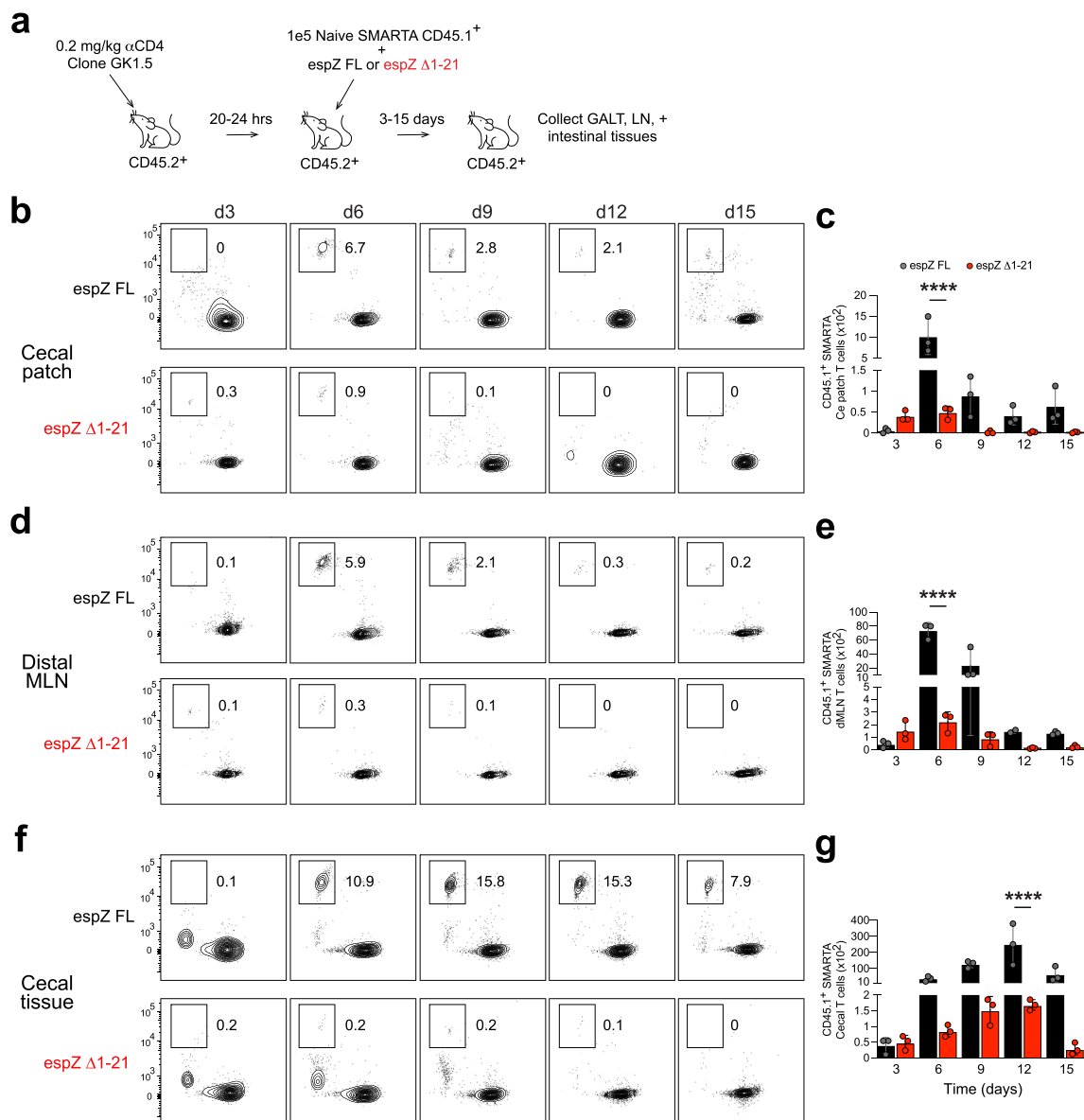
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Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | Generation of epitope-tagged *C. rodentium* strains and optimization of adoptive transfer model. **a**, Schematic of *Cr* DBS100 genome depicting relative positions of LEE-associated genes targeted for gp66 epitope-HA tag insertion (eae/intimin, tir, and espZ within LEE2 and LEE5 regions) and non-LEE-associated gene nleA into the DBS100 parent strain, as well as the xylE pseudogene locus used for insertion of espZ FL-gp66 and espZ Δ 1–21-gp66 constructs that replace GFP coding sequence in *Cr*-GFP parent strain. The xylE locus is distant the endogenous espZ locus and under distinct regulatory control, ensuring that expression from this site does not perturb endogenous espZ expression or *Cr* pathogenicity. Cartoon not drawn to scale. **b**, 3D ribbon diagram of *C. rodentium* strain DBS100 sepZ/espZ protein generated with AlphaFold3 with per-residue confidence metric (pLDDT) very high (pLDDT>90; dark blue), confident (90>pLDDT>70; cyan), low (70>pLDDT>50; yellow), and very low (pLDDT<50; orange). **c**, Full-length espZ protein sequence with T3SS secretion signal (green), linker (black), LCMV gp66-80 epitope sequence (red), and FLAG tag (blue) annotated. **d**, Adoptive transfer model used throughout this study whereby recipient C57BL/6 CD45.2⁺ recipient mice are administered 0.2 mg/kg anti-CD4 antibody 20–24 hours prior to receiving 1e5 naïve CD45.1⁺ SMARTA T cells and infection with espZ-gp66 (red) to create niche space in the endogenous T-cell repertoire for engrafted SMARTA cells. **e**, Optimization of anti-CD4 (GK1.5) antibody administration across 0.1–100 μ g doses on recovered CD4⁺ T cells from blood (blue), mesenteric lymph node (mLN; red), or spleen (green). **f**, Number of colonic CD4⁺ T cells recovered after depletion with anti-CD4 antibody or isotype control. Data are mean $\pm$ s.d. with individual mice shown; n = 6 (isotype control), 6 (50 μ g anti-CD4) and 4 (500 μ g anti-CD4) mice. Data are pooled from 2 independent experiments. One-way ANOVA with Tukey's post hoc

test; * P < 0.05 and ** P < 0.01. **g**, **h**, Number of colonic CD45.1⁺ SMARTA T cells (**g**) and endogenous CD45.2⁺ T cells (**h**) recovered from mice depleted with 0.2–1.8 mg/kg anti-CD4 or isotype control 14 days after adoptive transfer and infection with espZ-gp66. Data are mean $\pm$ s.d. with individual mice shown; n = 4 (isotype control), 4 (0.2 mg/kg), 4 (0.3 mg/kg), 4 (0.6 mg/kg), 4 (1.0 mg/kg) and 3 (1.8 mg/kg) mice for both **g** and **h**. Data are from one experiment representative of 2 independent experiments. One-way ANOVA with Tukey's post hoc test; * P < 0.05 and ** P < 0.01. **i**, Number of colonic CD45.1⁺ SMARTA T cells recovered from mice depleted with 0.2 mg/kg anti-CD4 14 days after adoptive transfer with 1e4–1e6 naïve CD45.1⁺ SMARTA cells and infection with espZ-gp66. Data are mean $\pm$ s.d. with individual mice shown; n = 4 (1e6), 8 (1e5) and 8 (1e4) naïve SMARTA cells transferred. Data are pooled from 2 independent experiments. One-way ANOVA with Tukey's post hoc test; *** P < 0.001 and **** P < 0.0001. Western blot for HA tag and GFP loading control performed on *Cr*-GFP parent strain and intimin-gp66, tir-HA, nleA-gp66, and espZ-gp66 *Cr*-GFP derivative bacterial cell pellets (**j**) and bacterial supernatants (**k**) from culture in DMEM. **l**, Western blot for HA tag and β -actin loading control performed on sorted *Cr*-GFP-bound IECs infected with *Cr*-GFP parent strain and intimin-gp66, tir-HA, nleA-gp66, and espZ-gp66 *Cr*-GFP derivatives. Panel **l** is comprised of two Western blot membranes. Asterisks indicate the expected HA-tagged fusion protein in each lane; all other visible bands represent non-specific background. Sorted *Cr*-GFP-bound IECs were pooled from 5–6 mice per group to obtain sufficient protein for analysis. Molecular weight markers are indicated in **j**–**l**; uncropped scans are provided as Supplementary Data. Western blot experiments in **j**–**l** were performed twice as independent biological replicates; representative blots are shown.

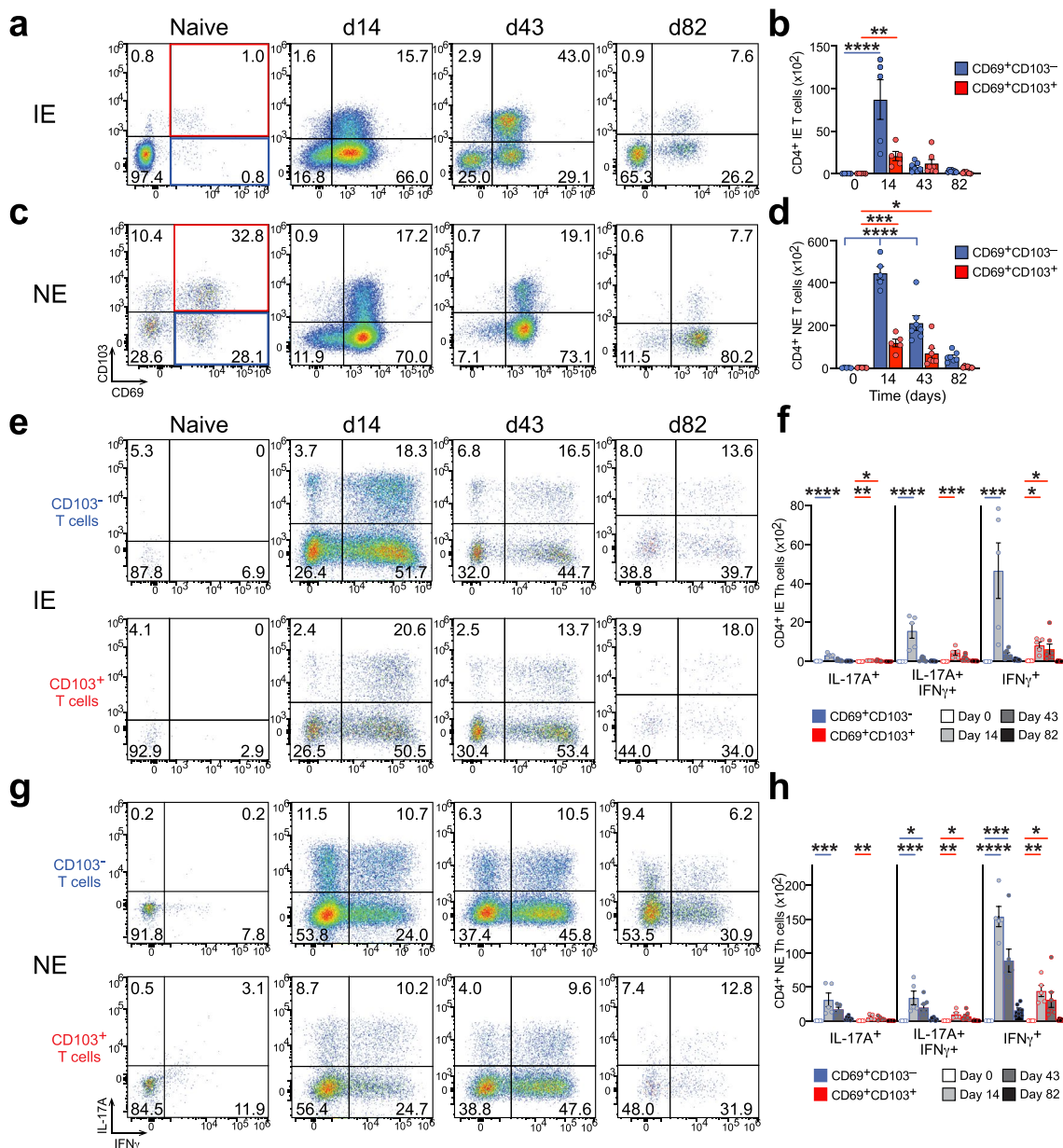


Extended Data Fig. 2 | Kinetics and tissue distribution of SMARTA T cell responses to translocated versus bacterially-associated espZ antigens.

C57BL/6 CD45.2⁺ mice received 0.2 mg/kg anti-CD4 20-24 hours prior to adoptive transfer of 1×10^5 naïve CD45.1⁺ SMARTA T cells and infection with either espZ FL-gp66 or espZ Δ 1-21-gp66 strains. **a**, Recovered colonic CD4⁺TCR β ⁺ cells were analyzed by flow cytometry for CD45.1 or CD45.2 expression across GALT (cecal

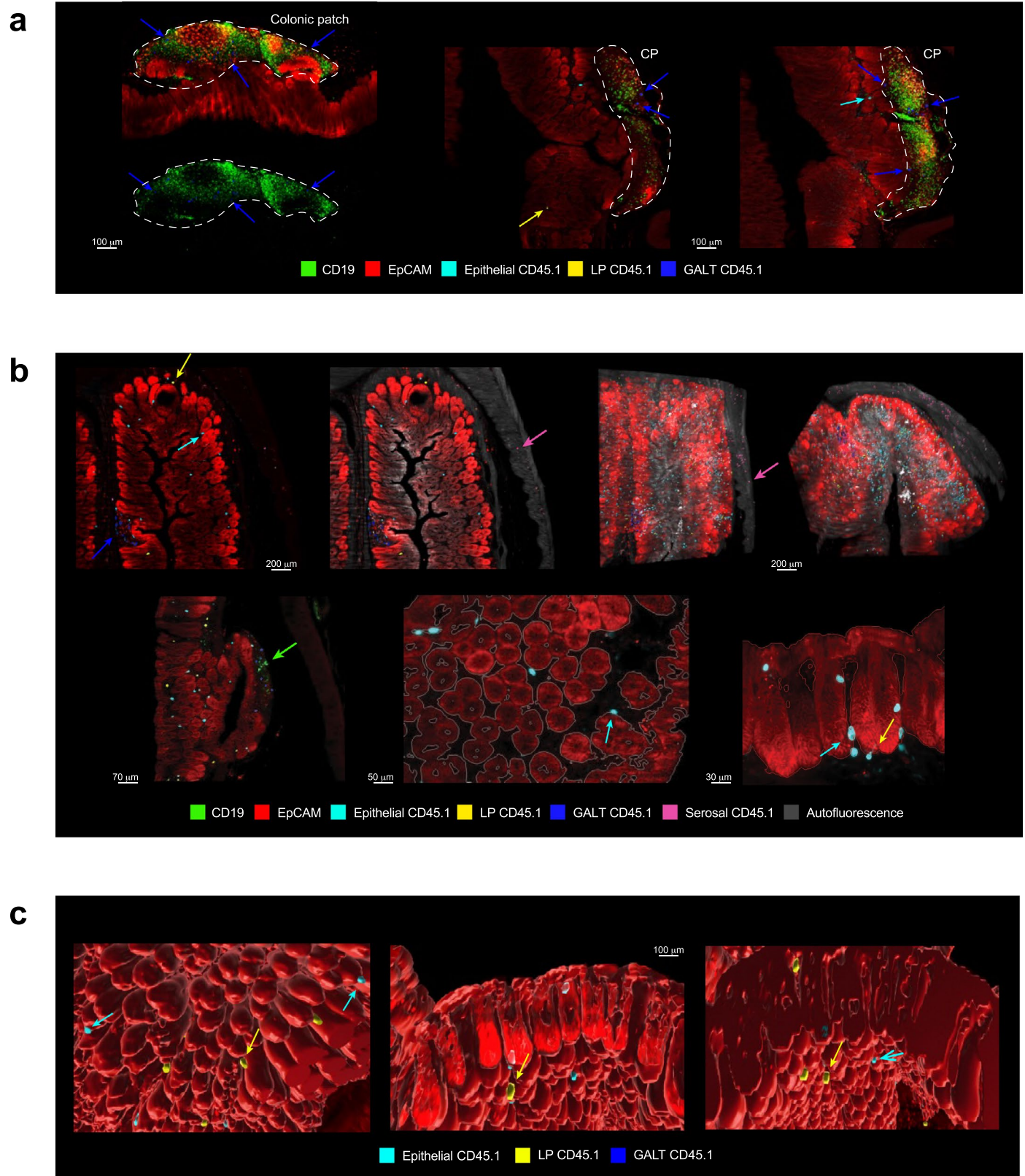
patch and distal mesenteric LN) and cecal tissue at days 3-15 post-infection.

b-g, Number of recovered CD45.1⁺ SMARTA T cells from cecal patch (**b, c**), distal MLN (**d, e**), or cecal tissue (**f, g**). Data are mean $\pm$ s.d. with individual mice shown; $n = 3$ mice per group per timepoint for all panels. Data are from one experiment representative of 2 independent experiments. One-way ANOVA with Tukey's post hoc test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.



Extended Data Fig. 3 | Phenotypic and functional characterization of endogenous CD4⁺ T cells during *C. rodentium* infection. a-h. Colonic CD4⁺TCRβ⁺ cells isolated from colonic epithelium (IE) and non-epithelial tissue (NE) were collected from naïve or *Cr*-DBS100-infected C57BL/6 mice at day 14, 43, or 82 post-infection. Recovered cells were stimulated for 3-4 hours with PMA and ionomycin and stained for surface CD4, TCRβ, CD69, and CD103 followed by intracellular staining for IL-17A and IFNγ and analysis by flow cytometry. Data are mean ± s.d. with individual mice shown. For **b** and **d**, n = 5 CD69⁺CD103⁻ and 5

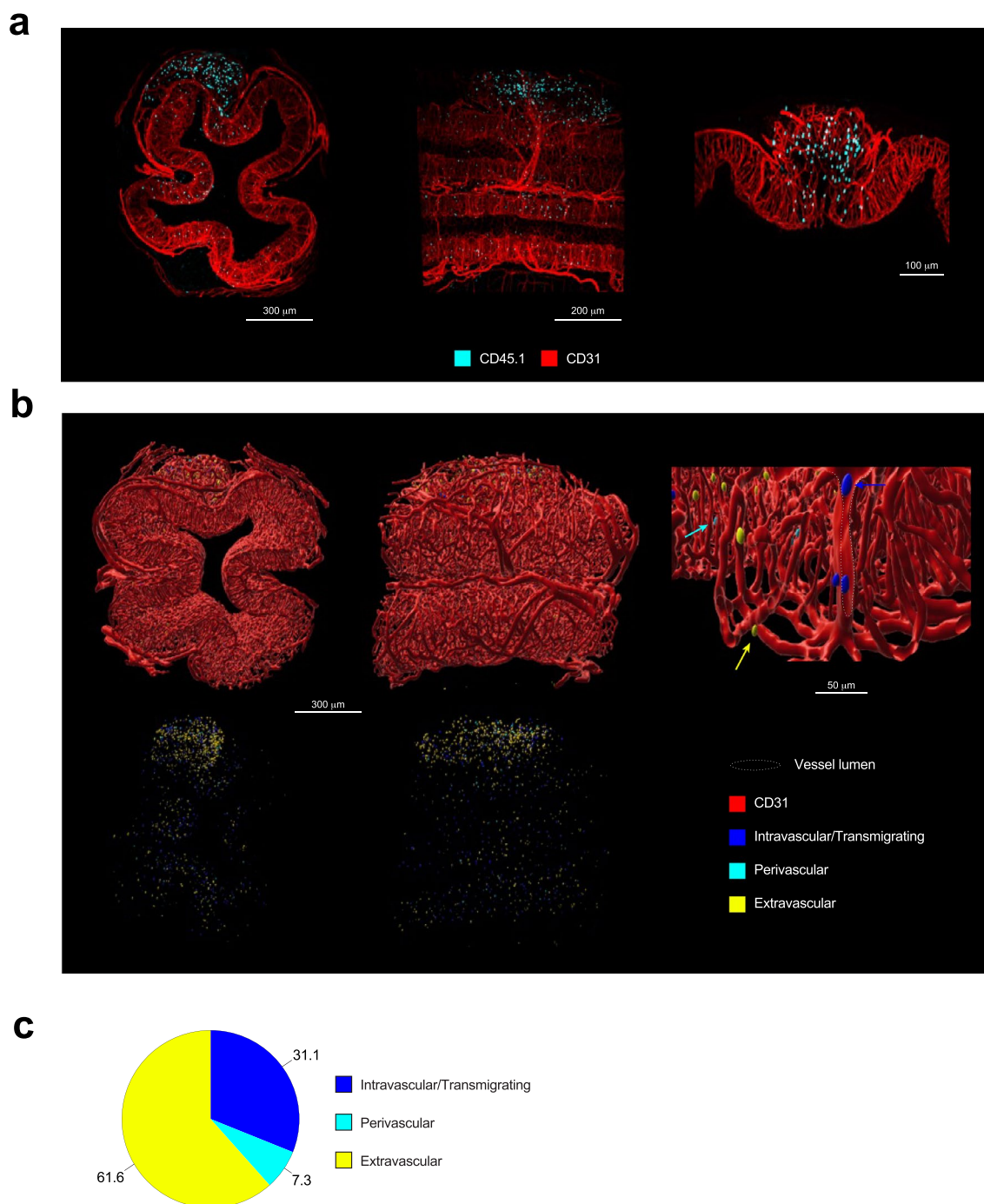
CD69⁺CD103⁺ (naïve), 5 and 5 (day 14), 7 and 7 (day 43), and 7 and 6 (day 82) mice. For **f**, n = 5 and 5 (naïve), 5 and 5 (day 14), 5 and 5 (day 43), and 7 and 7 (day 82) mice. For **h**, n = 5 and 5 (naïve), 5 and 7 (day 14), 5 and 5 (day 43), and 7 and 7 (day 82) mice. Data are from one experiment representative of 2 independent experiments. One-way ANOVA with Tukey's post hoc test, comparing CD69⁺CD103⁻ populations (blue) and CD69⁺CD103⁺ populations (red) separately; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.



Extended Data Fig. 4 | See next page for caption.

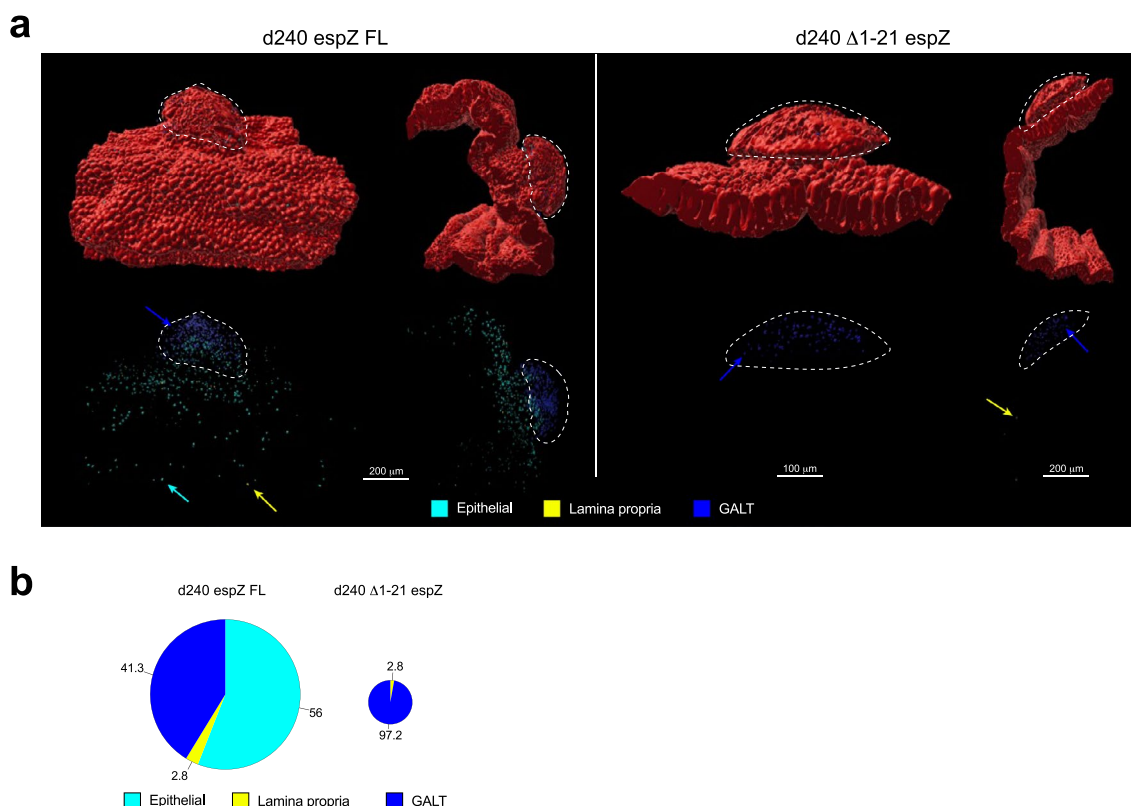
Extended Data Fig. 4 | Light sheet fluorescence microscopy-based assignment of SMARTA CD45.1⁺ T cell microanatomical localization within colonic mucosa. **a**, Representative LSFM images (100 μ m optical section) of distal colonic tissue from *C. rodentium* Δ 1–21 espZ-infected mice that received CD45.1 + SMARTA T cells, harvested at day 14 post-infection. CD19 staining (green) delineates colonic patch (GALT) boundaries (dashed white outline). EpCAM (red) defines the epithelium. Colored arrows indicate CD45.1⁺ cells assigned to the epithelial-associated (cyan), lamina propria (yellow), or GALT-resident (blue) based on their spatial relationship to EpCAM and CD19 surfaces. **b**, LSFM images at decreasing optical section depths (200, 70, 50, and 30 μ m)

illustrating the criteria used to assign compartmental identity to individual CD45.1⁺ cells. Epithelial association was defined as distance to EpCAM surface <0 μ m (cyan); lamina propria assignment was defined as distance to EpCAM surface >0 μ m (yellow); GALT assignment on the basis of colonic patch and ILF morphology, with CD19 as a confirmatory marker (blue). Serosal CD45.1⁺ cells (pink) and tissue autofluorescence (grey) are shown for reference. **c**, Three-dimensional surface renderings of EpCAM (red) and CD45.1⁺ cells showing showing epithelial (cyan) and lamina propria (yellow) assignments in relation to colonic crypt architecture. Images are representative of $n = 3$ mice per group at day 14 post-infection.



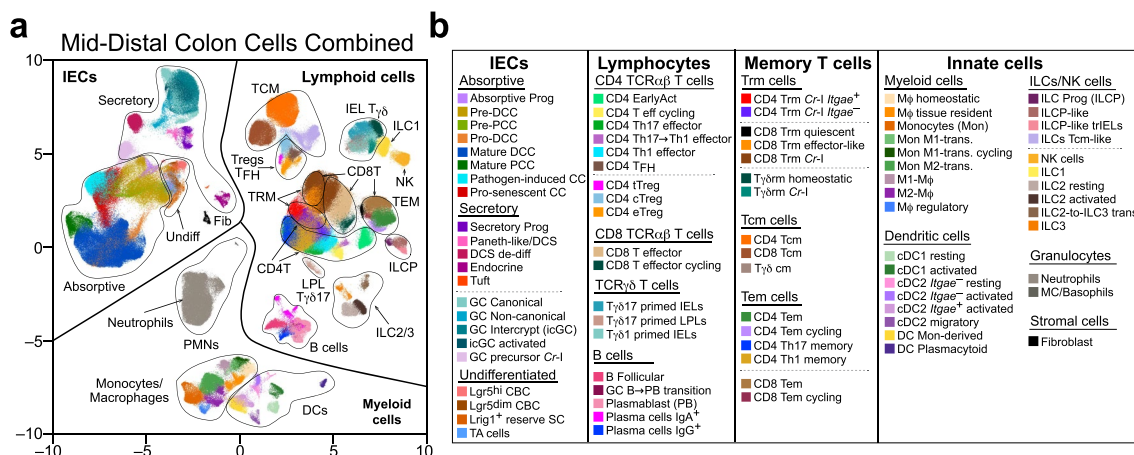
Extended Data Fig. 5 | Vascular association of SMARTA T cells at day 14 of *Cr* infection. **a**, Representative LSFM images at decreasing optical section depths (300, 200, and 100 μm) of distal colon tissue from *C. rodentium* espZ FL-infected mice that received CD45.1⁺ SMARTA T cells, harvested at day 14 post-infection. CD31 identifies the intestinal vasculature (red); CD45.1⁺ SMARTA T cells are shown in cyan. Transverse section (left and right panels) and sagittal section (middle panel) highlight abundant SMARTA cells in submucosa and mucosa. **b**, Three-dimensional surface renderings of CD31 and CD45.1⁺ cells from the specimen shown in **a**, illustrating the spatial relationship between SMARTA

T cells and vessel lumens (dashed outline) at 300 and 50 μm optical sections. CD45.1⁺ cells are pseudocolored to highlight vascular association: intravascular/transmigrating (blue), perivascular (cyan), and extravascular (yellow). **c**, Pie chart showing the relative distribution of CD45.1⁺ SMARTA T cells by vascular compartment – intravascular/transmigrating (31.1%, blue), perivascular (7.3%, cyan), and extravascular (61.6%, yellow) – quantified from the full intestinal sample of a single mouse at day 14 post-infection ($n = 1$); this analysis was performed on one specimen and was not repeated independently.



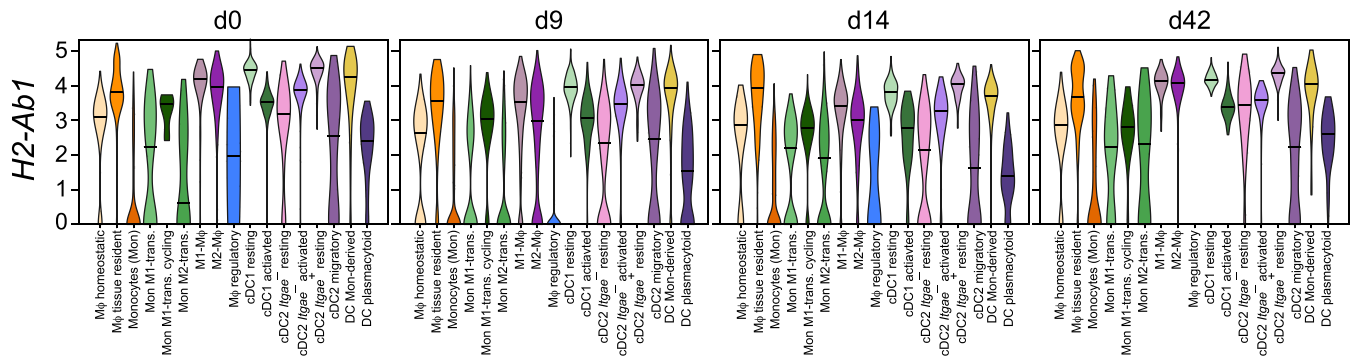
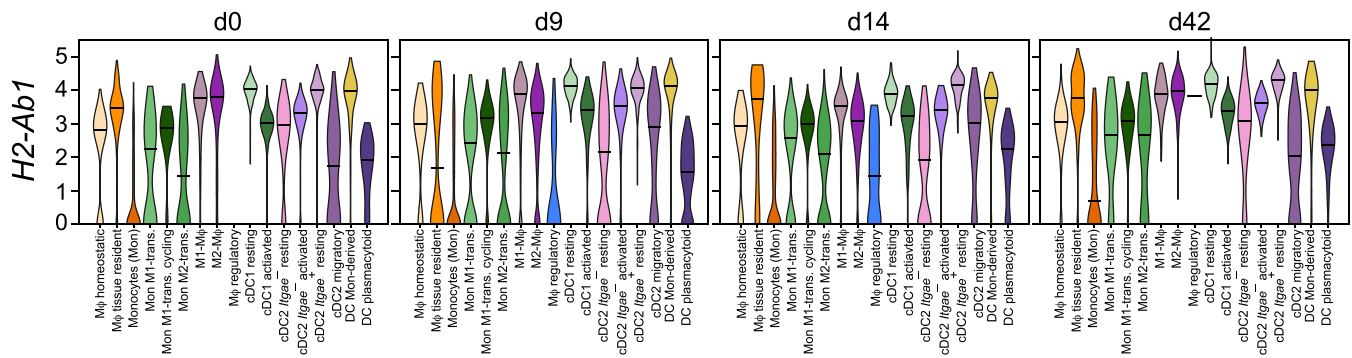
Extended Data Fig. 6 | Injected bacterial antigen induces durable tissue-resident memory in CD4⁺ T cells. a, Representative LSFM images combined with three-dimensional surface renderings of distal colon tissue from espZ FL- (left panel) and espZ $\Delta 1-21$ -infected mice (right panel) at day 240 post-infection. Colonic patch (GALT) boundaries are indicated by dashed white outlines. CD45.1⁺ SMARTA T cells are pseudocolored by compartment: epithelial (cyan),

lamina propria (yellow), and GALT (blue). **b**, Pie charts showing the relative distribution of SMARTA CD45.1⁺ T cells across epithelial, lamina propria, and GALT compartments at day 240 post-infection in espZ FL and espZ $\Delta 1-21$ -infected mice (in ~ 1.6 mm³ FOV). Pie chart area is scaled proportionally to total CD45.1⁺ cell number per FOV. Data are representative of $n = 5$ mice per group.



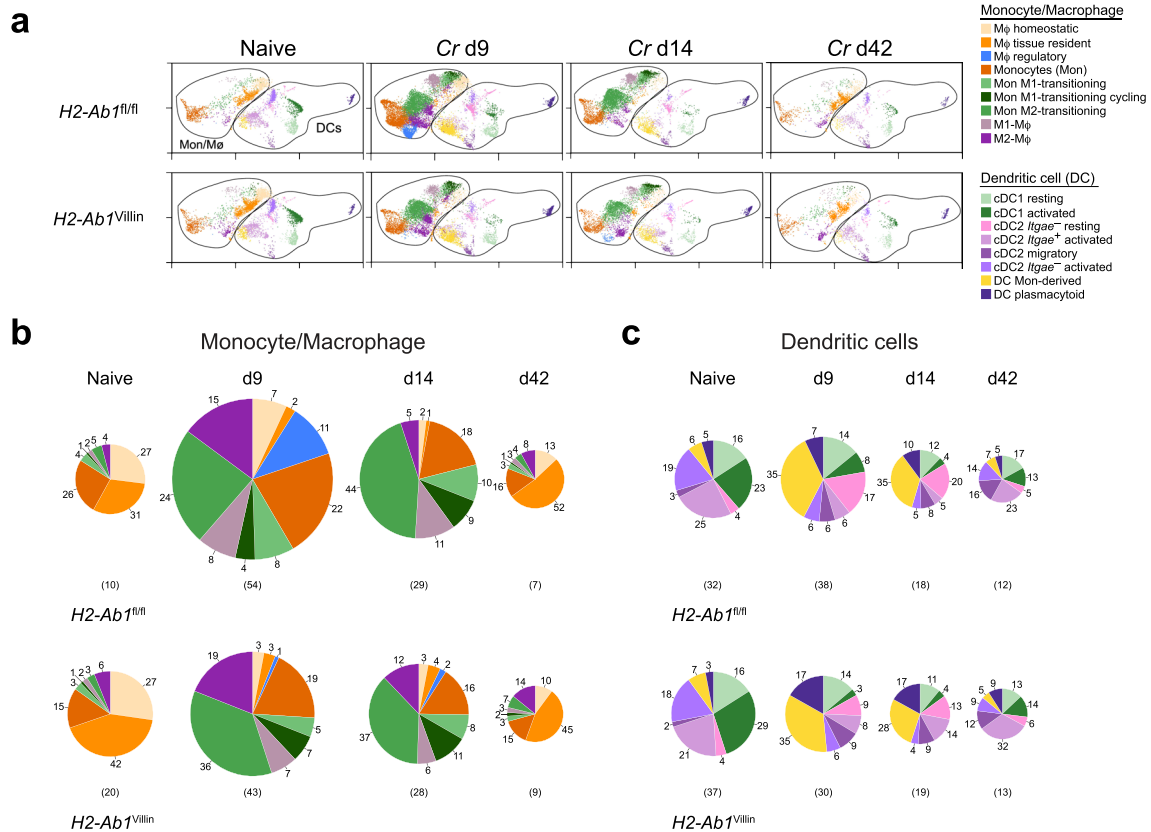
Extended Data Fig. 7 | Composite single-cell transcriptomic atlas of colonic immune and epithelial cells from naive and *Cr*-infected *H2-Ab1*^{fl/m} and *H2-Ab1*^{vilin} mice. **a, b**, UMAP analysis of total mid-distal colon cells isolated from naive or DBS100 *Cr*-infected *H2-Ab1*^{fl/m} or *H2-Ab1*^{vilin} mice on day 9, 14 or 42 post-infection. EpCAM⁺ and CD45⁺ intraepithelial (IE) and non-epithelial (NE) cells were sorted for scRNA-seq analysis. Approximately 600,000 cells were resolved into 87 individual clusters: 22 IEC clusters (8 absorptive lineage, 10 secretory lineage, and 4 undifferentiated populations); 35 lymphocyte clusters (19 effector populations and 16 memory populations spanning CD4, CD8, γδ T cells, and B cells); and 30 innate cell populations spanning

monocytes/macrophages, dendritic cells, ILCs/NK cells, granulocytes, and stromal fibroblasts. Abbreviations: prog, progenitor; DCC, distal colonocyte; PCC, proximal colonocyte; CC, colonocyte; DCS, deep crypt secretor; de-diff, de-differentiated; GC, goblet cell; *Cr-I*, *C. rodentium*-induced; CBC, crypt base columnar; TA, transit amplifying; EarlyAct, early activated; T eff, T effector; Tfh, T follicular helper; tTreg, thymic Treg; cTreg, central Treg; eTreg, effector Treg; GC B, germinal center B cell; PB, plasmablast; Trm, tissue resident memory T cell; Tcm, central memory T cell; Tem, effector memory T cell; ILCP, innate lymphoid cell precursor; trIELs, tissue resident intraepithelial lymphocytes; NK, natural killer; MC, mast cell.

a*H2-Ab1^{f/f}***b***H2-Ab1^{Villⁱⁿ}*

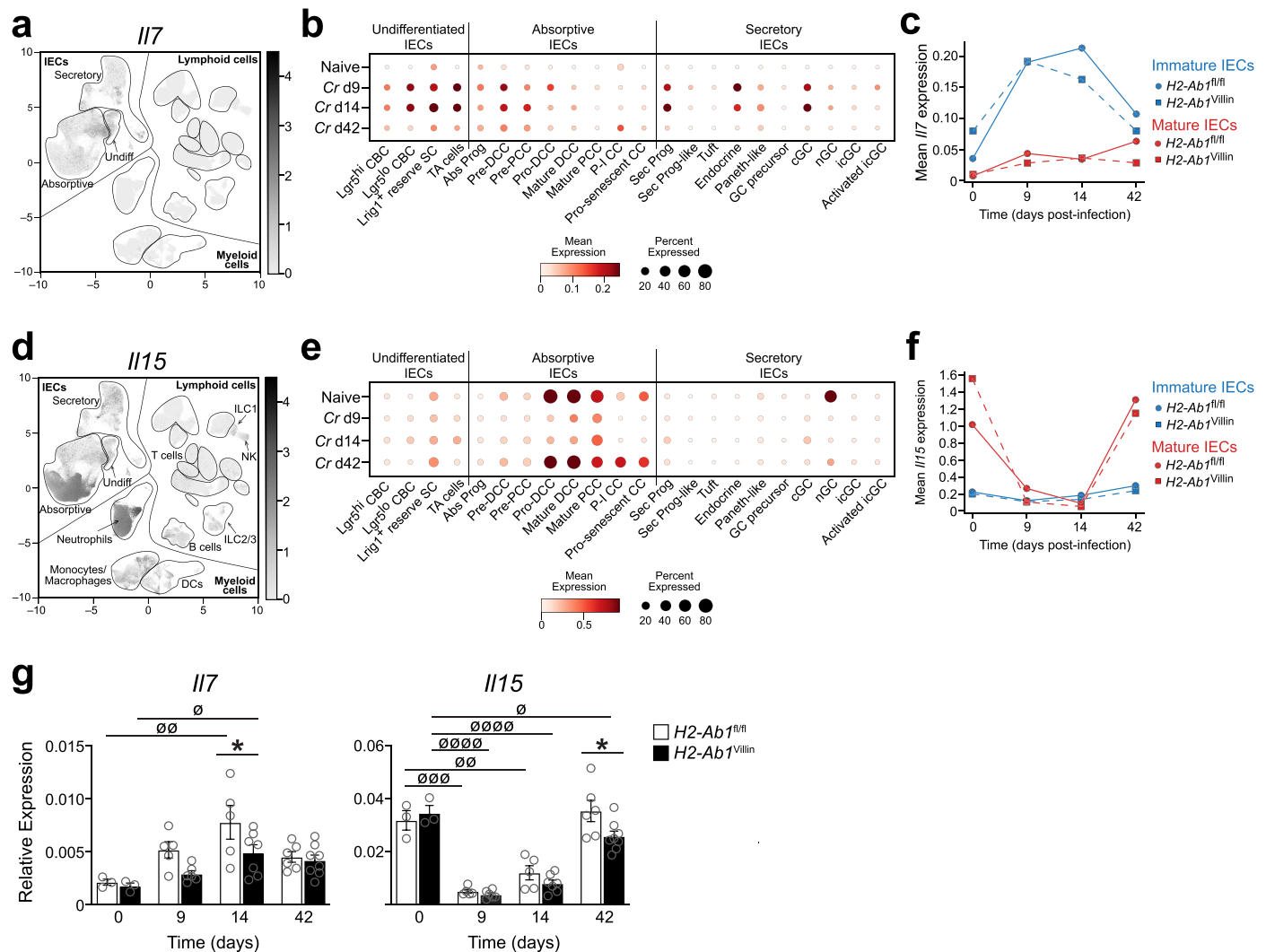
Extended Data Fig. 8 | Temporal expression of *H2-Ab1* across professional antigen-presenting cells in *H2-Ab1^{f/f}* and *H2-Ab1^{Villⁱⁿ}* mice. a–b, Violin plots of *H2-Ab1* expression across monocyte/macrophage and dendritic cell clusters in

naïve or DBS100 *Cr*-infected *H2-Ab1^{f/f}* or *H2-Ab1^{Villⁱⁿ}* mice on day 9, 14 or 42 post-infection. Abbreviations: DC, dendritic cell; Mφ, macrophage; trans, transitioning; Mon, monocyte.



Extended Data Fig. 9 | Distribution of myeloid cell populations across intraepithelial and non-epithelial compartments in *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice. **a, UMAPs of total monocyte/macrophage and dendritic cell populations isolated from mid-distal colon of naïve or DBS100 *Cr*-infected *H2-Ab1^{fl/fl}* or *H2-Ab1^{Villin}* mice on day 9, 14 or 42 post-infection. **b, c**, Pie charts of the number and percentages of monocytes/macrophages (**b**) and dendritic cells (**c**) at indicated**

times, highlighting that while total monocyte/macrophage numbers and subset composition are substantially altered within and between genotypes during active infection (d9), total DCs and DC subsets do not undergo major shifts in numbers or subset composition between *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice. Pie chart areas are normalized to largest population within monocyte/macrophage or DC pools.



Extended Data Fig. 10 | Epithelial *IL7* and *IL15* expression shows reciprocal temporal regulation and cellular source during *Cr* infection. **a**, UMAP analysis of *IL7* expression in scRNA-seq dataset. **b**, Dot plot of *IL7* expression across time points and cell types of *H2-Ab1^{fl/fl}* mice during *Cr* infection, highlighting significant *IL7* expression by undifferentiated IECs (Lgr5^{dim} CBCs, Lrig1⁺ reserve SCs, and TA cells), immature absorptive IECs (pre-DCCs and pre-PCCs), and immature secretory IECs (secretory progenitors), along with endocrine and canonical GCs, during active infection (days 9–14). **c**, Comparison of expression of *IL7* by immature and mature IECs of *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice over infection time course, highlighting similar pattern of expression by immature IECs of both genotypes that is maximal during active infection (days 9 and 14). **d**, UMAP analysis of *IL15* expression in scRNA-seq dataset. **e**, Dot plot of *IL15* expression across time points and cell types of *H2-Ab1^{fl/fl}* mice during *Cr* infection, highlighting significant *IL15* expression by mature absorptive IECs (pro-DCC, mature DCC, mature PCC, P-I CC, and pro-senescent CC) as well as some non-canonical GCs at homeostatic time points (naïve and day 42) but diminished expression during acute infection (days 9–14)—displaying a reciprocal expression pattern from that of *IL7*. **f**, Comparison of expression of *IL15* by immature and mature IECs of *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice over infection time course, highlighting enhanced expression of *IL15* in mature IECs compared to immature IECs at homeostatic time points and similar pattern of expression between genotypes. **g**, *IL7* and *IL15* expression from sorted cECs isolated from mid-distal colon of naïve or DBS100 *Cr*-infected *H2-Ab1^{fl/fl}* or *H2-Ab1^{Villin}* mice on day 9, 14

or 42 post-infection was detected by RT-qPCR, confirming the pattern shown in the scRNA-seq dataset. Data are mean $\pm$ s.d. with individual mice shown; $n = 3$ *H2-Ab1^{fl/fl}* and 3 *H2-Ab1^{Villin}* (naïve), 5 and 6 (day 9), 5 and 7 (day 14), and 6 and 8 (day 42) mice. Data are from one experiment representative of 2 independent experiments. Two-way ANOVA with Tukey's post hoc test; * $P < 0.05$ (comparing *H2-Ab1^{fl/fl}* and *H2-Ab1^{Villin}* mice); $\emptyset P < 0.05$, $\emptyset\emptyset P < 0.01$, $\emptyset\emptyset\emptyset P < 0.001$ and $\emptyset\emptyset\emptyset\emptyset P < 0.0001$ (comparing timepoints). In view of the fact that IL-7 and IL-15 are key common γ -chain cytokines that support survival of different Trm subsets, our data indicate that there are temporal and spatial expression differences between the two cytokines that emerge from the scRNA-seq dataset: *IL7* transcripts were predominantly expressed by immature, undifferentiated cECs (CBCs, TA cells, pre-DCCs, and some secretory cells) at days 9–14 post-infection, whereas *IL15* transcripts were predominantly expressed by mature IECs (pro-DCCs, mature DCCs, and pro-senescent CCs) in naïve cECs and at day 42 post-infection. The loss of *IL15* expression at days 9 and 14 likely reflected more rapid epithelial turnover and alterations in DCC maturation that accompany active *Cr* infection, as mature cells at mucosal surface are rapidly shed and replaced by immature populations. These findings suggest that IL-15 plays a key role in maintenance of intraepithelial T cells at homeostasis, and when the cellular source of IL-15 is lost during acute infection, IL-7 from proliferating immature cells—and possibly IL-2 produced by activated T effector cells—may compensate for this loss as new memory cells are generated and occupy the epithelial niche.

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ 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

Cell Ranger (v9.0.1; 10x Genomics)
ImSpector (v7.7, LaVision Biotec)
storm-control (v2019.06.28)

Data analysis

Scanpy (v1.9.8)
scvi-tools (v1.1.2)
Leiden (v0.10.2)
pyDeseq2 (v0.4.12)
LIANA+ (v1.5.1)
pySCENIC (v0.12.1)
Imaris (v10.1.0)
Fiji (v2.16.0)

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Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The scRNA-seq data used in this study has been deposited in the Gene Expression Omnibus database under the accession number GSE314593. All other data generated in this study are provided within the article and its Supplementary Information. The shell, R and Python scripts that enabled the main steps of the analyses performed in this project are available on request.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Reporting on race, ethnicity, or other socially relevant groupings

Population characteristics

Recruitment

Ethics oversight

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

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

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Life sciences study design

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

Sample size	In vivo animal experiments use group sizes of 4-6 animals and are repeated. This typical group size is based on empirical power analysis, using one-way ANOVA and empirical data; analyzing at least 4 mice/group provides 90% power to detect an effect size of 1.33 (or a 30% change) where $P < 0.05$. scRNA-seq data were generated from cells sorted from pools of tissues representing 2-3 animals per group and n=2 biological replicates. Experiments that required sorting of epithelial cells from pools of tissues from 2-3 animals were run in duplicate with a maximum of 4 groups to limit cell death after sorting.
Data exclusions	Data exclusion was considered when animals appeared sick.
Replication	All experimental findings were repeated and successful. Where possible, data represents first and repeat experiments combined.
Randomization	Cohorts of adult mice of similar age group (8-12 weeks old) were randomly assigned to either control or infected groups of at least 4 mice per group.
Blinding	Blinding is not possible due to safety reasons and potential cross-contamination between infected and non-infected naive animals.

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
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

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

Antibodies

Antibodies used

Rat anti-mouse Ly6G-APC (1A8); Biolegend 127614
 Donkey Anti-goat Alexa Fluor488; ThermoFisher A-11008
 Donkey Anti-goat Alexa Fluor647; ThermoFisher A-21447
 SA-Alexa Fluor594; ThermoFisher S32356
 Rabbit Anti-C.r-LPS; Accurate Chemical and Scientific of Denka Seiken Co., Ltd YCC312-012
 Rabbit Anti-GFP; ThermoFisher A-11122
 Goat Anti-rabbit Alexa Fluor488; ThermoFisher A-11008
 Goat Anti-rabbit Alexa Fluor594; ThermoFisher A-11037
 Rat anti-mouse CD45 PE (30-F11); Biolegend 103106
 Rat anti-mouse EpCAM PE-Cy7 (G8.8); ThermoFisher 25-5791-80
 Rat anti-mouse MHCII eFluor450 (M5/114.15.2); (I-A/I-E); Fisher 50-163-69
 Rabbit anti-pSTAT3 (Tyr705; Clone D3A7); Cell Signaling 9145P
 Rat anti-mouse CD4 FITC (RM4-5); BioLegend 100510
 Armenian hamster anti-mouse/human TCR β (H57-957); ThermoFisher 17-5961-82
 Rat anti-mouse CD44 BV510 (IM7); BioLegend 103044
 Rat anti-mouse CD45.1 PE-Cy7 (A20); ThermoFisher 25-0453-82
 Mouse anti-mouse CD45.2 BV711 (104); BD Biosciences 563685
 Armenian hamster anti-mouse/human CD69 (H1.2F3); ThermoFisher 12-0691-82
 Armenian hamster anti-mouse/human CD103 (2E7); ThermoFisher 14-1031-82
 Rat anti-mouse CD19 (1D3/CD19); Biolegend 152410
 Rat anti-mouse/human CD11b (M1/70); Biolegend 101226
 Armenian hamster anti-mouse CD11c (N418); Biolegend 117324
 Rat anti-mouse CD62L (MEL-14); Biolegend 104441
 Rat anti-mouse IFN γ eFluor450 (XMG1.2); ThermoFisher 48-7311-82
 Rat anti-mouse IL-22 PE (1H8PWSR); ThermoFisher/eBioscience 12-7221-82
 Rat anti-mouse IL-17A FITC (eBio17B7); ThermoFisher/eBioscience 11-7177-81
 Rat anti-mouse IL-17A PerCP-Cy5.5 (TC11-18H10); BD Biosciences 560666
 Live/Dead Fixable Near-IR dead cell dye (ThermoFisher)
 Goat anti-HA Tag (polyclonal); Fisher/Novus Biologicals NB600362
 Streptavidin-Alexa Fluor 594; ThermoFisher S32356
 Prolong Diamond antifade mountant with DAPI; ThermoFisher P36962

Validation

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals

C57BL/6 (WT; stock #000664), C3H/HeJ (stock #000659), H2-Ab1 floxed (stock #013181), SMARTA-1 CD45.1 (stock #030450), Villin-cre (stock #021504), and Villin-cre/ERT2 (stock #020282) mice were purchased from Jackson Laboratory. Rosa26uLIPSTIC mice were a gift from Gabriel D. Vitoria. MHCII-EGFP mice were a gift from Hidde L. Ploegh. Experimental adult animals (8-12 wk old) were co-caged in groups of 2-7 mice.

Wild animals

This study did not involve wild animals.

Reporting on sex

Both sexes were used per experimental group whenever possible.

Field-collected samples

This study did not include field-collected samples.

Ethics oversight

All mouse strains were bred and maintained at UAB in accordance with IACUC guidelines.

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

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ 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

Intestinal tissue was flushed, cut into 4 cm mid-distal colon, opened longitudinally and then cut into strips of 1 cm length. Tissue pieces were incubated for 20 min at 37°C with 1 mM DTT (Sigma), followed by 2 mM EDTA (Invitrogen) in H5H media (1x HBSS, 5% FBS, 20 mM Hepes, and 2.5 mM 2-β-ME). Tissue pieces were vortexed briefly after each 20 min incubation, followed by washing with H5H prior to centrifugation at 1800 rpm for 10 min at 4°C. IECs were then purified on a 40%/75% Percoll gradient by centrifugation for 20 min at 25°C and 2000 rpm with no brake. For analysis of C.r-GFP attached to IECs, tissue pieces from 4 cm of mid-distal colon were incubated for 20 min at 37°C with 1 mM EDTA in H5H media, followed by gentle mixing and washing with H5H. Colonic IECs were stained with Fc Block (Clone 2.4G2) followed by staining with fluorescent-labeled antibodies in IEC buffer (1x PBS with 5% FBS and 2mM EDTA to reduce cell clumping) on ice in 1.5 ml microcentrifuge tubes. For intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Bioscience). Colonic lamina propria CD4 T cells were isolated by flushing colons with 1x PBS, opening longitudinally and cutting into small pieces and placed in H5H media. Tissue was then minced in 1.5 ml microcentrifuge tubes for 2-3 min before being transferred into scintillation vials with 10 ml of complete R10 media (1x RPMI 1640, 10% FBS, 1x Pen/Strep, 1x NEAA, 1mM Sodium pyruvate, 2 mM L-glutamine, and 2.5 mM 2-β-ME) with collagenase IV (Sigma, 100 U/ml) and DNase I (Sigma, 20 mg/mL). Tissue was digested at 37°C for 40 min with stirring followed by filtering over a 70 μm filter and washing with complete R10 media. Cells were then centrifuged at 1750 rpm at 4°C for 10 min and then purified on a 40%/75% Percoll as described above. Where indicated, cells were stimulated with PMA (50 ng/ mL) and Ionomycin (750 ng/mL) at 37°C for 4 hrs in the presence of GolgiPlug (BD Biosciences). Samples were acquired on an Attune NxT flow cytometer (Life Technologies) or sorted on either a BD FACS Aria or Aria II (BD Biosciences).

Instrument

FlowJo 10.8.1

Software

Cell population abundance

A portion of sorted cells were resorted to confirm >95% purity.

Gating strategy

FSCSSC included all cells (lymphocytes and epithelial cells) except small debris

IEC gating strategy:

1. Live cells were gated as LIVE/DEAD Fixable Near-IR Dead Cell dye (ThermoFisher L34976) negative, live cells were gated 2CD45-PE negative, EpCAM1-PE-Cy7 positive epithelial cells were analyzed for C.r-GFP, Ly6G-APC, or MHCII-EGFP.

Colonic T cell gating strategy:

1. Colonic LP CD4 T cells were gated as LIVE/DEAD dye negative, CD4+ live cells were gated
 2. TCRβ+ cells from the live CD4+ population were gated
 3. TCRβ+ cells were then gated on CD69+CD103- or CD69+CD103+
 4. CD69+CD103- and CD69+CD103+ cells were then gated on IFNγ+, IL-17A+, or IL-17A+IFNγ+ by quadrant gate
- Colonic T cell gating strategy from SMARTA-1 CD45.1+ adoptive transfer experiments:
1. Colonic LP CD4 T cells were gated as LIVE/DEAD dye negative, CD4+ live cells were gated
 2. TCRβ+ cells from the live CD4+ population were gated
 3. TCRβ+ cells were then gated on CD45.1+; CD45.2+ cells were excluded

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