Intestinal fibroblastic reticular cell niches control innate lymphoid cell homeostasis and function

Hung-Wei Cheng1,3, Urs Mörbe1,3, Mechthild Lütge1, Céline Engetschwiler1, Lucas Onder1, Mario Novkovic1, Cristina Gil-Cruz1, Christian Perez-Shibayama1, Thomas Hehlgens2, Elke Scandella1 & Burkhard Ludewig1

Innate lymphoid cells (ILCs) govern immune cell homeostasis in the intestine and protect the host against microbial pathogens. Various cell-intrinsic pathways have been identified that determine ILC development and differentiation. However, the cellular components that regulate ILC sustenance and function in the intestinal lamina propria are less known. Using single-cell transcriptomic analysis of lamina propria fibroblasts, we identify fibroblastic reticular cells (FRCs) that underpin cryptopatches (CPs) and isolated lymphoid follicles (ILFs). Genetic ablation of lymphotoxin-β receptor expression in Ccl19-expressing FRCs blocks the maturation of CPs into mature ILFs. Interactome analysis shows the major niche factors and processes underlying FRC-ILC crosstalk. In vivo validation confirms that a sustained lymphotoxin-driven feedforward loop of FRC activation including IL-7 generation is critical for the maintenance of functional ILC populations. In sum, our study indicates critical fibroblastic niches within the intestinal lamina propria that control ILC homeostasis and functionality and thereby secure protective gut immunity.

1Institute of Immunobiology, Kantonsspital St. Gallen, St. Gallen, Switzerland. 2Leibniz Institute of Immunotherapy (LIT), Chair for Immunology, University of Regensburg, Regensburg, Germany. 3These authors contributed equally: Hung-Wei Cheng, Urs Mörbe. *Email: burkhard.ludewig@kssg.ch
The intestinal immune system harbors diverse immune cell populations that are instructed within discrete lymphoid structures to contain the microbiome and to regulate immune responses against food antigens, while staying alert and functional in response to the invasion by pathogens. Intraepithelial lymphoid cells (ILCs) operate independently of specific antigen receptor signaling and are particularly abundant in the intestinal lamina propria. ILCs can be grouped into functionally distinct subsets including natural killer cells with cytotoxic potential, ILC1; natural killer-like cells with cytotoxic potential, ILC2; and ILC3, which produce anti-bacterial cytokines IL17 and IL22. ILCs are generated from a common progenitor (ILCp) that is present in the fetal liver during embryogenesis or in the bone marrow of adult individuals. Fetal ILCp seed tissues, expand and locally differentiate concomitantly with the microbial colonization of the host. Adult ILCp in humans have been shown to migrate to different organs and contribute to local, tissue-resident ILC populations. Pathobiosis and cell fate-mapping experiments in mice have confirmed replenishment of ILCs from circulating ILCp or migratory mature ILCs and revealed increased ILCp and ILC recruitment during inflammation. Regardless of their origin, i.e., coming from tissue-resident or recently immigrated ILCp, the ILC populations acquire tissue-specific molecular traits that are imprinted by the local microenvironment. It is therefore important to characterize the key niche-forming cells that support ILC maintenance and functionality.

Results

Molecular characterization of SILT-underpinning FRCs. To define potential fibroblastic ILC niches, we performed a high-resolution single-cell RNA-sequencing (scRNA-seq) analysis of the intestinal immune system in uniform manifold approximation and projection (UMAP) to identify potential niches. At the chosen clustering resolution, the analysis revealed several distinct ILC populations expressing Cd34 and Pdgfra that can be separated by the expression of Cd81 and correspond to the previously identified Pdgfra+ fibroblast and Cd81+ trophocyte populations, respectively. The Pdgfra+ Cd81+ cluster with tenascin C (Tnc) expression represents telocytes that are situated mainly at the tip of the villi and epithelial niche factors such as R-Spondins and Wnt (Wnt2b and Wnt5a) were enriched in the Cd81+ trophocytes and the Pdgfra+ cells (Supplementary Fig. 1a, left panel). Two Acta2hi Cd34+ cell clusters could be detected that differed in Ncam1 expression (Fig. 1a). The expression of perivascular cell markers (Pdgfb, Rgs5, and Esam) (Supplementary Fig. 1a, middle panel) and co-staining for ACTA2 and NCAM1 in confocal microscopy analysis identified Acta2hi Ncam1+ cells as mural cells situated around blood vessels (Supplementary Fig. 1c). High expression of molecules mediating cell contractility (Tgln, Actg2, and Myh11) marked Ncam1+ muscularis mucosae myocytes (Supplementary Fig. 1a, middle panel) and document the molecular ILC-FRC interactome that is required for the establishment of intestinal immunity.

Fibroblastic reticular cells (FRCs) are the key cells that determine SLO compartmentalization and generate specialized niches for immune cell interactions. Here, we provide a detailed molecular characterization of SILT FRCs and elaborate on the mechanisms underlying ILC homeostasis and function. Single-cell transcriptomic analysis of intestinal fibroblasts identifies Ccl19expressing cells as SILT FRCs. Genetic ablation of lymphoxygenase-1 receptor (Ltbr) expression in Ccl19-Cre+ cells shows a crucial function of SILT FRCs for the transition of CPs to mILFs. The continued activation of SILT FRCs is required for the maintenance of effective ILC pools in the lamina propria and for protection against Citrobacter rodentium infection. These results further characterize the nature of highly specialized SILT FRCs and document the molecular ILC-FRC interactome that is required for the establishment of intestinal immunity.
to distinguish the different lamina propria fibroblast populations (Supplementary Fig. 2c). Consistent with the small fraction of SILT FRCs in the lamina propria fibroblast landscape, we found less than 2% EYFP+ cells in the lineage-negative lamina propria cells of Ccl19-EYFP mice (Fig. 1g). As expected, EYFP-positive cells were almost exclusively confined to the CLU+ FRC population (Fig. 1h and Supplementary Fig. 2d). Taken together, these results reveal the molecular identity of SILT FRCs within the heterogeneous intestinal fibroblast landscape (Fig. 1i).

FRCs control SILT remodeling and maturation. Signaling via the LTβR represents the first key signal for FRC differentiation and activation in lymph nodes30, the splenic white pulp32 and in Peyer’s patches35. To assess to what extent LTβR-mediated
activation of FRCs affects SILT formation, we crossed Ccl19-EYFP mice to Ltbβfl/fl mice (Ccl19-EYFP Ltbβfl/fl). Whereas CPs and imILFs were still formed in the absence of LTβR signaling in SILT FRCs, such as Mfge8-expressing FRCs, such as Fig. 3a). The expression of other genes that are expressed by SLO cells from the small intestine of Ccl19-EYFP and Ccl19-EYFP littermates resulted in significant reduction in cell numbers in the lamina propria under conditions of Ltbβ-deficiency in Ccl19-Cre+ cells (Fig. 3c) and Supplementary Fig. 5b). Assessment of ILC proliferation and cell death markers revealed that Ltbβ-deficiency in SILT FRCs mainly affected the survival of ILC3 as shown by the significantly elevated expression of active caspase 3/7 in the GATA3+ RORC+ subset (Supplementary Fig. 5c, d). These data indicate that FRC-mediated SILT maturation is crucial for the maintenance of ILC pools in the intestinal lamina propria.

Next, we assessed the functional consequences of Ltbβ-deficiency in SILT FRCs and found significantly reduced numbers of IL17- or IL22-secreting ILC3 in the lamina propria of Ccl19-Cre Ltbβfl/fl mice in comparison to co-housed Cre-negative littermates (Fig. 3d and Supplementary Fig. 5e). Since LTβ-signaling modulates the IL22 production by ILCs35 and IL22 induces the production of antimicrobial peptides in intestinal epithelial cells36,37, we examined the expression of genes encoding for antimicrobial peptides. We found significantly reduced expression of RegIIIγ, S100a8, and S100a9 in the small intestine of Ccl19-Cre Ltbβfl/fl mice (Fig. 3e). Mice with Ltbβ-deficient SILT FRCs showed reduced numbers of functional ILC3 and diminished expression of antimicrobial peptides that most likely led to the increased susceptibility to infection with the bacterial pathogen C. rodentium (Fig. 3f). Impaired pathogen control in Ccl19-Cre Ltbβfl/fl mice resulted in significantly reduced weight gain (Fig. 3g), reduced colon length (Fig. 3h), and increased inflammation (Fig. 3i) when compared to co-housed Cre-negative littermates. These data support the notion that SILT FRCs generate microenvironmental niches that are crucial for the maintenance of functional ILC populations to secure efficient control of intestinal pathogens.

Persistent SILT FRC-ILC crosstalk sustains intestinal immunity. To assess whether sustained communication between FRCs and ILCs is required for the maintenance of mature SILT structures and resistance to pathogen challenge, we utilized the Ccl19-iTA inducible mouse model32 crossed with R26R-EYFP and Ltbβfl/fl mice (abbreviated as Ccl19-iEYFP Ltbβfl/fl) (Supplementary Fig. 6a). Due to the expression of the tetracycline transactivator in Ccl19-expressing cells, Cre recombinase activity can be regulated through the provision of doxycycline35. Here, we used the non-antibiotic doxycycline derivative 4-epidoxycycline (abbreviated as Dox)38 to concomitantly regulate the expression of the EYFP marker and ablation of Ltbβ expression by first keeping pregnant dams and the offspring on Dox until the age of 8 weeks and subsequent withdrawal for 2 or 8 weeks (Fig. 4a). In the absence of Dox treatment, the Ccl19-iEYFP model faithfully recapitulated the phenotype of the Ccl19-EYFP mouse model with genetic targeting of FRCs underpinning all SILT stages.
(Supplementary Fig. 6b). As expected, the formation of SILTs was impaired in Ccl19-iEYFP Ltbr<sup>fl/fl</sup> mice in the absence of Dox treatment, i.e., with active Cre recombinase expression (Fig. 4b, no Dox). Consistent with data from the Ccl19-Cre model<sup>15</sup>, continued ablation of the Ltbr gene in Ccl19-iEYFP Ltbr<sup>fl/fl</sup> mice (no Dox) led to reduced Peyer’s patch formation (Supplementary Fig. 6c). Dox treatment of pregnant dams and postnatal provision of Dox via the drinking water (Dox on) prevented Cre-mediated Ltbr-deletion and EYFP-expression and hence allowed for SILT maturation and Peyer’s patch formation (Fig. 4b and Supplementary Fig. 6c, d). At the age of 8 weeks, all ILC subsets were reduced in untreated Ccl19-iEYFP Ltbr<sup>fl/fl</sup> mice compared to Ltbr-competent Ccl19-iEYFP mice (Supplementary Fig. 6e). Blocking of Cre-mediated Ltbr-ablation through Dox treatment restored ILC homeostasis in Ccl19-iEYFP Ltbr<sup>fl/fl</sup> mice (Supplementary Fig. 6e). To reactivate Cre-mediated recombination in Dox-treated Ccl19-iEYFPLtbr<sup>fl/fl</sup> mice, we ceased the provision of Dox via the drinking water at the age of 8 weeks (Fig. 4a). After Dox removal for 2 weeks (Dox off 2 wk), Cre-mediated recombination had started and first EYFP<sup>+</sup> cells
could be detected in the SILTs (Fig. 4b). While mILF could still be detected after 2 weeks of Dox removal, mILCs were not detectable after prolonged cessation of Dox exposure (Dox off 8 wk) in Ccl19-iEYFP $\text{Ltbr}^{fl/fl}$ mice when compared to co-housed littermate controls (Fig. 4b, c). Quantitative image analysis confirmed that the maturation of SILT structures required continued LTβR signaling in Ccl19-expressing FRCs (Fig. 4c). In 16-week-old Ccl19-iEYFP $\text{Ltbr}^{fl/fl}$ mice, Dox removal for 8 weeks led to a significant reduction of ILC3 and ILC1 numbers (Fig. 4d). Moreover, we observed significant weight loss (Fig. 4f) and significantly reduced colon length (Fig. 4g) after C. rodentium infection under conditions of induced $\text{Ltbr}$-deficiency in adult SILT FRCs. These data indicate that SILT FRCs constantly interrogate their environment and receive crucial signals via the LTβR to nourish ILC niches and sustain SILT structures.

**Interacome analysis of FRC-ILC crosstalk.** To elaborate on the mechanisms underlying SILT FRC-mediated ILC sustenance, we analyzed the transcriptome of small intestinal CD127$^+$ ILCs from Ccl19-EYFP $\text{Ltbr}^{fl/fl}$ mice and co-housed littermate controls. The merged scRNA-seq datasets identified 8 main CD127$^+$ ILCs clusters that could be categorized based on particular gene expression pattern and known canonical markers as $\text{Ncr}^+$ $\text{Rorc}^+$ ILC3, $\text{Ccr6}^+$ $\text{Rorc}^+$ ILC3, $\text{Gata3}^+$ $\text{Ilr1l}^+$ ILC2, $\text{Gata3}^+$ $\text{Ilr1l}^+$ ILC2 and $\text{Tbx21}^+$ ILC1 (Fig. 5a and Supplementary Fig. 7a). In addition, we identified two clusters that co-expressed the ILC2 marker $\text{Gata3}$ and the ILC3 marker $\text{Rorc}$, and showed elevated expression of key transcription factors found in ILC progenitors (ILCp) such as $\text{Rorc}$ marker and known canonical markers as $\text{Nfia}$$^{40,41}$, $\text{Tcf7}$$^{42}$, and $\text{Tex}$$^{43}$ (Fig. 5a, b). These data suggest that cells in the ILCp cluster possess the potentials for mixed-lineage contribution$^{44}$. One of the two ILCp clusters expressed ILC2 activation markers such as $\text{Tbx21}$ and $\text{Klrg1}$ suggesting a poised ILC2 lineage differentiation$^{45}$. These results provide a mechanistic explanation for our observation that ILC sustaining mechanisms in SILT FRCs are disrupted upon $\text{Ltbr}$ deficiency, leading to reduced ILC numbers and compromised ILC functions.
comparable ILC subset composition (Fig. 5a), a result that confirmed our flow cytometry analysis shown in Fig. 3a.

To assess the full array of molecular interactions between SILT FRCs and ILCs, we used the CellPhone-DB algorithm. In this analysis, pairs of known interacting ligands and receptors are derived from public databases and cell types are analyzed for enriched receptor–ligand interactions based on the expression of a receptor by one cell type and a ligand by another cell type. Using this approach, we found that the number of detected ligand and receptor interactions between FRCs and all ILC subsets except for ILC2p were enriched under Ltbfr-proficient conditions (Fig. 5c) suggesting that distinct molecular circuits in SILT FRC niches drive the interaction with ILCs. The subsequent in silico interactome analysis indicated the major predicted SILT FRC niche factors (IL7, IL15, Dll1, KITL, chemokines (CXCL13 and CXCL12), and adhesion molecules (VCAM1) that direct interaction with the different ILC subsets (Fig. 5d) and Supplementary Fig. 7b). Moreover, the algorithm identified ILC-derived ligands such as lymphotixin-alpha (LTα) that contribute to the reciprocal crosstalk between the cell populations (Fig. 5d). In accordance with our experimental observations of IL7 downregulation in the absence of LTβR signaling (Fig. 2e, f), the predicted interactions in the IL7-IL7R axis were substantially reduced in some or all ILC subsets (Fig. 5d). Since IL7 availability was shown to be important for sustained ILC programming, we hypothesized that IL7 provided by SILT FRCs may act as one of the key niche factors for ILC homeostasis. We therefore crossed Ccl19-EYFP mice with Il7fl/− mice and found that conditional ablation of Il7 in FRC did not affect ILC maturation (Supplementary Fig. 7c), but significantly reduced accumulation of RORC+ ILCs in imILFs and mLFs (Fig. 5e and Supplementary Fig. 7c). Flow cytometric analysis of ILC populations revealed a substantial reduction in total ILC numbers and in all ILC subsets in the lamina propia of Ccl19-Cre Il7fl/− mice in comparison to co-housed Cre-negative littermate controls (Fig. 5f). Consistent with the data from mice with Ltbfr-deficient SILT FRC niches, the overall ILC composition remained comparable under Il7-proficient and -deficient conditions (Supplementary Fig. 7d). However, conditional ablation of Il7 gene in SILT FRCs did not affect T cell subset composition in the lamina propria of Ccl19-Cre Il7fl/− mice when compared to co-housed littermate controls (Supplementary Fig. 7e–g). The infection of Ccl19-Cre Il7fl/− mice with C. rodentium further showed shortened colon length (Supplementary Fig. 7h) and increased bacterial concentration in the faeces and colon (Supplementary Fig. 7i). Taken together, the FRC-ILC interactome analysis unveiled critical niche factors and molecular

---

**Fig. 4 SILT maturation and ILC sustenance in the absence of persistent LTβR signaling in FRCs.**

- **a** Schematic timeline of non-antibiotic Dox treatment regime. Biological images of SILT structures in different conditions analyzed by confocal microscopy after staining with the indicated antibodies.
- **b** Representative images of SILT structures in different conditions analyzed by confocal microscopy after staining with the indicated antibodies.
- **c** Number and size of SILT structures detected in the small intestine from Ccl19-iEYFP Ltbfr/− mice and co-housed littermate controls after Dox withdrawal for 8 weeks. **d** Cell number of ILC subsets from Ccl19-iEYFP Ltbfr/− mice and co-housed littermate controls after Dox withdrawal for 8 weeks. **e** Bacterial concentration in faeces and colonic tissue on day 11 after C. rodentium infection of Ccl19-iEYFP Ltbfr/− mice and co-housed littermate controls after Dox withdrawal for 8 weeks. **f, g** Weight change and colon length on day 11 after C. rodentium infection in Ccl19-iEYFP Ltbfr/− mice and co-housed littermate controls after Dox withdrawal for 8 weeks. **b** Images are representative of at least four mice. **c** Number of ILC subsets (Fig. 5a), a result that confirmed our flow cytometry analysis shown in Fig. 3a. **d** Statistical analyses were performed using non-parametric two-tailed Mann–Whitney test (c, e–g) and unpaired two-tailed Student’s t test (d).
interaction partners that support ILC sustenance and function in SILTs.

Discussion

The intestinal immune system has to react to the constantly evolving microbiome with varying composition of commensals, changes in pathobiont communities, and occasional exposure to pathogens. While the number and location of Peyer’s patches in the small intestine are programmed during embryogenesis, the postnatal development of SILTs provides the flexibility to establish additional lymphoid organs on demand. The data presented here show that number, size, structure, and function of the inducible lymphoid organs in the small intestine depend on the interaction between Ccl19-expressing SILT FRCs and ILCs. The combination of inducible gene ablation and transcriptomics-based interactome analysis revealed the tonic nature of the SILT FRC-ILC crosstalk. SILT FRCs integrate signals from ILCs to generate and maintain the niche environment required for ILC sustenance. Disruption of the core
signaling pathways underlying FRC-ILC interaction led to the dismantling of SILT structures and impaired ILC-mediated immune protection. Thus, a small (<2%) fraction of highly specialized fibroblasts in the intestinal lamina propria controls ILC homeostasis and function.

Signaling via the LTβR is the key process underlying the initiation of SILT formation during embryogenesis and post-natal generation of SILTs. However, a large array of immune cells and non-hematopoietic cells exhibit Ltb expression and recent studies have shown that LTβR-positive stromal cell populations contribute to different degrees to lymphoid organ development. Our findings support the notion that SILT formation and maturation is regulated to a large extent by LTβR-signaling in Ccl19-Cre- FRCs and their progenitors. This scenario is highly likely because LTβR-signaling in neither intestinal epithelial cells nor dendritic cells contributes to SILT formation. Thus, SILT development and the steering of immune cell interactions in SILTs are controlled by a sequence of events that starts with an enabling “signal 1” in Ccl19-Cre-expressing FRCs, i.e., LTβR-signaling, and a series of further molecular FRC-ILC interactions that facilitate the full functionality of these lymphoid tissues.

A recent scRNA-seq-based analysis of fibroblastic stromal cells from patients with inflammatory bowel disease has revealed the presence of a CCL19-expressing cell population in the inflamed tissues. Cells in this particular cluster express the SILT FRC signature genes CCL21 and CCL5. It is still unclear whether CCL19-expressing cells in the human lamina propria are located exclusively in the SILTs as demonstrated here for the mouse small intestine. Nevertheless, the increase of organized lymphoid structures in numbers and size in Crohn’s disease patients suggests that chronic intestinal inflammation is associated with SILT formation in humans. Furthermore, aberrant ILC composition and increased IL23-mediated IL17A production by ILCs were shown to be associated with Crohn’s disease supporting the notion that ILC activity in the human intestine might be controlled in organized lymphoid structures. It is noteworthy that Ccl19-Cre- FRCs do not only underpin the fibroblastic infrastructure of SLOs but participate in the formation of inducible tertiary lymphoid tissues such as bronchus-associated lymphoid tissues in the lung during chronic immune activation. It will be important to elaborate in future studies on whether and to what extent the excessive formation of SILTs and/or tertiary lymphoid tissues in the intestinal lamina propria can be modulated to attenuate inflammatory reactions in human disease. Clearly, the data presented here show that an impairment of the ILC niche formed by SILT FRCs bears the danger of reducing the ability of the intestinal immune system to cope with pathogens. The bone marrow niche concept delineates the micro-environmental conditions that facilitate the maintenance and differentiation of hematopoietic stem cells. Recent advancements of the concept with consideration of peripheral immune processes highlight the general relevance of niche conditions for immune cells such as macrophages and ILCs: provision of scaffold, supply of trophic factors, induction of tissue imprinting, and reciprocal benefits during cell-cell interactions. SILT FRCs fulfill all criteria of a critical niche-forming cell. The dense cellular FRC network generates a distinct space for immune cell interactions and provides ample surface for direct FRC-ILC communication. The interactome analysis based on single-cell transcriptomics data from both FRCs and ILCs revealed the major pathways involved in the communication between the two cell populations. LTβR-dependent ILC stimulation by FRCs includes the production of IL7 to sustain the size of the ILC pools. In addition, the interactome analysis suggests that several LTβR-independent reciprocal interactions are operational, e.g., IL33-IL1RL1, KIT- and CXCL16-CXCR6, to mold the niche microenvironment. It remains to be determined which cell type provides the stimuli that initiate FRC-ILC interaction in CPs prior to LTβR-dependent cascade. It is possible that glial cells, which protrude into the SILT structures and can be found in close contact with ILCs, generate neurotrophic factors that contribute to these processes. Further elaboration of the initiation of FRC-ILC interaction in SILTs and the means of their reciprocal interaction will be important to better understand the basis of intestinal immunity.

In summary, our study disentangles core processes that highlight immune cell interactions in the intestinal lamina propria and control of pathogens. A distinct fraction of highly specialized fibroblasts appears to be at the nexus of immune cell interactions in the lamina propria and thereby controls the balance between immunity and immunopathology. Hence, the identification of druggable targets in SILT FRCs can guide the development of therapeutic approaches to attenuate excessive inflammatory processes in the gut.

Methods
Mice. All mouse strains were on a C57BL/6Ncrl genetic background and maintained in individually ventilated cages under specific pathogen-free conditions. C57BL/6N-Tg[Ccl19-Cre]489Bat (Ccl19-Cre), C57BL/6N-Tg[Ccl19-TA]888BAT (Ccl19-TA), and Ltb1milk1The (LTβRfl/fl) strains were described previously. C57BL/6N-(IL7Rβflox/flox) (IL7Rβflox/flox) are kindly provided by Koichi Ikuta (Kyoto University, Japan). B6.129 × 1-Gt(Rosa)26Sortm1(EYFP)Crg (R26R-EYFP) mice were purchased from The Jackson Laboratories and the LC1 strain was kindly provided by Dr. Fendler (Max Delbrück in Center of Medicine, Berlin, Germany). Non-antibiotic 4-Epoxydioxycycline (Dox) was kindly provided by Dr. Rodewald at the University of Leipzig, Germany. 4-Epoxydioxycycline was administered to pregnant dams in the drinking water (50 μg/ml) and maintained after weaning. All mice were housed in the Institute of Immunobiology, Kantonsspital St. Gallen under specific pathogen-free conditions at 22 ± 2°C and 50 ± 5% humidity in a 12:12 h light/dark cycle and provided ad libitum access to food and water. Experiments were performed in accordance with Swiss federal and cantonal guidelines (Tierschutzgesetz) under the permission numbers SG01/18, SG08/18, SG04/20, and SG24/20 granted by the Veterinary Office of the Canton of St. Gallen.

Antibodies. Anti-Mouse CD3ε Biotin (Clone:145-2C11, 100303, 1:100), Anti-Mouse GR1 Biotin (Clone:RB6-8C5, 108403, 1:100), Anti-Mouse CD19 Biotin (Clone:6D5, 115903, 1:100), Anti-Mouse Ter119 Biotin (Clone:Ter119, 116203, 1:100), Anti-Mouse CD5 Biotin (Clone:5.3-7.3, 100603, 1:100), Anti-Mouse CD127/IL7R BV711 (Clone:ATR734, 135035, 1:50), Anti-Mouse CD45.2 BV605 (Clone:104, 109941, 1:100), Anti-Mouse CD335/Nkpe BV421 (Clone:29A1.4, 137612, 1:100), Anti-Mouse T-bet PE-Cy7 (Clone:4B10, 644823, 1:100), Anti-Mouse CD8 BV717 (Clone:6B6, 107074, 1:100), Anti-Mouse CD4 BV421 (Clone:104, 109831, 1:100), Anti-Mouse CD54.2 BV421 (Clone:104, 109831, 1:100), Anti-Mouse CD3ε PerCP (Clone:145-2C11, 100325, 1:100), Anti-Mouse/Human GL-7 AlexaFluor488 (Clone:GL-7, 144611, 1:100), Anti-Mouse CD11c APC-Cy7 (Clone:N418, 117523, 1:100), Anti-Mouse 1-A/E-4 BV421 (Clone:MS5/114.15.2, 107631, 1:100), Anti-Mouse/Human CD11b AlexaFluor488 (Clone:M1/70, 102199, 1:100), Anti-Mouse ly6G PE (Clone:1A8, 127607, 1:100), Anti-Mouse F4/80 PE-Cy7 (Clone:BM8, 123113, 1:100), Anti-Mouse/Human K67 AlexaFluor488 (Clone:11F6, 115120, 1:100), Anti-Mouse IL7Rα Biotin (Clone:1C8, 115120, 1:100), Anti-Mouse IL7A-7 AlexaFluor488 (Clone:TC11-18H10.1, 506990, 1:100), Anti-Mouse CD81 PerCP-Cy5.5 (Clone:Eat-2, 104991, 1:100), Anti-Mouse CD3ε PerCP-Cy7 (Clone:12H7, 12000, 1:100), Anti-Mouse CD90.2 BV785 (Clone:30-H12, 105331, 1:100), Anti-Mouse TER-119/Erythroid Cells BV605 (Clone:TER-119, 116239, 1:100), Anti-Mouse CD34 BV421 (Clone:MEC147, 119321, 1:100), Anti-Mouse CD31 PE-Cy7 (Clone:390, 104218, 1:100), Anti-Mouse CD326 (EpCAM) PE-Cy7 (Clone:G9.8, 118216, 1:100), Anti-Mouse/Human CD45R0/B220 AlexaFluor488 (Clone:RA3-682, 102356, 1:100), Syrian Hamster Anti-Mouse Podoplanin (Clone:8.1.1, 127401, 1:100), Anti-Mouse CD3ε AlexaFluor488 (Clone:MEC133, 102515, 1:100), Biotin Anti-Mouse CD90.2 (Thy1.2) (Clone:53-2.1, 140314, 1:500), Anti-Mouse TCR γδ APC (Clone:GL3, 118115, 1:100) were purchased from The Jackson Laboratories and the LC1 strain was kindly provided by Dr. Fendler (Max Delbrück in Center of Medicine, Berlin, Germany). Anti-Mouse/Rodent IFNγ PE-Cy7 (Clone:AFKKS-9, 14-6988-82, 1:50), Anti-Mouse EOMES AlexaFluor488 (Clone:Dan11mag, 12-9966-42, 1:100).
For lymphocytes staining, cell suspensions were incubated with fluorochrome-conjugated antibodies against CD45.2, CD3, CD4, CD8, B220, and Gr-1. This cell fraction was further treated with FoxP3/transcription factor staining buffer and subsequently stained for IgA or FoxP3. For Th17 staining, cell suspensions were stimulated with PMA/ionomycin (Sigma-Aldrich, P8139/I0634) and 10 µg/ml Brefeldin A in the RPMI medium with 1 mM HEPES, 1% sodium pyruvate, 10% FCS and 1% NEAA for 4 h at 37 °C. After stimulation, the cell fraction was centrifuged and was first stained with antibodies against CD127, CD45.2, Nk4p6, CCR6 together with FoxP3, anti-CD19 and anti-CD122. Cells were washed either with MACS buffer or with permeabilization buffer, acquired with a BD LSR Fortessa (BD Biosciences) in software FACSDiva (BD Biosciences), and analyzed using FlowJo software v10.1r5 (Tree Star Inc.).

**Histology.** Organs were fixed overnight with 4% paraformaldehyde (Merck-Milipore, 100050) in PBS under agitation at 4 °C. Fixed samples were further washed with PBS containing 1% Triton-X100 (Sigma-Aldrich, S7877) and 2% FCS (Sigma-Aldrich) overnight at 4 °C. Samples were then embedded in paraffin, cut into 5 µm sections, and stained with hematoxylin and eosin (H&E, Leica VT1200). Sections were then placed in 1% Triton-X100 (Sigma-Aldrich), 10% fetal calf serum (Sigma-Aldrich) and 1 mM anti-Fcy receptor (BD Biosciences, AB_394656) at 4 °C for 2 h and further incubated with antibodies: with or without permeabilization (saponin). Unbound single-cell suspensions were stained with MACS anti-CD45 (Miltenyi Biotec, 130-052-301) and anti-Ter119 microbeads followed by rat anti-RORC (eBioscience) antibody, anti-ACTA2 (Sigma-Aldrich), anti-EYP (Clontech), anti-Clusterin (R&D), anti-NCAM1 (R&D) and anti-CCCL3 (R&D) and visualized with anti-FITC, Alexa488 and Cy3. For anti-FITC and anti-ACTA2 staining, cells were detected with the following secondary antibodies: Alexa488-conjugated anti-rabbit-IgG (71-546-1500), Alexa647-conjugated anti-goat-IgG (11-547-152), Alexa488-conjugated anti-rabbit-IgG (71-546-1500), Alexa647-conjugated anti-goat-IgG (11-547-152) and Alexa488-conjugated anti-rabbit-IgG (71-546-1500), respectively. For anti-EYP stained cells, samples were incubated with Cy3-conjugated streptavidin and anti-B220 (eBioscience) for 2 h. Microscopy analysis was performed using a confocal microscope (Zeiss LSM-710) and images were processed with ZEN 2010 software (Carl Zeiss Inc.).

**Quantification of CP and ILF structures.** Samples from the distal ileum were fixed, cut, and stained as described above. For the quantification of CPs and ILFs, the DAPI area of three to six sections per mouse was recorded. CPs were defined as clusters of PDP+ fibroblastic stromal cells and RORC+ cells, containing less than 10 B220+ B cells. ImILFs were defined as clusters of RORC+ ILCs with more than 10 B cells. mILFs were defined as cell accumulations with a large central B-cell cluster and a corona of RORC+ ILCs. The sum of counted CPs and ILFs was normalized to an area of 1 cm² DAPI area. The area covered by CPs and ILFs was calculated on the basis of RORC+ cell signal within CPs and ILFs using the ZEN 2010 software (Carl Zeiss Inc.). Quantification of RORC+ ILC3 numbers was evaluated through the colocalization of DAPI and RORC signals within defined SILT structures reconstructed in 3D in Imaris (Bitplane) using an automatic threshold.

**Infection of Citrobacter rodentium.** *C. rodentium* cultures (strain ICC169, kindly provided by K. Maloy, Oxford, UK) were grown from a single colony overnight in LB broth supplemented with 50 µg/ml nalidixic acid (Sigma-Aldrich). Mice were fed ~5×10⁹ bacteria by oral gavage in 200 μl PBS. For the analysis of bacterial burdens, mice were sacrificed and samples were taken 11 days post infection from cecum, colon, and feces. Colon length was measured and the collected samples were mechanically homogenized and cultured overnight on LB agar plates supplemented with 50 µg/ml nalidixic acid. Samples from the colon were fixed in 4% formalin and embedded in paraffin. Samples were cut into 1.5-µm sections and stained with hematoxylin and eosin. To determine the histopathological score, the following parameters were considered and graded by two blinded examiners on a scale from 0 (none) to 3 (severe) for a maximal histology score of 12: (1) cellular infiltration, (2) erosion, (3) crypt architectural distortion, and (4) edema.

**Quantitative real-time PCR.** Total cellular RNA was extracted from the sorted cells using Quick-RNA microprep kit (Zymo Research, R1050) or from tissue using Direct-zol RNA Miniprep kit (Zymo Research, R2051) following the commercial protocol. cDNA was prepared using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4368814) and quantitative PCR was performed using PowerSYBR Green master mix (Applied Biosystems, A25741) on a QuantStudio 5 machine (Applied Biosystems) and analyzed with the QuantStudioTM Design & Analysis Software (Applied Biosystems). Expression levels were measured by using
the following primers: IL7 (QT01013180, Qiagen), Cd19 (QTO02532173, Qiagen), Cua (QTO0164714, Qiagen), IgE3 (QTO015175, Qiagen). RegHilg (fw: AGGCCCTTATTGATTCCG, rv: GCCGCCCTTATTGATTCCG) was one of the used primers.

**References**

1. Mowat, R. M. & Agace, W. W. Regional specialization within the intestinal immune system. *Nat. Rev. Immunol.* 14, 667–685 (2014).

2. Diefenbach, A., Gnafakis, S. & Shimrat, O. Invasive lymphoid cell epithelial cell modules sustain intestinal homeostasis. *Immunity* 52, 452–463 (2020).

3. Bal, S. M., Golebski, K. & Spits, H. Plasticity of innate lymphocyte cell subsets. *Nat. Rev. Immunol.* 20, 552–565 (2020).

4. Yehezkel, E. et al. Innate lymphocyte cell 10 years on. *Cell* 174, 1054–1066 (2018).

5. Artis, D. & Spits, H. The biology of innate lymphoid cells. *Nature* 517, 293–301 (2015).

6. Serafini, N., Vossenranch, C. A. & Di Santo, J. P. Transcriptional regulation of innate lymphoid cell fate. *Nat. Rev. Immunol.* 15, 415–428 (2015).

7. Schneider, C. et al. Tissue-resident group 2 innate lymphoid cells differentiate from layered ontogeny of cell line marking in the skin in perinatal priming. *Immunity* 50, 1425–1438.e1425 (2019).

8. Bando, J. K., Liang, H. E. & Locksley, R. M. Identification and distribution of developing innate lymphoid cells in the fetal mouse intestine. *Nat. Immunol.* 16, 153–160 (2015).

9. Satoh-Takayama, N. et al. Microbial flora drives inter leukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* 29, 958–970 (2008).

10. Sano, S. L. et al. ROIRgammat and commensal microflora are required for the differentiation of mucosal inter leukin 22-producing NKp46+ cells. *Nat. Immunol.* 10, 83–91 (2009).

11. Lim, A. I. et al. Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell* 168, 1086–1100.e1010 (2017).

12. Moro, K. et al. Interferon-γ and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat. Immunol.* 17, 76–86 (2016).

13. Gasteiger, G., Fan, X., Dikty, S., Lee, S. Y. & Rudensky, A. Y. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science* 350, 981–985 (2015).

14. Huang, Y. et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science* 359, 114–119 (2018).

15. Ricardo-Gonzalez, R. R. et al. Tissue signals imprint ILC2 identity with anti-hepatitis potential. *Nat. Immunol.* 19, 1093–1099 (2018).

16. Lehmann, F. M. et al. Microbiota-induced tissue signals regulate ILC3-mediated antigen presentation. *Nat. Commun.* 11, 1794 (2020).

17. Yudanin, N. A. et al. Spatial and temporal mapping of human innate lymphoid cells reveals elements of tissue specificity. *Immunity* 50, 505–519.e504 (2019).

18. Eberl, G. Inducible lymphoid tissues in the adult gut: recapitulation of a fetal developmental pathway? *Nat. Rev. Immunol.* 5, 413–420 (2005).

19. van de Pavert, S. A. & Mebius, R. E. New insights into the development of lymphoid tissues. *Nat. Rev. Immunol.* 10, 664–674 (2010).

20. Kurebayashi, S. et al. Retinoic-related orphan receptor gamma (ROIRgammat) is essential for lymphoid organogenesis and controls apoptosis during lymphopoiesis. *Proc. Natl Acad. Sci. USA* 97, 10132–10137 (2000).

21. Eberl, G. et al. An essential function for the nuclear receptor ROIRgammat in the generation of fetal lymphoid tissue inducer cells. *Nat. Immunol.* 5, 64–73 (2004).

22. Randall, T. D. & Mebius, R. E. The development and function of mucosal lymphoid tissues: a balancing act with micro-organisms. *Mucosal Immunol.* 7, 455–466 (2014).

23. Lee, J. S. et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid development via pathways dependent on and independent of Notch. *Nat. Immunol.* 13, 144–151 (2011).

24. Qiu, J. et al. Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. *Immunity* 39, 386–399 (2013).

25. Taylor, R. T. et al. Lympho Poetic-dependent expression of TNF-receptor activation-induced cytokine by stromal cells in cryptopatches, isolated lymphoid follicles, and Peyer’s patches. *J. Immunol.* 178, 5659–5667 (2007).

26. Kiss, E. et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science* 334, 1561–1565 (2011).

27. Boukra, D. et al. Lymphoid tissue genesis is induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* 456, 507–510 (2018).

28. Krishnamurty, A. T. & Turley, S. J. Lymph node stromal cells: cartographers of the immune system. *Nat. Immunol.* 21, 569–580 (2020).

29. Pikor, N. B., Cheng, H. W., Onder, L. & Ludewig, B. Development and immunological function of lymph node stromal Cells. *J. Immunol.* 206, 257–263 (2021).

30. Lütge, M., Pikor, N. & Ludewig, B. Diffentiation and activation of fibroblastic reticular cells. *Immunol. Rev.* 302, 32–46. https://doi.org/10.1111/imr.12981 (2021).

31. L. S. C. C. T. E. N. A. R. T. I. E. Articles

**Data availability**

The scRNA-seq data generated in this study have been deposited in the arrayexpress database (www.ebi.ac.uk/arrayexpress) under access code E-MTAB-10638 and E-MTAB-10645. Source Data are provided with this paper.

Received: 21 July 2021; Accepted: 21 March 2022; Published: 19 April 2022
32. Cheng, H. W. et al. Origin and differentiation trajectories of fibroblastic reticular cells in the splenic white pulp. Nat. Commun. 10, 1739 (2019).
33. Picelli, S. et al. Barcoding of light and dark zone follicular dendritic cells governs germinal center responses. Nat. Immunol. 21, 649–659 (2020).
34. Chai, Q. et al. Maturation of lymph node fibroblastic reticular cells from myofibroblastic precursors is critical for antiviral immunity. Immunity 38, 1013–1024 (2013).
35. Prasad, A. et al. Fibroblastic reticular cell lineage convergence in Peyer’s patches governs intestinal immunity. Nat. Commun. 22, 510–519 (2021).
36. Tumanov, A. V. et al. Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge. Cell Host Microbe 10, 44–53 (2011).
37. Sonnenberg, G. F., Monticelli, L. A., Elloso, M. M., Fouser, L. A. & Artis, D. CD45(+) lymphoid tissue-inducer cells promote innate immunity in the gut. Immunity 34, 122–134 (2011).
38. Eger, K. et al. 4-Epidoxycycline: an alternative to doxycycline to control gene expression in conditional mouse models. Biochem. Biophys. Res. Commun. 323, 979–886 (2004).
39. Gury-BenAri, M. et al. The spectrum and regulatory landscape of intestinal innate lymphoid cell subsets. J. Exp. Med. 211, 1723–1731 (2014).
40. Seillët, C. et al. IL-35 is required for the development of all innate lymphoid cell subsets. J. Exp. Med. 211, 1733–1740 (2014).
41. Harly, C. et al. The transcription factor TCF-1 enforces commitment to the innate lymphoid cell lineage. Nat. Immunol. 20, 1150–1160 (2019).
42. Seehus, C. R. et al. The development of innate lymphoid cells requires TOX-dependent generation of a common innate lymphoid cell progenitor. Nat. Immunol. 16, 599–608 (2015).
43. Wallrapp, A. et al. The neuropetide NMU amplifies ILC2-driven allergic lung inflammation. Nature 549, 351–356 (2017).
44. Yu, Y. et al. The transcription factor Bcl11b is specifically expressed in group 2 innate lymphoid cells and is essential for their development. J. Exp. Med. 212, 865–874 (2015).
45. Elfenbeinova, M., Vento-Tormo, M., Teichmann, S. A. & Vento-Tormo, R. CellPhoneDB: inferring cell-cell communication from combined expression of multi-subunit ligand-receptor complexes. Nat. Protoc. 15, 1484–1506 (2020).
46. Vonarbourg, C. et al. Regulated expression of nuclear receptor ROXyR confers distinct functional fates to NK cell receptor-expressing ROXyR(+) innate lymphocytes. Immunity 33, 736–751 (2010).
47. Satoh-Takayama, N. et al. IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKP46(+) cells from Id2-dependent precursors. J. Exp. Med. 207, 273–280 (2010).
48. Upadhyay, V. & Fu, Y. X. Lymphotixin signalling in immune homeostasis and disease. Nat. Rev. Immunol. 13, 270–279 (2013).
49. Onder, L. et al. Endothelial cell-specific lymphotixin-β receptor signaling is critical for lymph node and high endothelial venule formation. J. Exp. Med. 210, 465–473 (2013).
50. Onder, L. et al. Lymphatic endothelial cells control initiation of lymph node organogenesis. Immunity 47, 80–92.e184 (2017).
51. Maeho-T Fernandez, E. et al. Lymphotixin beta receptor signaling limits mucosal damage through driving IL-23 production by epithelial cells. Mucosal Immunol. 8, 403–413 (2015).
52. Guendel, F. et al. Group 3 innate lymphoid cells program a distinct subset of IL-22BP-producing dendritic cells demarcating solitary intestinal lymphoid tissues. Immunity 53, 1015–1032.e1018 (2020).
53. Kinchen, J. et al. Structural remodeling of the human colonic mesenchyme in inflammatory bowel disease. Cell 175, 372–386.e317 (2018).
54. Sura, R., Colombel, J. F. & Van Kruiningen, H. J. Lymphatics, tertiary lymphoid organs and the granulomas of Crohn’s disease: an immunohistochemical study. Aliment. Pharm. Ther. 33, 930–939 (2011).
55. Jegersen, P. B. et al. Identification, isolation and analysis of human gut-associated lymphoid tissues. Nat. Protoc. 16, 2051–2067 (2021).
56. Bernink, J. H. et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. Nat. Immunol. 14, 221–229 (2013).
57. Geremia, A. et al. IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. J. Exp. Med. 208, 1127–1133 (2011).
58. Perez-Shibayama, C. et al. Fibroblastic reticular cells initiate immune responses in visceral adipose tissues and secure peritoneal immunity. Sci. Immunol. 3, eaar4539 (2018).
59. Cupovic, J. et al. Adenovirus vector vaccination reprograms pulmonary fibroblastic niches to support protective infiltrating memory CD8+ T cells. Nat. Immunol. 22, 1042–1051 (2021).
60. Morrison, S. J. & Schedden, D. T. The bone marrow niche for haematopoietic stem cells. Nature 505, 327–334 (2014).
61. Guilliams, M., Thierry, G. R., Bonnardel, J. & Bajenoff, M. Establishment and maintenance of the macrophage niche. Immunity 52, 434–451 (2020).
62. Ibaæa, S. et al. Glial-cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. Nature 535, 440–443 (2016).
63. Wimmer, N. et al. Lymphotixin beta receptor activation on macrophages induces cross-tolerance to TLR4 and TLR9 ligands. J. Immunol. 188, 3426–3433 (2012).
64. Liang, B. et al. Role of hepatocyte-derived IL-7 in maintenance of intrathoracic NKT cells and T cells and development of B cells in fetal liver. J. Immunol. 189, 4444–4450 (2012).
65. Schonig, K., Schwenk, F., Rajewsky, K. & Bujard, H. Stringent doxycycline dependent control of CRE recombinase in vivo. Nucleic Acids Res. 30, e134 (2002).
66. Groman, K., Kofoed-Nielsen, M. & Diefenbach, A. Isolation and flow cytometry analysis of innate lymphoid cells from the intestinal lamina propria. Methods Mol. Biol. 1559, 255–265 (2017).
67. Maccosko, E. Z. et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell 161, 1202–1214 (2015).
68. Zheng, G. X. et al. Massively parallel digital transcriptional profiling of single cells. Nat. Commun. 8, 14049 (2017).
69. McCarthy, D. J., Campbell, K. R., Lun, A. T. & Willis, Q. F. Scater: pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R. Bioinformatics 33, 1179–1186 (2017).
70. Stuart, T. et al. Comprehensive integration of single-cell data. Cell 177, 1888–1902.e1821 (2019).

Acknowledgements
We thank Dr Stefan Freigang (Institute of Pathology, University of Bern, Switzerland) for providing CD1d/ePPB5–Tetramer. This study received financial support from the Swiss National Science Foundation (grants 182583 and 159188 to B.L., and 151370 to E.S.), the Deutsche Forschungsgemeinschaft (HE3116/9-1 to T.H. and B.L.). The funders had no role in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

Author contributions
B.L. designed the study, discussed data, and wrote the paper. H.-W.C. and U.M. performed experiments, analyzed data, and wrote the paper. L.O., M.N., C.E., C.G.-C., C.P.-S., M.L., and E.S. performed experiments, analyzed and discussed data. T.H. provided produced transgenic mice and discussed data.

Competing interests
B.L., L.O., H.-W.C., C.G.-C., and C.P.-S. are cofounders and shareholders of Stromal Therapeutics. H.-W.C. is a part-time employee of Stromal Therapeutics. The remaining authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-29734-2.

Correspondence and requests for materials should be addressed to Burkhard Ludewig.

Peer review information Nature Communications thanks Cecile Benzech and the other anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article that are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022