A committed precursor to innate lymphoid cells

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Innate lymphoid cells (ILCs) specialize in the rapid secretion of polarized sets of cytokines and chemokines to combat infection and promote tissue repair at mucosal barriers1–9. Their diversity and similarities with previously characterized natural killer (NK) cells and lymphoid tissue inducers (LTi) have prompted a provisional classification of all innate lymphocytes into groups 1, 2 and 3 solely on the basis of cytokine properties10, but their developmental pathways and lineage relationships remain elusive. Here we identify and characterize a novel subset of lymphoid precursors in mouse fetal liver and adult bone marrow that transitively express high amounts of PLZF, a transcription factor previously associated with NK T cell development11,12, by using lineage tracing and transfer studies. PLZFhigh cells were committed ILC progenitors with multiple ILC1, ILC2 and ILC3 potential at the clonal level. They excluded classical LTi and NK cells, but included a peculiar subset of NK1.1+/NKp46−/DX5− ‘NK-like’ cells residing in the liver. Deletion of PLZF markedly altered the development of several ILC subsets, but not LTi or NK cells. PLZFhigh precursors also expressed high amounts of ID2 and GATA3, as well as TOX, a known regulator of PLZF-independent NK and LTi lineages13. These findings establish novel lineage relationships between ILC, NK and LTi cells, and identify the common precursor to ILCs, termed ILCP. They also reveal the broad, defining role of PLZF in the differentiation of innate lymphocytes.

To study the expression pattern of Zbtb16 encoding the transcription factor PLZF, which directs the developmental acquisition of the innate effector program of NK T cells14,15, we inserted a sequence coding for a fusion of enhanced green fluorescent protein (GFP) and Cre downstream of an IRES after the last exon of Zbtb16 (Extended Data Fig. 1a). As expected, GFP was selectively expressed in the NK T lineage, with early developmental stages 1 and 2 showing higher levels than mature stage 3 cells, but was not found in the bone marrow common lymphoid precursor (CLP), T cells or B cells of PLZFGFPcre+−/− mice (Fig. 1a). In PLZFGFPcre−/− mice carrying the ROSA26-floxstop-yellow fluorescent protein (YFP) fate-mapping allele, nearly all NK T cells expressed YFP, as expected, although approximately 35% of cells in all lymphoid and myeloid lineages were also labelled (Extended Data Fig. 1b, c and data not shown). Because haematopoietic stem cells (HSC) did not express GFP but were already labelled by YFP, this 'background' reflected some expression of PLZF before the HSC stage, probably in multipotent embryonic cells. Indeed, after transfer of fluorescence-activated cell sorting (FACS)-sorted YFP-negative bone marrow cells into lethally irradiated recipients, 94% of NK T cells still expressed YFP, whereas donor-derived CLPs, B cells and T cells were unlabelled (Fig. 1a).

Thus, the experiments shown in Fig. 1 were conducted with such bone marrow chimaeras, although all results were confirmed in non-chimaeric mice. CLP and iILC2 from bone marrow (BM); B and T cells and NK cells from spleen; NK cells from thymus (GFP), divided into early stages 1, 2 and late stage 3, and from spleen (YFP), ILC2 from lung, DX5+ and DX5− NK cells from liver; ILC1 from intestinal intraepithelial lymphocytes (IEL); LTi and ILC3 from LPL. BM iILC2 and lung ILC2 were identified as Lin−CD3e−CD19−KLRG1+; LPL NCR1+ILC1 as CD3e−CD19−NKp46−NK1.1+; LPL CD4+ LTi cells as CD3e−CD19−IL-7Rα−KLRG1−CCR6+CD4−; LPL CD4+ LTi cells as CD3e−CD19−CCR6−CD4+; and IEL ILC1 as CD3e−CD19−NKp46−NK1.1−CD160+. FACS identification of the remaining subsets is defined in the methods section. Summary of results (mean ± s.e.m.). Data representative of 3–9 individual mice analysed in at least 2 independent experiments.

Figure 1 | ILC lineage tracing in PLZFGFPcre reporter mice. a–c, Top rows, expression of GFP by indicated cell-types of PLZFGFPcre+− mice (filled grey) and wild type (WT; open). Bottom rows, YFP expression in radiation chimaeras reconstituted with YFP−Lin−Sca−1−cKit− (LSK) bone marrow cells from PLZFGFPcre+−/− ROSA26-YFP mice. CLP and ILC2 from bone marrow (BM); B and T cells and NK cells from spleen; NK cells from thymus (GFP), divided into early stages 1, 2 and late stage 3, and from spleen (YFP), ILC2 from lung, DX5+ and DX5− NK cells from liver; ILC1 from intestinal intraepithelial lymphocytes (IEL); LTi and ILC3 from LPL. BM iILC2 and lung ILC2 were identified as Lin−CD25−IL-7Rα−Thy1.2+; LPL ILC2 as CD3+CD19−CD25−KLRG1+; LPL NCR1+ILC1 as CD3e−CD19−NKp46−NK1.1+; LPL CD4+ LTi cells as CD3e−CD19−IL-7Rα+KLRG1+CCR6+CD4−; LPL CD4+ LTi cells as CD3e−CD19−CCR6−CD4+; and IEL ILC1 as CD3e−CD19−NKp46−NK1.1−CD160+. FACS identification of the remaining subsets is defined in the methods section. d, Summary of results (mean ± s.e.m.). Data representative of 3–9 individual mice analysed in at least 2 independent experiments.

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whereas few splenic NK cells expressed YFP, intestinal intraepithelial NK-like cells (termed ILC1) were prominently labelled (Fig. 1b). In the liver the recently described non-recirculating DX5+CD49a+ subset of CD3ε+ NK1.1+ cells was heavily labelled, whereas classical recirculating DX5+CD49a+ NK cells were mostly negative. Different subsets of group 3 innate lymphocytes in the LP also showed markedly different patterns of tracing (Fig. 1c and Extended Data Fig. 2). CD4+ and CD4+ LTi were not labelled, whereas NCR+ILC3 prominently expressed YFP. In summary, PLZF lineage-tracing labelled not only ILC2 but also the subsets of group 1 and group 3 cells that are most clearly distinguishable from classical NK and LTi cells, respectively, and will hereafter be termed ILC1 and ILC3.

Searching for the PLZF-expressing precursor of ILCs, we identified a rare subset of PLZFnhigh cells in fetal liver and adult bone marrow. They showed a homogeneous lineageLN−IL-7Rα−cKit−α4β7high phenotype (Fig. 2a, b), similar to the CLP-derived subset previously suggested to contain precursors for LTi17–20. The PLZFnhigh population represented approximately 5% of Lin−IL-7Rα−cKit− cells and approximately 30% of the α4β7high fraction (Fig. 2b, c). It expressed Thy1 but lacked expression of markers associated with mature ILCs, NK or LTis such as CD4, CXCR6, CD25, IL-17RB, T1/ST2, Sca-1, CD122, NK1.1, CCR6 and NKp46 (Fig. 2c, d and data not shown). Interestingly, the PLZFnhigh cells included a fraction of CD62LhighICOSlow cells, probably representing the earliest developmental stage after PLZF expression, because PLZF characteristically induces the downregulation of CD62L and upregulation of ICOS15,21. Transcriptional analysis of purified PLZFnhigh cells compared with bone marrow CLP, ILC2 and NK progenitors (NKp), and with ILC3-enriched lamina propria lymphocytes (LPL), confirmed the very high amounts of Zbtb16 mRNA encoding PLZF, as well as of other key transcription factors such as Id2, Gata3 and Rorc, which are required for ILC2 development22–25 (Fig. 2e and Extended Data Fig. 3) and Tcf7, a target of Notch required for ILC2 and ILC326,27. Notably, they expressed very high amounts of Tox, which is required for the development of both NK and LTi cells13, and low but detectable amounts of Tbx21 and Rorc, the transcription factors associated with ILC1 and ILC3, respectively14,28. Single-cell analysis by intracellular flow cytometry was performed after MACS-depletion of Lin− cells in fetal liver and adult bone marrow, gating on Lin−IL-7Rα−α4β7high cells (Fig. 2f). PLZFnhigh cells, which represented up to 40% of this population, characteristically co-expressed high amounts of both GATA3 and TOX. Interestingly, a small fraction of these PLZFnhigh cells also expressed RORγt, although these were mostly found in the fetal liver and were rare in adult bone marrow. A distinct population consisting of RORγthigh PLZF− cells, which was more abundant in the fetal liver, co-expressed high levels of TOX but not GATA3, probably representing LTi precursors. Finally, a fraction of Lin−IL-7Rα−α4β7high cells lacked both PLZF and RORγt, but expressed TOX. These may be earlier undifferentiated precursors to the ILC and LTi lineages, and may also include precursors to classical NK cells.

To determine precursor-product relationships, we injected a 1:1 mixture of PLZFnhigh cells and CLP cells into Rag2−/−Il2rg−/− mice. The PLZFnhigh cells, which were purified from a ROSA-foxstop-tdTomato ×
Significantly more peripheral ILC2 were derived from the PLZF<sup>high</sup> background to further ascertain their history of PLZF expression based on tdTomato expression, gave rise to a diversity of innate lineages, with the notable exception of CD4<sup>+</sup> LT<sup>1i</sup> (Fig. 3a). Both progenitors gave rise to similar amounts of CD3ε<sup>−</sup> NK1.1<sup>−</sup> group 1 cells, but CLP mostly generated the classical DX5<sup>+</sup> CD49a<sup>−</sup> NK1.1<sup>−</sup> NK cells, whereas the PLZF<sup>high</sup> cells primarily generated a distinct DX5<sup>−</sup> CD49a<sup>−</sup> NK1.1<sup>−</sup> ILC1 fraction at 5–7 weeks post-injection (Fig. 3a, c).}

### Figure 3 | PLZF<sup>high</sup> cells are ILC progenitors

- **a-c**: CD45.2 Rag<sup>−/−</sup> Il2rg<sup>−/−</sup> mice were injected with equivalent numbers of tdTomato<sup>+</sup> PLZF<sup>high</sup> cells and CD45.1 CLP (800–1,200 of each) and the progeny of these populations analysed 5–7 weeks later by FACS, as indicated. Summary bar graph of mean percentages ± s.e.m. in c, with significant differences between PLZF<sup>high</sup>- (black bar) and CLP-derived (white bar) shown by * or **. LPL ROR<sup>γ</sup><sup>−</sup> NCR<sup>−</sup> cells were identified as CD3ε<sup>−</sup> CD19<sup>−</sup> ROR<sup>γ</sup><sup>+</sup> Nkp46<sup>−</sup> CD4<sup>−</sup>; LPL ROR<sup>γ</sup><sup>−</sup> NCR<sup>−</sup> cells as CD3ε<sup>−</sup> CD19<sup>−</sup> ROR<sup>γ</sup><sup>−</sup> Nkp46<sup>−</sup> CD4<sup>−</sup>; and LPL ROR<sup>γ</sup><sup>−</sup> CD4<sup>−</sup> cells as CD3ε<sup>−</sup> CD19<sup>−</sup> ROR<sup>γ</sup><sup>−</sup> CD4<sup>−</sup>. Remaining populations were gated as indicated in Fig. 1. Data representative of 6–9 chimaeras analysed in at least 2 independent experiments.

- **d**: FACS analysis of Lin<sup>−</sup> BM and cultured on OP9 or OP9-DL1 for 48 h. Data representative of 3 independent experiments.

- **e**: FACS analysis of sorted PLZF<sup>high</sup> BM cells cultured on OP9 for indicated periods. Data representative of at least 4 replicate cultures for each time point from 2 or more independent experiments.

- **f**: FACS analysis of PLZF<sup>high</sup> or CLP cells from adult BM or fetal liver cultured on OP9 for 4 days. Data representative of at least 4 replicate cultures from 2 or more independent experiments. g, FACS analysis of representative colonies originating from single fetal liver PLZF<sup>high</sup> cells that were sorted and cultured into 96-well plates containing irradiated OP9 stromal cells for 5–6 days. ILC1 were characterized as ICOS<sup>lo</sup>– ROR<sup>γ</sup>– NK1.1<sup>−</sup> populations, ILC2 as ICOS<sup>hi</sup>– ROR<sup>γ</sup>– NK1.1<sup>−</sup>, and ILC3 as ICOS<sup>hi</sup>– ROR<sup>γ</sup>– NK1.1<sup>−</sup>. The table summarizes the analysis of thirteen 96-well plates analysed in four independent experiments (three plates in experiments 1, 2 and 4; four plates in experiment 3), with the average colony size ± s.e.m. as indicated. In experiment 4, we mixed a 1:1 ratio of CD45.1.5/2 and CD45.2 PLZF<sup>Cre</sup><sup>/</sup><sup>−</sup>/<sup>+</sup> fetal liver cells before single-cell sorting. All 122 colonies were either CD45.1.5/2 (n = 59) or CD45.2 (n = 63), ruling out doublet contamination as an explanation for the presence of mixed ILC colonies (P < 0.01 Chi-square). The cloning efficiency was 40% on average, with all but 17 colonies (not shown in the table) unambiguously assigned to defined ILC lineages.

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subset in the liver (Fig. 3b, c). PLZF<sup>high</sup>-derived NK1.1<sup>+</sup>CD3ε<sup>–</sup>TCRβ<sup>–</sup> cells were observed in the spleen and lungs, and expressed significantly more NKp46 than CLP-derived NK cells (Extended Data Fig. 4). Unlike CLP, PLZF<sup>high</sup> cells did not generate B cells, consistent with their expression of Tcf7<sup>+</sup>, suggesting that they had already received a Notch signal in vivo. They also gave rise to far fewer T cells than CLP after transfer into Rag2<sup>–/–</sup>Il2rg<sup>–/–</sup> mice in vivo (Fig. 3a, c) or after coculture with fetal thymic stroma in vitro (Extended Data Fig. 5), indicating that PLZF<sup>high</sup> cells had also largely lost T cell potential. Thus, the PLZF<sup>high</sup> cell is an ILC precursor (ILC) heavily committed to innate lymphoid lineages, excluding classical NK and LTi cells.

When cultured on OP9 stromal cells, the GFP<sup>+</sup> fraction of Lin<sup>–</sup>IL-7Rα<sup>+</sup> cKit<sup>–</sup>/Zbtb16<sup>high</sup> cells generated a small fraction of GFP<sup>+</sup> cells within 2 days of culture, which was increased upon culture on OP9-DL1 cells (Fig. 3d), confirming the precursor–product relationship and its promotion by Notch signals. To study the short-term fate of ILCP in vitro, we sorted Lin<sup>–</sup>IL-7Rα<sup>+</sup>cKit<sup>–</sup>/Zbtb16<sup>high</sup> PLZF<sup>high</sup> cells from either adult bone marrow or fetal liver and cultured them on OP9 stromal cells, without Notch ligands, in the presence of non-polarizing IL-7 and stem-cell factor (SCF). By 24 h, a substantial fraction of the PLZF<sup>high</sup> population had already begun to downregulate GFP and to upregulate either ICOS or CD122 (Fig. 3e), and by 4 days, the cells had resolved into three separate populations of ILC1 (ICOS<sup>–</sup>IL-17RB<sup>–</sup>T1/ST2<sup>–</sup>CD25<sup>–</sup>RORγt<sup>+</sup>GATA3<sup>+</sup>Zbtb16<sup>+</sup>CD122<sup>–</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup>), ILC2 (ICOS<sup>high</sup>IL-17RB<sup>–</sup>T1/ST2<sup>–</sup>CD25<sup>–</sup>RORγt<sup>+</sup>GATA3<sup>+</sup>Zbtb16<sup>+</sup>CD122<sup>–</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup>), and ILC3 (ICOS<sup>±</sup>IL-17RB<sup>–</sup>T1/ST2<sup>–</sup>CD25<sup>–</sup>RORγt<sup>+</sup>GATA3<sup>+</sup>Zbtb16<sup>+</sup>CD122<sup>–</sup>NK1.1<sup>+</sup>NKp46<sup>low</sup>)(Fig. 3f and Extended Data Fig. 6). This transient expression of PLZF before ILC differentiation may explain why fate-mapping by the ROSA-YFP allele in Fig. 1 did not quite reach 100% for these populations as in NK T cells where fate-mapping may explain why fate-mapping by the ROSA-YFP allele in Fig. 1 did not quite reach 100% for these populations as in NK T cells where fate-mapping.

Notably, ILC3 cells consistently arose with higher frequency from fetal liver than from adult bone marrow cells (Fig. 3f). In single-cell cultures with OP9 stromal cells, fetal PLZF<sup>high</sup> cells generated mixtures of ILC lineages, including triple and double ILC1, ILC2 and ILC3, in 58 out of 500 wells (12%) as well as single ILC1, ILC2 or ILC3 populations in the remaining wells (Fig. 3g). Mixing CD45 allele-marked fetal liver cells before single-cell sorting confirmed that the mixed ILC colonies did not result from cell aggregates escaping exclusion during cell-sorting (Fig. 3g, experiment 4) and post-sorting microscopic assessment confirmed single-cell seeding in each of 96 wells. These results indicated that the PLZF<sup>high</sup> cells contained a common ILC progenitor characterized by multiple ILC lineage potential at the clonal level, and that subsequent commitment to individual ILC1, ILC2 or ILC3 lineages occurred rapidly during the PLZF<sup>high</sup> stage of ILC development. Wells containing mixtures of ILC lineages had more growth than those with single lineages, indicating that multipotent ILC progenitors retained greater proliferative capacity.

We then assessed the contribution of PLZF to ILC development in lethally irradiated recipients of a mixture of bone marrow from congenically labelled Zbtb16<sup>12/12</sup> and Zbtb16<sup>–/–</sup> littermate donors (Fig. 4a). The production of ILC2 was markedly decreased in the PLZF-deficient compartment of all tissues, demonstrating a cell-intrinsic requirement of PLZF. Furthermore, residual immature ILC2 in the bone marrow expressed significantly less ICOS and more CD62L in the absence of PLZF (Fig. 4b, c), a phenotype entirely consistent with that of PLZF-deficient NK T cells<sup>3,12,15</sup>, which accounts for altered recirculating properties and reduced frequencies in peripheral tissues. LTi cells were unaffected by the genetic deletion of PLZF, as expected (Fig. 4a). Other ILC3 cells, including NCR<sup>+</sup> ILC3, were unaffected, despite their expression of PLZF during development. However, liver ILC1 (DX5<sup>–</sup>CD49a<sup>+</sup>) were profoundly affected, similar to ILC2, whereas classical (DX5<sup>–</sup>CD49a<sup>–</sup>) NK cells were not (Fig. 4a).

Our studies have identified the elusive CLP-derived common progenitor to the ILC1, ILC2, and ILC3 lineages and demonstrated close but distinct lineage relationships with classical NK and LTi cells. We have shown that a committed PLZF<sup>high</sup> ILC precursor emerges from within the
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METHODS

Mice. C57BL/6j (stock no. 000664), B6.SL-Ptgprc Pecept-B(Boy) (CD45.1; stock no. 002104), B6.129X1-Gt(Rosa)26Sor1tm14(CAG-GFP)BoyJ (stock no. 006148), B6.Cg-Gt(Rosa)26Sor1tm4(CAG-tdTomato)BoyJ (stock no. 007914), and B6.Cg-Tg(ACFLPe)2905Dym/J (stock no. 007503) mice were obtained from The Jackson Laboratory, whereas B6.Rag2±/−Il2rg−/− (stock no. 4111) mice were obtained from Taconic. Plzf−/− mice were a gift from P. P. Pandolfi and were backcrossed to C57BL/6J for at least nine generations. Mice were housed in a specific-pathogen-free environment at the University of Zurich and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Generation of the PLZFGFP reporter strain. The PLZFGFP strain was generated as follows. A sequence encoding an IRES and GFP–Cre fusion protein was cloned from the pGcn2 plasmid and inserted immediately following the Zbtb16 stop codon. The linearized construct was transfected into C57BL/6j embryonic stem cells and neomycin-resistant clones were screened by PCR. Clones that had undergone homologous recombination were injected into albino C57BL/6j blastocysts and the resulting chimeric mice were crossed with ACTB-FLPe mice to excise the neomycin resistance cassette. The following PCR primers were used to isolate the LPL fraction.

Preparation of cell suspensions. Spleen, liver and lymph nodes were mechanically dissociated through 70-µm filters and bone marrow was isolated by gently crushing femurs and tibiae before filtration. Following dissociation, each liver was centrifuged at 400 g for 5 min, resuspended in 5 ml of 40% Percoll (Sigma-Aldrich), and then centrifuged at 800g for 10 min. The supernatant was aspirated and the cell pellet was resuspended in HBSS (Gibco) containing 0.25% BSA (Sigma-Aldrich) and 0.65 mM 1-sodium azide (Sigma-Aldrich).

For the isolation of lung lymphocytes, mice were anaesthetized with ketamine/xylazine and approximately 1 ml of PBS (Sigma-Aldrich) was injected into the right ventricle to perfuse the lung tissue. Pairs of lungs were diced and incubated in 37 °C incubator (Thermo Scientific) with 5% CO2. Stromal cells were 70% confluency when lymphocytes were plated at 103–105 cells/ml for 3 days and CD3+ cells were identified and sorted as Lin−CD3−CD4−CD8−. In addition, post-sorting microscopic assessment confirmed that the nature of CD45 allele marking was systematically verified during FACS analysis.

Flow cytometry. Cell suspensions were incubated with purified anti-CD16/32 (clone 93) for 10 min on ice to block Fc receptors. Fluorochrome- or biotin-labelled monoclonal antibodies (clones denoted in parenthesis) against 2B4 (2B4), 4a7f (DATK32), B220 (RA3-6B2), CCR6 (29-2L17), CD3 (17A2), CD5 (145-2C11), CD4 (RM-4-5 or GK1.5), CD8α (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (6D3), CD25 (PC6), CD27 (LG799), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD49a (1H4), CD64 (3.2.1), CD66g (14.1), CD122 (SH14 or TM-1β), CD160 (7H11), CD161 (7B2), CXCR2 (241002), DX5 (DX3), F4/80 (A20), Gr-1 (RB-68C5-1), Ly6C (5E10/BoyJ), Ly6G (10G12), MHC class II (M5/114.15.2), ICOS (398.4A), IL-7R (752101), IL-7R (A734), KLRG1 (2F1), NK.1.1 (PK136), Nkp46 (2A9.14), Sca-1 (D7), T1/ST2 (D1H9), Ter119 (H57-597), Ter-119 (TERR-119), and Thy1.2 (S3-3.2) were purchased from BD Biosciences. Biolegend, eBioscience or R&D Systems. CD1d-PBS57 tetramer was from the NIH tetramer facility. For pre-enrichment of fetal liver and BM PLZFhigh cells, samples were stained with allopurinol (APC-conjugated anti-4a7f) antibody, bound to anti-APC microbeads (Miltenyi Biotec), and subjected to double-column enrichment on an autoMACS (Miltenyi Biotec). For the isolation of HSC, CLP, BM iILC2 and lung ILC2, lineage− cells were depleted by staining with biotin-conjugated antibodies against B220, CD3, CD4, CD8α, CD11b, CD11c, CD19, Gr-1, NK.1.1, TCRβ and Ter119, followed by an incubation with SAv microbeads (Miltenyi Biotec). Fetal liver progenitors were similarly enriched, except the biotin-conjugated lineage antibodies used were against B220, CD3, CD11c, CD19, NK.1.1, Nkp46 and Ter119. To exclude dead cells, 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) was added to all live samples. Cells were run on an LSRII (BD Biosciences) or sorted using a FACS Aria II (BD Biosciences) and analysed using FlowJo software (Tree Star). Collected events were gated on DAPI− lymphocytes and doublets were excluded.

To generate PLZF−/−/WT mixed chimaeras, 1–2 million bone marrow lymphocytes were injected into lethally irradiated (1,000 rad) C57/129.1 mice. Mixing Chimaeras were re-analysed 5–7 weeks after reconstitution by gating on CD45.2− cells to exclude residual host cells.

To remove the ‘background’ YFP staining (that is, the YFP staining occurring before the HSC stage) of bone marrow cells from YFP−/−/WT mixed chimaeras, 1–2 million bone marrow lymphocytes were injected into lethally irradiated (1,000 rad) C57/129.1 mice. Mixing Chimaeras were re-analysed 5–7 weeks after reconstitution by gating on CD45.2− cells to exclude residual host cells.

In vivo transfers. 800–1,200 tdTomato+ PLZFhigh cells and CD45.1 CLPs were sorted and injected retro-orbitally into 6–10 week-old sub-lethally irradiated (400 rad) C57/129.2−/−/IL2rγ−/− mice. Recipient mice were analysed 5–7 weeks post-transfer.

Bone marrow chimaeras. To remove the ‘background’ YFP staining (that is, the YFP staining occurring before the HSC stage) of bone marrow cells from YFP−/−/WT mixed chimaeras, 1–2 million bone marrow lymphocytes were injected into lethally irradiated (1,000 rad) C57/129.1 mice. Mixing Chimaeras were re-analysed 5–7 weeks after reconstitution by gating on CD45.2− cells to exclude residual host cells.

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analysed 5–7 weeks post-reconstitution. Populations of interest were normalized to the reconstitution ratio of each chimaera, as determined by the CD45.2°/CD45.1° ratio of BM lymphocytes or splenic B cells.

**Quantitative PCR with reverse transcription.** RNA was isolated from $5 \times 10^3$–$10 \times 10^3$ PLZF<sup>high</sup>, CLP, iILC2, NK and ILC3-enriched LPL using the Arcturus PicoPure RNA Isolation kit (Applied Biosystems) and reverse transcribed using the USB First-Strand cDNA Synthesis kit (Affymetrix). The following TaqMan primers (Applied Biosystems) were used (Probe ID in parenthesis): Gata3 (Mm00484683_m1), Hprt1 (Mm00446968_m1), Id2 (Mm00711781_m1), Rora (Mm00443103_m1), Rorc (Mm01261022_m1), Tbx21 (Mm00450960_m1), Tcf7 (Mm00493445_m1), Tox (Mm00455231_m1) and Zbtb16 (Mm01176868_m1). Quantitative PCR with reverse transcription was performed on an Mx3005p machine (Stratagene).

**Statistical analysis.** Two-tailed Student’s $t$-test was performed using Prism (GraphPad Software) to determine whether data differed from the expected value. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

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Extended Data Figure 1 | PLZF expression and lineage tracing in PLZF<sup>GFPCre</sup> mice.  

**a**, A sequence encoding an IRES and a GFP-cre fusion protein was inserted immediately after the Zbtb16 stop codon in C57BL/6J ES cells and knock-in mice were bred to ACTB-FLPe mice to excise the neomycin resistance cassette and generate the PLZF<sup>GFPCre</sup> allele.  

**b**, FACS analysis of the indicated populations from PLZF<sup>GFPCre+/−</sup> ROSA26-YFP mice.  

**c**, Summary of data (mean ± s.e.m.) from 2–5 mice analysed in 2 or more independent experiments.
Extended Data Figure 2 | Gating strategy for analysis of ILC and LTi among LPL. ILC2 cells were identified as IL-7Rα⁻ KLRG1⁺ among CD3ε⁻ CD19⁻ LPL (top left), and then gated Thy1.2⁻ (not shown). CD3ε⁻ CD19⁻ LPL were gated as IL-7Rα⁻ KLRG1⁺ (top left) and then subsetted into CCR6⁻ CD4⁺ (CD4⁺ LTi cells) and CCR6⁺ CD4⁺ (CD4⁺ LTi cells) (bottom left). NCR⁻ ILC3 were identified as CD3ε⁻ CD19⁻ LPL that expressed NKp46 but not NK1.1 (top right).
Extended Data Figure 3 | Transcription factor expression by PLZFhig bone marrow precursors. Quantitative PCR with reverse transcription analysis for Tbx21 and Rora as indicated. NKP are Lin−CD27−IL-7Rα−Flt3−CD122− BM cells. Mean ± s.e.m. of data from 2–3 independent experiments.
Extended Data Figure 4 | PLZF<sup>high</sup>-derived NK1.1<sup>+</sup> cells are distinct from CLP-derived NK1.1<sup>+</sup> cells. CD45.2 Rag<sup>2−/−</sup> Il2rg<sup>−/−</sup> mice were injected with equivalent numbers of CD45.2 PLZF<sup>high</sup> cells and CD45.1 CLP (800 of each) and the resulting NK1.1<sup>+</sup> CD3<sup>e−</sup> TCR<sup>β−</sup> cells present in the spleen were analysed 5–7 weeks later by FACS, as indicated. Note that PLZF<sup>high</sup>-derived cells expressed higher amount of surface NKp46, whether they were identified as CD45.2<sup>+</sup> or as CD45.1<sup>+</sup> in reciprocal staining experiments. Similar results were obtained for lung NK1.1<sup>+</sup> cells. Data representative of 5 chimaeras from 2 independent experiments.
Extended Data Figure 5 | FTOC of PLZF<sup>high</sup> cells. FACS analysis of PLZF<sup>high</sup> and CLP cells (100 of each) co-cultured for 15 days in FTOC (a). The percentages of PLZF<sup>high</sup>- or CLP-derived cells that are CD3<sup>e</sup><sup>+</sup> are summarized in the bar graph (b). Data representative of 7 independent cultures.
Extended Data Figure 6 | Additional characterization of PLZF$\text{high}$ cells after culture on OP9 cells. a, FACS analysis of PLZF$\text{high}$ or CLP cells from adult BM cultured on OP9 for 4 days showing expression of T1/ST2 on ICOS$\text{high}$ cells. Data representative of 4 replicate cultures from 2 independent experiments. b, FACS analysis of fetal liver PLZF$\text{high}$ cells after culture on OP9 for 7 days, showing expression of GATA3 by ICOS$\text{high}$ cells and RORγt by ICOS$\text{int}$ cells. Data representative of 2 independent experiments.
Extended Data Figure 7 | Proposed model of ILC development.
A CLP-derived IL-7Rα+ε4β7+ population bifurcates into RORγt<sup>hi</sup> LTi precursors (LTiP) and PLZF<sup>hi</sup> ILCP, the latter of which gives rise to all ILC lineages. Whether NKP cells develop directly from CLPs or progress through an IL-7Rα+ε4β7+ stage has yet to be determined.