HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2016 April 01.

Published in final edited form as:

Nat Immunol. 2015 October ; 16(10): 1044–1050. doi:10.1038/ni.3248.

TCF-1 upregulation identifies early innate lymphoid progenitors in the bone marrow

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Abstract

The cellular and molecular events that drive early innate lympoid cell (ILC) development remain poorly understood. We show that transcription factor TCF-1 is required for the efficient generation of all known adult ILC subsets and their precursors. Using novel reporter mice, we identified a new subset of early ILC progenitors (EILP) that expressed high amounts of TCF-1. EILP lacked efficient T and B lymphocyte potential, but efficiently gave rise to NK cells and all known adult helper-ILC lineages, indicating that they are the earliest identified ILC-committed progenitors. Our results suggest that upregulation of TCF-1 expression denotes the earliest stage of ILC fate specification. The discovery of EILP provides a basis to decipher additional signals that specify the ILC fate.

Introduction

Innate lymphoid cells (ILC) lack adaptive antigen receptors, but functionally and transcriptionally resemble subsets of effector T cells[1–4]. They include conventional Natural Killer (NK) cells and three subsets of cytokine-producing helper cells, ILC1, ILC2, and ILC3[5]. ILC are important players in tissue homeostasis, host defense and tumor

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Accession Codes

Microarray data were deposited at GEO with accession number GSE69789.

Contributions

Q.Y. performed most of the experiments with the help of C.H., L.Y., X.X., H.W., and X.W.; F.L., S.X., S.Y., and X.Z. provided mice, reagents and input on the manuscript; M.C. analyzed the micro-array data; Q.Y., H.X., and A.B. designed the study, analyzed the data; and wrote the manuscript.

surveillance[2]. However, the cellular and molecular events that underlie ILC fate specification and commitment remain poorly understood.

ILC derive from bone marrow (BM) lymphoid progenitors[6–8], but little is known about their further lineage progression steps. Previous studies have identified several candidate ILC progenitor subsets in the BM, but none efficiently gave rise to all four ILC lineages at the clonal level[9–11]. A developmental history of PLZF expression was detected in several cytokine-producing helper-ILC subsets but not conventional NK cells[11]. BM $PLZF^+Thy\text{-}1^+IL\text{-}7R\alpha^+\alpha_4\beta_7^+$ progenitors efficiently matured into several cytokine-producing helper-ILC subsets, but not conventional $DX5^+$ NK cells and $CD4^+$ LTi-like cells[11]. A similar BM progenitor subset, identified as $Id2^+Thy\text{-}1^+IL\text{-}7R\alpha^+\alpha_4\beta_7^+Lin^-$ cells, are termed common helper-innate lymphoid cells progenitors (CHILP). CHILP contain both $PLZF^+$ and $PLZF^-$ progenitors. CHILP gave rise to all helper-ILC subsets, but not conventional NK cells[10]. The majority of single BM $PLZF^+$ progenitors or CHILP gave rise to one or two ILC lineages, but lacked multi-ILC lineage potential when assessed *in vitro*, indicating that they may represent more mature ILC progenitor stage(s)[10,11]. A different progenitor subset defined as $CXCR6^+$ $IL\text{-}7R\alpha^+$ alpha-LP may give rise to conventional NK and also helper ILC subsets[9]. But these $CXCR6^+$ $IL\text{-}7R\alpha^+$ alpha-LP were extremely rare (around 50 cells per entire mouse), and only 2.5% of them (1 cell per entire mouse) efficiently gave rise to all four ILC lineages at the clonal level[9]. Hence, physiological early ILC progenitors for all four adult ILC lineages remain to be identified.

T cell factor-1 (TCF-1, encoded by the *Tcf7* gene) is a sequence-specific high-mobility group (HMG) transcription factor. TCF-1 was cloned from T cells[12,13], and it plays an essential role in T cell lineage specification and differentiation[14–19]. Recent work from us and others also implicated TCF-1 in the biology of ILC[20–24]. Mucosal ILC2 were greatly reduced in $Tcf7^{-/-}$ mice, and therefore these mice can not clear nematodes or mount innate type-2 immune reponses to airway protease challenge[20,21]. The number of small intestinal laminal propria (SiLP) ILC3 was also significantly reduced in $Tcf7^{-/-}$ mice. In addition, the number of NK cells was modestly reduced in $Tcf7^{-/-}$ mice, and $Tcf7^{-/-}$ NK cells did not express the receptor Ly49a[22–24]. Hence, TCF-1 is an essential controller in the generation or function of ILC. The precise role of TCF-1 in early ILC develpoment, however, remains largely unknown.

Here we report that TCF-1 promotes early ILC development in the BM, and further identify BM early innate lymphoid cell progenitors that express TCF-1 and efficiently develop into all known ILC lineages at the clonal level. We showed that TCF-1 is required for the efficient generation of all known adult mature ILC subsets as well as the ILC precursors CHILP and NKP. Using newly-generated $Tcf7^{GFP}$ reporter mice, we identified novel early innate lymphoid progenitors that express high levels of TCF-1 but lack surface markers of adaptive and innate lymphocyte lineages. These early innate lymphoid progenitors closely resemble bone marrow lymphoid progenitors at the transcriptome level, but lack efficient T and B lymphocyte potential. Instead, they efficiently gave rise to various ILC lineages, including conventional NK cells as well as cytokine-producing helper ILC at the clonal level, indicating that they are the earliest identifiable ILC progenitors. Our data implicate TCF-1 in very early stages of BM ILC specification. Identification of early innate lymphoid

progenitors will provide a basis to understand the molecular signals that drive early ILC development.

Results

TCF-1 promotes early ILC development

To understand the specific developmental stage(s) at which TCF-1 acts to promote ILC development, we examined the previously described PLZF-expressing common helper ILC progenitors (CHILP)[10,11] and also CD122-expressing NK cell progenitors (NKP)[25] in the bone marrow (BM) of *Tcf7*$^{-/-}$ mice (Exon VII targeted)[19]. Both PLZF^{+}CHILP and NKP were greatly reduced in *Tcf7*$^{-/-}$ mice, indicating that TCF-1 was required for early ILC development in the bone marrow (Fig. 1a, b, c). The previously described pre-NKP[26], rNKP[26], and pre-pro-NKP[27] were also greatly reduced in *Tcf7*$^{-/-}$ mice (Supplementary Fig. S1a). Despite the near absence of CHILP and NKP in *Tcf7*$^{-/-}$ mice, some mature NK cells, ILC1 and ILC3 persisted in the peripheral tissues of these mice[21,23] (Supplementary Fig. S1b, c). To more precisely define the requirement for TCF-1 in ILC development, we purified BM multipotent LSK progenitors (Lin^{-}Sca-1^{+}Kit^{+}Thy-1^{-}) from donor *Tcf7*$^{-/-}$[19] and control littermates (B6 mice, CD45.2) as well as competitor CD45.1 wild-type (WT) mice, and generated competitive LSK chimeras in irradiated recipient B6 congenic CD45.1 mice. *Tcf7*$^{-/-}$ LSK-derived donor populations were nearly devoid of all known mature ILC subsets in the peripheral tissues (Fig. 1d, e, Supplementary Fig. S1d, e), indicating that TCF-1 is expressed in hematopoietic precursors of all these lineages but that the requirement is most strict when competition is present. The results further established that the requirement for TCF-1 in the generation of CHILP and NKP was cell-intrinsic (Fig. 1f, g). Together, these data indicated a role for TCF-1 in promoting early ILC development in the bone marrow, and suggested that TCF-1 might be expressed and act at an early stage of innate cell fate specification.

Identification of an early innate lymphoid progenitor

Recent work has described several subsets of IL-7Rα-expressing BM ILC progenitors, but none of them efficiently gave rise to all four ILC lineages at the clonal level[9–11]. To search for TCF-1-expressing progenitors, we generated a *Tcf7*-GFP reporter allele by inserting GFP-coding sequences in the first intron of the *Tcf7* gene (called *Tcf7*^{GFP}, Supplementary Fig. S2a,b,c). In *Tcf7*^{GFP} mice, GFP was specifically expressed in T lymphocytes that expressed high levels of TCF-1, but not in B lymphocytes that lacked TCF-1 expression (Supplementary Fig. S2d). Mature ILC at peripheral sites also expressed various levels of TCF-1 (Supplementary Fig. S2e).

Because ILC development does not require the thymus, we initially searched for possible novel TCF-1^{+} ILC progenitors in the BM. The Lineage-negative (Lin^{-}) cells in the BM of *Tcf7*^{GFP} mice contained rare but distinct GFP^{+} subsets (Fig. 2a). We fractioned the GFP^{+}Lin^{-} BM progenitors based on the surface expression of IL-7Rα and Thy-1. Consistent with previous reports[9–11,28], the IL-7Rα^{+}Thy-1^{+}GFP^{+} subsets included previously described CHILP and ILC2P, which expressed TCF-1. Interestingly, we also identified a novel BM subset that expressed high levels of TCF-1, but were negative or low for surface expression

of IL-7Rα and Thy-1 (Fig. 2a), and thus differed from previously described candidate progenitor populations[9–11,25]. Unlike previously described alpha-LP, NKP, pre-NKP, rNKP, pre-pro NKP, or CHILP[9–11,25–27], these IL-7Rα$^{neg/lo}$Thy-1^{-}TCF-1^{+}Lin^{-} cells did not express detectable CD122 or CXCR6 on the cell surface (Fig. 2b), and only expressed low levels of mRNAs for *Il7r* and *Zbtb16* (encoding PLZF) expression (Fig. 2c), confirming that they are a previously unrecognized cell subset. These candidate early innate lymphoid progenitors (EILP) developed into all four ILC lineages on OP9 stroma, but lacked efficient B or T cell potential *in vitro* (Supplementary Fig. S3 a, b, c). EILP lacked expression of *Cd3e*, confirming that they were not T cells (Fig. 2c); and of B cell lineage transcription factors such as *Ebf1* (Fig. 2c). EILP, however, expressed high amounts of *Nfil3* and *Tox* (Fig. 2c), two other transcription factors implicated in early ILC development[9,29–32], and so exhibited features of early innate lymphoid cell progenitors.

We compared EILP with other early hematopoietic progenitors. Like CLP, EILP expressed low levels of Kit and Sca-1 (Fig. 3a). EILP also expressed a low level of Flt3 (Fig. 3a). We performed genome-wide microarray analysis to compare the transcriptome of EILP with those of other early progenitors, including HSC, LMPP, Flt3^{+} CMP[33], Flt3^{-} CMP[33], CLP, ETP, and DN3. Genetic profiling indicated that EILP are closer to CLP than other early progenitor populations at the transcriptome level (Fig. 3b). We next compared LMPP, CLP and EILP for their ability to develop into myeloid and lymphoid cell lineages *in vitro*. Similar to CLP, EILP lacked efficient myeloid differentiation activity (Fig. 3c, d), but rapidly developed into dendritic cells (DC) *in vitro*, at an early time point (day 4) when DC differentiation from LMPP was not yet evident (Fig. 3e, f). Hence, EILP resemble CLP in phenotype, gene expression profile, and differentiation activity, indicating that EILP have a close developmental relationship with CLP.

EILP are clonogenic progenitors to all known adult ILC subsets

To determine the *in vivo* lineage potential of EILP, we intravenously transferred EILP or TCF-1^{+} CHILP together with competitor CLP into unirradiated *Rag2*$^{-/-}$*gc*$^{-/-}$ recipient mice. We analyzed lymphocyte reconstitution at 3–6 weeks post-transplantation. EILP failed to generate B lymphocytes, and generated very few T cells (Fig. 4a, b). They, however, gave rise to all four ILC lineages examined *in vivo* (Fig. 4a, b). The ILC progeny derived from EILP included liver DX5^{+} Eomes^{+} NK cells, Eomes^{-} DX5^{-} ILC1, intestinal KLRG-1^{+}Sca-1^{+}ICOS^{+} ILC2 and RORγt^{+} ILC3 including some CD4^{+} LTi-like cells (Fig. 4c). Consistent with previous reports[10,11], TCF-1^{+} CHILP efficiently gave rise to ILC1, 2, 3, but not conventional NK cells, indicating that they were more downstream helper ILC progenitors (Fig. 4a, b). Together, these data established that EILPs are ILC-committed progenitors possessing the capability to give rise to all known adult ILC lineages *in vivo*.

We considered whether the inability of EILP to reconstitute T lineage cells after intravenous transfer might be due to their incapability to home to the thymus, rather than lack of T cell potential. To better determine the T lineage potential of EILP, we performed intrathymic transfer of EILP or control CLP. Whereas CLP generated CD4^{+}CD8^{+} (double-positive, DP) thymocytes as expected, EILP lacked T lineage potential even when placed inside the thymus, (Fig. 3d, e). These data, together with our observation that EILP failed to give rise

to T cells on OP-DL1 stroma (Supplementary Fig. S3b), indicated that EILP lack T cell lineage potential.

We next sought to determine the lineage potential of EILPs at the clonal level *in vitro*. We tested a number of different cytokine conditions based on previous reports[8–11,34] to identify a cytokine combination (SCF, IL2, IL-7) with which EILP displayed the greatest proliferative expansion (Supplementary Fig. S4a). We plated single EILP isolated by cell sorting on OP9 stromal cells in each well of 96-well plates. Plating efficiencies in these experiments were approximately 50% (Supplementary Fig. S4b). The majority of positive wells contained all four ILC lineages, including RORγt$^+$ ILC3, GATA-3$^+$ ILC2, as well as cells with the phenotype of conventional NK cells (NK1.1$^+$NKp46$^+$DX5$^+$)[11,35–37] and ILC1 (NK1.1$^+$NKp46$^+$DX5$^-$)[11,35–37] (Fig. 5a, b). The remaining wells contained a wide variety of combinations of ILC progeny, including a few ILC1–3 tri-lineage helper ILC progenitor that may overlap with the previously described PLZF$^+$ progenitors ([10,11], Fig. 5a). The downstream TCF-1$^+$ CHILP chiefly gave rise to one or two lineages *in vitro*, consistent with previous work, and suggesting that they were likely a mixture of intermediate stages of progenitors ([10,11], data not shown). The earlier progenitors LMPP, CLP and ETP did not efficiently gave rise to ILC under these conditions, indicating that additional signals are required *in vitro* for ILC fate specification and commitment (Supplementary Fig. S4c). Together, these results established that EILP efficiently develop into all four ILC lineages *in vivo* and *in vitro* at the clonal level, indicating that they likely represent the earliest ILC progenitors yet identified.

Previous reports indicated that Notch signaling promotes the earliest stages of ILC development, but is dispensable for ILC maturation at later stages[6,10,20,34]. Our experiments indicated that EILP do not require Notch signals for further maturation *in vitro*, likely because they already express TCF-1 and possibly other Notch target genes. To examine whether Notch can modulate ILC development after the EILP stage, we compared the differentiation of EILP on OP9 and OP9-DL1 stroma. The Notch ligand DL1 enhanced the generation of ILC2, but repressed the development of NK cells from EILP (Fig. 5c). Nevertheless, all ILC subsets clearly developed on OP9 stroma in the absence of Notch signals, indicating that Notch signaling is not strictly required for the further development of EILP into ILC lineages (Fig. 5c).

Our identification of EILP in the bone marrow indicated that the earliest stages of ILC specification and commitment occur at this site. To understand whether the thymus might also be a suitable site for early ILC development, we searched for EILP in the thymus. A few thymocytes displayed the phenotype of EILP (Lin$^-$TCF-1$^+$Thy-1$^-$IL-7Rα$^{neg/lo}$, Supplementary Fig. S4d), but they failed to efficiently generate ILC *in vitro* (Supplementary Fig. S4e). Hence, functional EILP are rare in the thymus, if they exist. These data indicated that although upregulated expression of TCF-1 occurs both during early ILC development in the BM and early T cell development in the thymus, other important differences must exist between lymphocyte development at these sites.

Id2 is dispensable for the generation of EILP

In addition to TCF-1, the transcriptional inhibitor of E proteins Id2 is also required for the generation of all known ILC[38–40]. However, previous studies suggested that ILC fate specification might initiate independently of Id2[9,10,41]. Indeed, NKP remained present in *Id2*$^{-/-}$ mice[41], but were greatly reduced in *Tcf7*$^{-/-}$ mice, suggesting that TCF-1 might act prior to Id2 during early ILC development (Fig. 1a,b). EILP expressed *Id2* mRNA, but the levels were lower as compared with CHILP and ILC2P (Fig. 2c). To determine whether *Id2* was required for EILP generation, we generated *Id2*$^{-/-}$*Tcf7*^{GFP} mice. EILP clearly persisted in *Id2*$^{-/-}$ mice, although their numbers were reduced 50% (Fig. 6a, b). CHILP and ILC2P were absent, as expected[10]. *Id2*$^{-/-}$ EILP expressed Nfil3 and TOX at a slightly lower level than *Id2*$^{+/+}$ EILP (Fig. 6c). Hence TCF-1 is expressed prior to the dependence on Id2 during the emergence of the earliest progenitors for NK cells and ILC in early BM hematopoiesis.

To determine whether Id2 might control the lineage commitment of EILP, we cultured *Id2*$^{-/-}$ EILP under B or T lymphocyte differentiation conditions with OP9 or OP9-DL1 stroma. *Id2*$^{-/-}$ EILP failed to give rise to B or T cells *in vitro*, indicating that other molecules can repress T/B lymphocyte potential in EILP (Supplementary Fig. S5a, b). We examined the expression of other Id proteins in *Id2*$^{-/-}$ EILP. In the absence of Id2, *Id1* expression was increased 3-fold, and *Id3* expression was increased 11-fold in EILP (Fig. 6c). Hence, Id2 possibly participates in ILC fate commitment, but other Id proteins may be up-regulated in their expression to compensate for the loss of Id2.

Discussion

The present study identifies an early innate lymphoid cell progenitor. EILP express TCF-1 and are clonogenic progenitors for both NK cells and all known adult helper-ILC lineages. EILP were identified in the BM, persisted in the absence of Id2, and were most closely related to CLP at the transcriptome level, indicating that TCF-1 might identify the earliest steps of BM ILC development, prior to the requirement of Id2. NKP and CHILP were nearly absent in adult *Tcf7*$^{-/-}$ mice, indicating that these innate progenitors are likely downstream of EILP, and that TCF-1 is required for their generation.

Interestingly, although CHILP and NKP were nearly completely lacking in adult *Tcf7*$^{-/-}$ mice, many mature ILC developed and were present in these mice in the absence of competition[20,21,24]. However, the remaining ILC In *Tcf7*$^{-/-}$ mice may be functionally compromised[20,21,24]. The remaining NK cells in *Tcf7*$^{-/-}$ mice do not express Ly49a[24]; the remaining *Tcf7*$^{-/-}$ ILC2 do not make IL-13 in responses to *in vivo* intranasal protease challenge[20,21]; and the development of Peyer's patches is severely compromised in *Tcf7*$^{-/-}$ mice despite a near-normal number of CD4^{+} LTi-like cells[21]. How these abnormal ILC developed in *Tcf7*$^{-/-}$ mice remains to be further determined; compensatory upregulation of Lef-1 expression is one possible mechanism[14,20,42]. Nevertheless, our data from competitive LSK chimeras indicated that the TCF-1-dependent pathway is the predominant pathway that replenishes normal, functional intact ILC in adult mice.

EILP are close to CLP at the transcriptome level, but EILP lack efficient T and B lymphocyte potentials and express very low levels of IL-7Rα. ETP, the earliest identified T-

lineage progenitors, are also low for *Il7r* mRNA expression; but most ETP were marked with a history of IL-7Rα expression in *Il7r*-Cre/Rosa YFP mice[43,44]. Whether EILP also have a history of IL-7Rα expression, and what controls the dynamic expression of IL-7Rα during early ILC and T lineage development, are interesting topics for future investigation. One possibility is that downregulation of IL-7Rα expression might help quench B cell-lymphoid fate to allow entry into the ILC as well as T cell lineages[45].

Previous studies indicated that NK cell fate specification initiates before the requirement for Id2 in NK development[9,10,41]. Consistently, we observed that Id2 is dispensable for the formation and lineage commitment of EILP. However, $Id2^{-/-}$ EILP exhibited increased expression of *Id1* and *Id3*, indicating that other Id proteins may compensate for the loss of Id2 during early ILC development. The role of Id proteins in the earliest stages of ILC fate specification and commitment, and the implication of other molecular signals, warrant further investigation.

TCF-1 is highly expressed in EILP in the BM as well as ETP[14] in the thymus, yet EILP and ETP differ significantly in their lineage potentials and their gene expression profiles. Hence other differences must exist between early ILC development versus early T cell development. EILP differ from ETP in their expression of several key transcription factors, such as Id2 and Nfil3 that may collaborate with TCF-1 to specify the ILC fate. How their expression is elicited in the BM but not the thymus is presently unknown. One possibility is that expression of other key transcriptional controllers in early innate lymphoid progenitors is elicited by signals distinct from those that induce TCF-1. Our discovery and characterization of EILP will facilitate identification of these signals, and will help decipher the mechanisms underlying innate lymphoid specification.

In summary, our results implicate TCF-1 in early stages of ILC development, promoting the development of all known adult ILC subsets and precursors. We showed that TCF-1 upregulation identifies the earliest known innate lymphoid progenitors in the bone marrow. Comparison of EILP with adaptive lymphocyte-biased progenitors may shed light on the unique pathways that determine the fate of each lymphocyte lineage.

Online Methods

Mice

C57BL/6 (B6, CD45.2) and B6-Ly5.2 (CD45.1) were purchased from National Institutes of Health or The Jackson Laboratory. $Rag2^{-/-}gc^{-/-}$ mice were purchased from Taconic. Other mice used include $Tcf7^{-/-}$ mice[15], $Tcf7^{GFP}$ mice, and $Id2^{-/-}Tcf7^{GFP}$ mice. All mice used were on the B6 background. Mice were used at 6–12 weeks old, of either sex. The sample size was empirically determined. No statistics was used to pre-determine the sample size. No samples were excluded from analysis. No randomization or bliding was used. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee policies at the University of Pennsylvania, the University of Iowa and the National Institutes of Health.

Generation of *Tcf7*-GFP reporter mouse strain

Targeting construct design: The *Tcf7* gene is expressed in multiple isoforms due to different promoter usage and alternative splicing. The full length TCF-1 isoforms (p45 and p42) are transcribed from an upstream promoter utilizing Exon 1, and the short isoforms (p33 and p30) are transcribed from Exon 3 (Extended Fig. 2a). To generate a *Tcf7*-GFP reporter allele, we inserted an EGFP expression cassette and a neomycin-resistant gene (NEO) cassette into the least conserved region in the first intron of the *Tcf7* gene. The EGFP expression cassette contains a strong *En2* gene splicing acceptor (En2-SA) to facilitate splicing of *Tcf7* exon 1 to the reporter, an internal ribosome entry site (IRES) to facilitate independent translation of EGFP, and the EGFP cDNA followed by poly-adenylation sequence. Two Frt sites were inserted to flank the EGFP and NEO cassettes One LoxP site was inserted immediately downstream of the second Frt site in intron 1, and another LoxP site was inserted into the least conserved region in intron 2. These features, although not used in this report, were designed to remove the EGFP and NEO cassettes with Flippase, converting the *Tcf7*-GFP reporter allele to *Tcf7* Exon 2-floxed allele, so as to conditionally target the TCF-1 long isoforms.

The targeting construct was assembled using the recombineering approach. All sequences surrounding the insertion sites were verified to ensure no unwanted mutations had occurred. The targeting construct was electroporated into C57Bl/6 embryonic stem (ES) cells, and the ES clones with expected homologous recombination were screened by Southern blotting (Extended Fig.2b and 2c). Blastocyst injection of the ES cells was performed at the Transgenic Animal Model Core, the University of Michigan. The chimeras containing the targeted allele were crossed with C57Bl/6 mice to achieve germline transmission.

Isolation of hematopoietic cells in periphery tissues

For isolation of lung hematopoietic cells, lungs were perfused by injecting 10 ml PBS into the right ventricle of the heart. Lungs were carefully cut into small fragments and digested in HBSS containing 0.025 mg/ml Liberase D (Roche Diagnostics) and 10 U/ml DNase 1 (Roche Diagnostics). Cells were filtered using a cell strainer.

For isolation of intestinal lymphocytes, payer's patches were removed from the small intestines. The small intestines were opened and the contents were emptied. The small intestines were cut into 1-cm pieces and were shaken at 37 °C for 30 mins in RPMI 1640 containing 1% FCS (Invitrogen), 1mM EDTA (Invitrogen), and 1mM DTT (Sigma-Aldrich).†The tissues were washed and subsequently digested with HBSS containing 0.025 mg/ml Liberase D (Roche Diagnostics) and 10 U/ml DNase 1 (Roche Diagnostics) by shaking at 37 °C for 30 mins. The digested tissues were filtered. Lamina Propria Lymphocytes were isolated by 40% Percoll.

Flow Cytometry and Cell Sorting

All antibodies (Abs) used in this study were purchased from eBioscience. Clones for Abs can be found in Supplementary Table 1. Flow cytometric analysis was performed on a LSR-II (BD Biosciences). Intracellular staining was performed using Fix/Perm Kit (eBioscience) according to manufacture's instructions.

Adoptive Transfer

For competitive LSK chimeras, 5000 Sorted bone marrow LSK progenitors from donor mice (CD45.2) were mixed with 5000 competitor wildtype LSK cells (CD45.1) and together injected into lethally irradiated (950 rads) wildtype recipient mice (CD45.1). Reconstitution of ILC in the recipient mice was examined at 12–16 weeks post-transfer.

For adoptive transfer of purified BM progenitors, 1000 EILP or TCF-1^{+}CHILP from *Tcf7*^{GFP} mice were mixed with bone marrow equivalent numbers (around 20,000) of competitor CLP (CD45.1), and together transferred intravenously into *Rag2*$^{-/-}$*gc*$^{-/-}$ mice. Reconstitution of lymphocytes were examined at 3–6 weeks post-transfer.

Cell Culture

Bone marrow progenitors were sorted by flow cytometric cell sorting. For ILC differentiation assays, progenitors were cultured in DMEM medium containing 20% FCS, 30ng/ml SCF, 30ng/ml IL-7 and 30ng/ml IL-2 on OP9-stromal cells. Cytokines were purchased from PeproTech or R&D. Progenitor differentiation was examined at 7–14 days after culture. For B cell differentiation assays, progenitors were co-cultured with OP9-stromal cells in the presence of IL-7 (10ng/ml) and FL (5ng/ml) for 7 days. For T cell differentiation assays, progentiors were cultured with OP9-DL1 stromal cells in the presence of IL-7 (1ng/ml or 10 ng/ml) and FL (5ng/ml) for 7 days. For myeloid differentiation, progenitors were co-cultured with OP9-stroma in the presence of IL-7 (1ng/ml) and FL(5ng/ml) for 4 days as desribed[46,47]. For assessing DC differentiation, progenitors were cultured in suspension with IL-1β (2 ng/ml), IL-3 (400 ng/ml), IL-7 (10ng/ml), SCF (10ng/ml), FL (100ng/ml), and TNF-α (1 ng/ml) for 4 days as previously described[48].

Microarray

BM HSC(Flt3^{-}CD150^{+}Lin^{-}Sca-1^{+}Kit^{+}), LMPP(Flt3^{hi}Lin^{-}Fca-1^{+}Kit^{+}), CLP (Lin^{-}Kit^{lo}Sca-1^{lo}Flt3^{+}IL-7Rα^{+}), Flt3^{+}CMP (Flt3^{+}Lin^{-}Sca-1^{-}Kit^{+}FcγR^{-}CD150^{-}CD34^{+})[33], Flt3^{-}CMP (Flt3^{+}Lin^{-}Sca-1^{-}Kit^{+}FcγR^{-}CD150^{-}CD34^{+})[33], EILP (Lin^{-}TCF-1^{+}Thy-1^{-}IL-7Rα$^{neg/lo}$) and thymus ETP (Lin^{-}Kit^{hi}CD25^{-}), DN3 (Lin^{-}Kit^{-}CD25^{+}) were isolated by flow cytometric cell sorting. Microarray were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual by the UPENN Microarray Core Facility. RNA was extracted with Trizol and amplified using Nugen PicoV2 kit (Nugen), and the quality of the RNA was tested on a bioanalyser. Biotinylated cDNA were prepared using Encore Biotin Module kit (Nugen) from 5.5 ug RNA according to the manufacture's instructions. 2.5ug/ul cDNA were hybrizied at 16 hours at 45 C on Affymetrix Mouse Gene 2.0 ST Array. The microarrays were then washed and stained with streptavidin-phycoerythrin. GeneChips were scanned using the GeneArray Scanner 3000 7G. The data were analysed using Affymetrix Expression Console with the default analysis settings. Gene signal values for the arrays were normalized and log2-transformed. Heatmaps were generated using the pheatmap R package (version 1.0.2)[49].

Statistics

Statistical analysis was performed on groups with limited variance. Comparison between two groups was performed using two-sided Student's t Test. The difference was considered significant with a p value lower than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Mark Greene and Dr. Gail Massey for essential support. We thank the laboratories of Dr. Angela Haczku, Dr. Yasmine Belkaid, Dr. Remy Bosselut, and Dr. David Artis for technical and scientific advice. OP9-DL1 stromal cell lines were obtained from Dr. Juan Carlos Zuniga-Pflucker from University of Toronto. Reagents for recombineering were kindly provided by Drs. Donald L. Court (NCI) and Neal G. Copeland (Houston Methodist Research Institute). We thank Dr. Thomas L. Saunders at the University of Michigan for blastocyst injection of the Tcf7-GFP ES cells. Supported by the American Cancer Society (RSG-11-161-01-MPC to H.-H.X.) and the National Institutes of Health (AI105351, AI112579, AI115149, and AI119160 to H.-H.X., AI059621, AI098428, HL11074103 to A.B). A.B. is supported by the Intramural Research Program of the National Institutes of Health, the National Cancer Institute, and the Center for Cancer Research.

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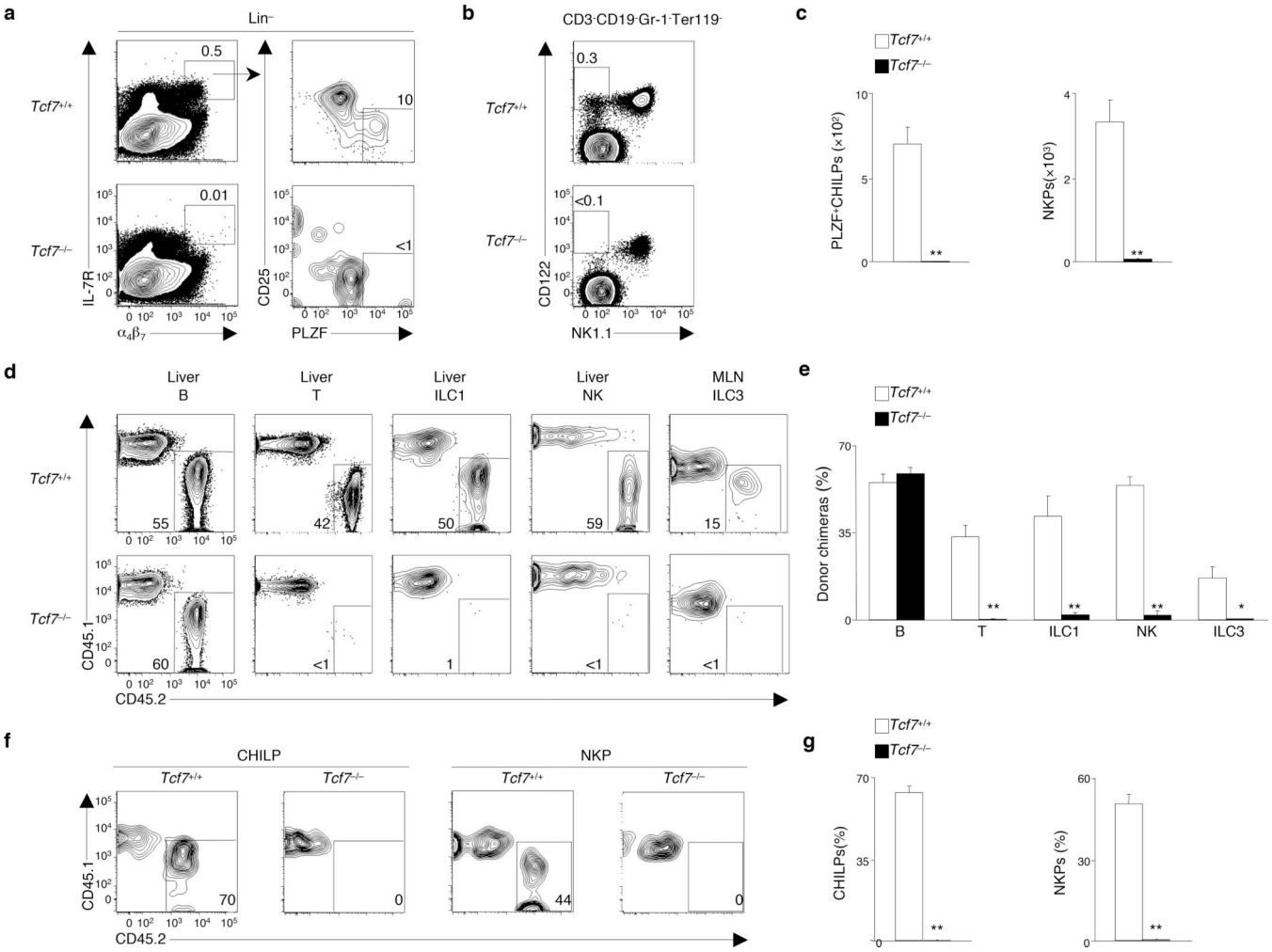

Figure 1. TCF-1 is required for the efficient generation of all known adult ILC and their progenitors

(**a)** Flow cytometry analysis of PLZF$^+$ common helper innate lymphoid cell progenitors (CHILP) in the bone marrow of *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ littermates. (**b**) Flow cytometry analysis of NK progenitors (NKP) NK1.1$^-$CD122$^+$ in pregated Lin$^-$ bone marrow cells from *Tcf7*$^{+/+}$ and *Tcf7*$^{-/-}$ littermates. (**c**) Quantification of the numbers of PLZF$^+$CHILP (left) and NKP (right) per *Tcf7*$^{+/+}$ or *Tcf7*$^{-/-}$ mouse analyzed as in **a** and **b**. (**d)** Flow cytometry analyzing mature ILC, B and T cells in the liver and mesenteric lymph nodes (MLN) of lethally irradiated chimeric mice (CD45.1) intravenously inoculated with *Tcf7*$^{-/-}$ or *Tcf7*$^{+/+}$ Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) bone marrow cells (CD45.2) equally mixed with wild-type competitor LSK cells (CD45.1) and analyzed at 12–16 weeks post-transplant. (**e)** Donor chimerism of mature ILC, B and T cells in the peripheral tissues of recipient mice as assessed as in **d**. (**f)** Flow cytometry analyzing bone marrow CHILP and NKP cells in the chimeric mice at 12–16 weeks post-transplant. (**g)** Donor chimerism of bone marrow CHILP and NKP as assessed in **f**. Data are representative of (**a**, **b**, **d**, **f**) or are pooled from three independent experiments with three mice per group (**c, e, g**). Error bar = mean ± SEM. *p<0.05. **p<0.01.

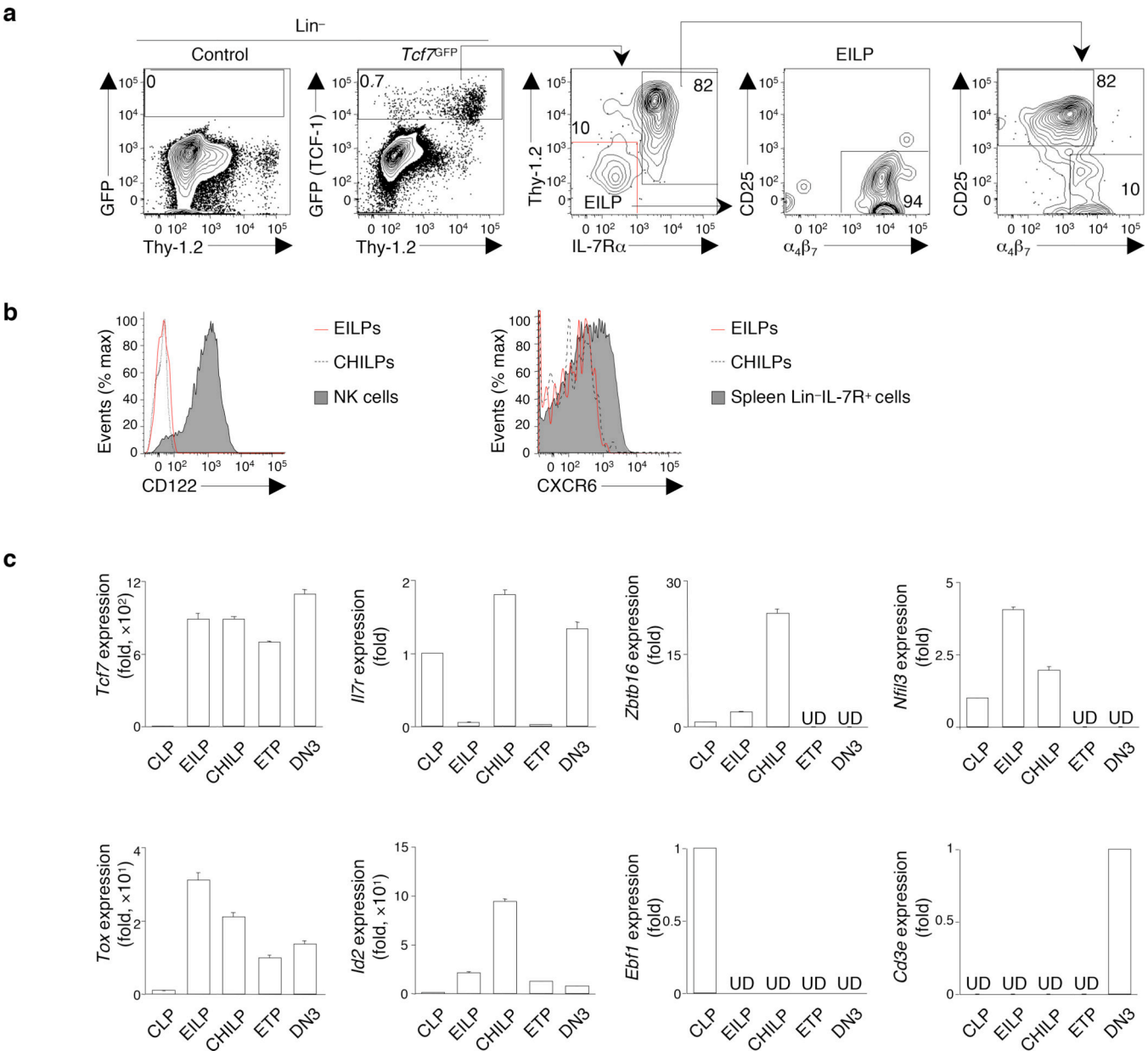

Figure 2. Identification of a novel TCF-1-expressing bone marrow cell population, termed early innate lymphoid progenitors (EILP)

(**a**) Flow cytometry analysis showing the profile of a novel Lin^{-}TCF-1^{+}IL-7R$\alpha^{neg/lo}$Thy-1^{-} cell population, termed EILP. Bottom panels depict the levels of surface CD25 and $\alpha_4\beta_7$ on EILP. (**b**) Flow cytometry analyzing the expression of surface CD122, and CXCR6 of the indicated subsets. (**c**) Expression of the indicated genes in bone marrow common lymphoid progenitors (CLP), EILP, CHILP, and thymus early T lineage progenitors (ETP) and double-negative 3 cells (DN3). Results are normalized to those of the control gene *Gapdh*. UD, undetectable. Data are representative of more than 10 experiments (**a**), or are representative of 3 independent experiments (**b**), or are from triplicate samples representative of 3−6 independent experiments (**c**). Error bar = mean ± SEM.

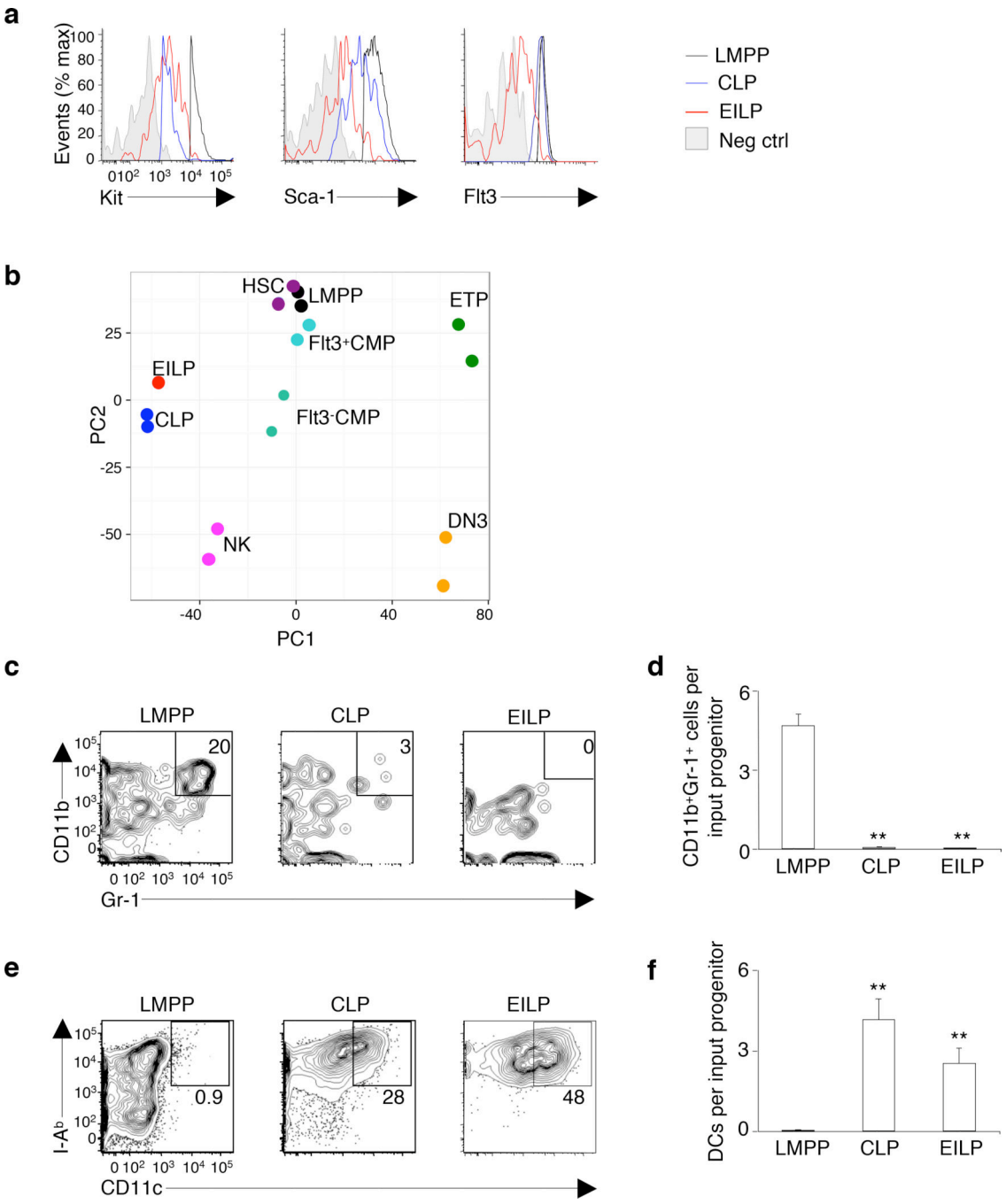

Figure 3. Comparison of EILP with bone marrow lymphoid progenitors

(a) Flow cytometry analyzing the expression of Kit, Sca-1 and Flt3 on EILP. LMPP, and CLP. Neg ctrl, negative control. (**b)** Genome-wide microarray analysis was performed with the indicated progenitor subsets. PCA analysis comparing the transcriptome of EILP with those of other progenitor subsets. (**c)** Flow cytometry analyzing $CD11b^+$ $Gr\text{-}1^+$ myeloid cells derived from LMPP, CLP and EILP cultured on OP9-stroma with FL and IL-7 for 4 days. (**d)** Quantification of the number of myeloid cells generated per progenitor input as assessed in **c**. (**e)** Flow cytometry analyzing $CD11c^+$ DC derived from LMPP, CLP or EILP

cultured on OP9-stroma in DC-differentiation cytokines for 4 days. **f**, Quantification of the number of DC generated per progenitor input as assessed in **e**. Data are representative of 3 independent experiments (**a**), or are representative of or from 3 experiments (**c, d**) or are representative of or from 4 experiments (**e,f**). Error bar = mean ± SEM. **p<0.01.

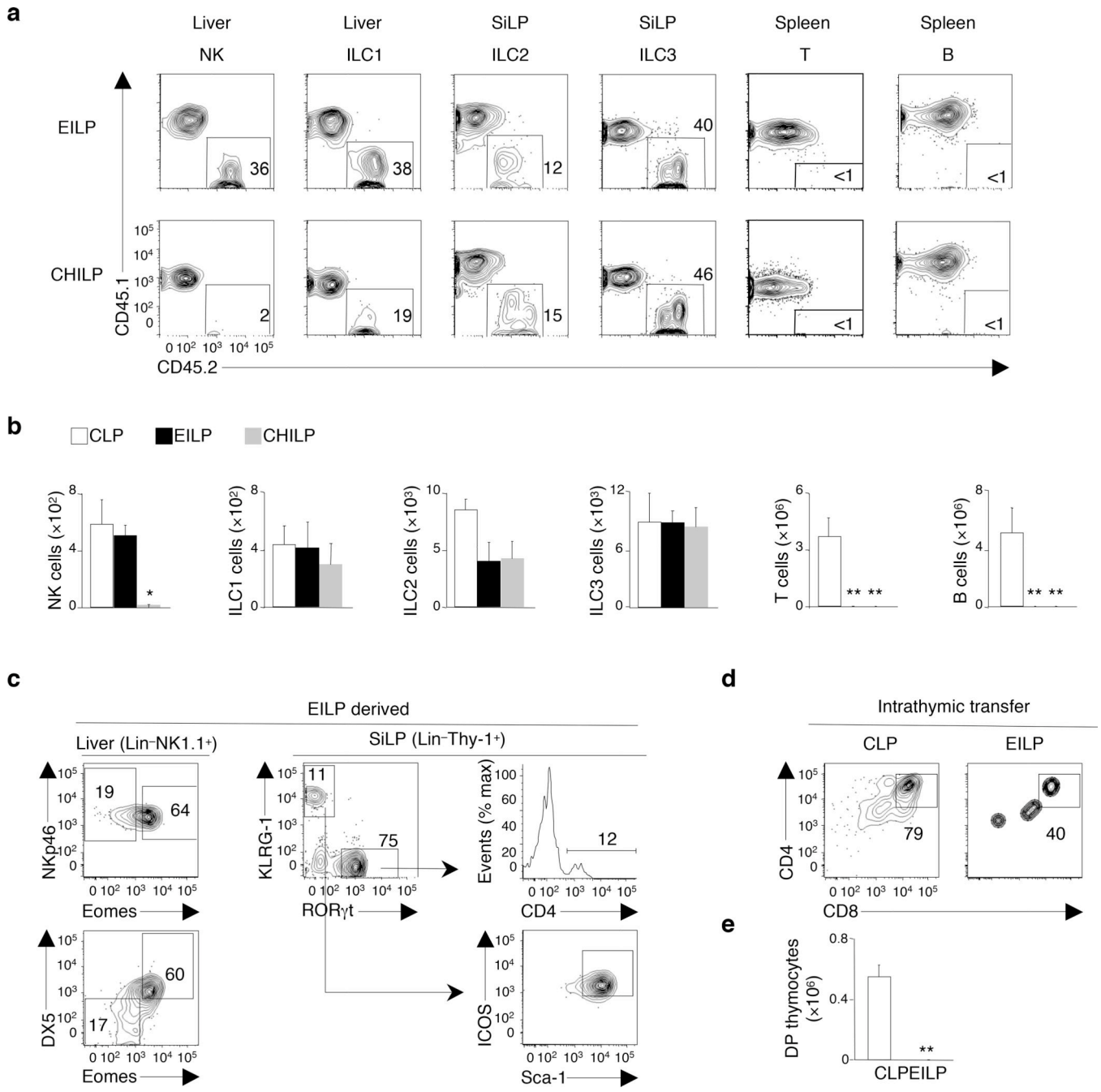

Figure 4. EILP efficiently give rise to all four ILC lineages *in vivo*

(**a**) Flow cytometry analysis of lymphocytes in the liver, small intestine lamina propria (SiLP), and spleen of *Rag2*$^{-/-}$*gc*$^{-/-}$ mice intravenously inoculated with EILP (CD45.2) mixed with bone marrow equivalent numbers of CLP (CD45.1) and analyzed at 3–6 weeks post-transplant. (**b**) Quantification of the number of donor-derived ILC, B and T cells in the peripheral tissues of the recipient mice as assessed in **a**. (**c**) Flow cytometry analyzing the phenotype of liver and SiLP ILC derived from EILP in the *Rag2*$^{-/-}$*gc*$^{-/-}$ recipient mice. (**d**) Flow cytometry analysis of donor-derived DP (double-positive, CD4^{+}CD8^{+}) thymocytes in sub-lethally irradiated recipient mice (CD45.1) intrathymically inoculated with CLP or EILP

(CD45.2) and analyzed at 14 days post-transfer. (**e**) Quantification of the number of donor-derived DP thymocytes in the recipient mice as assessed in **d**. Data are representatitve of or from four independent adoptive transfer experiments (**a, b, c**), or are representative of or from three independent experiments (**d,e**). Error bar = mean ± SEM. *p<0.05. **p<0.01.

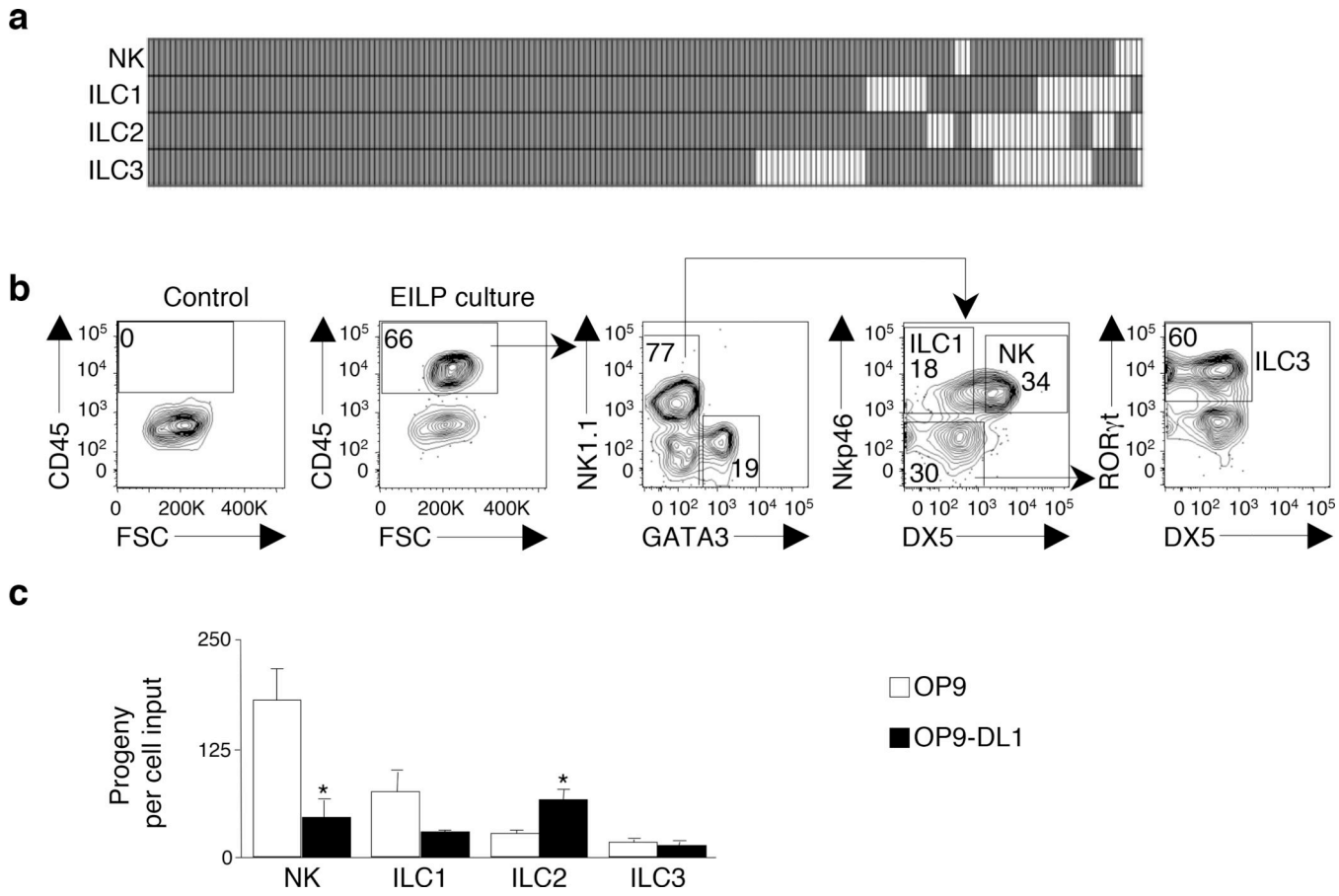

Figure 5. EILP efficiently give rise to all four ILC lienages at the clonal level

(**a**) Clonogenic differentiation assay showing the emergence of ILC progeny derived from single EILP cultured on OP9 stroma at one-cell per well in the presence of IL-2, IL-7 and SCF for 10 days. Each column represents one well with detected ILC lineages highlighted in grey. Only wells with positive ILC growth are shown. (**b)** Flow cytometry analyzing the daughter cells derived from EILP single cell culture as assessed in **a**. (**c**) Quantification of the number of ILC progeny derived from EILP that were cultured on OP9 or OP9-DL1 in the presence of SCF, IL-2 and IL-7 for 10 days. Data are pooled, or are representative of five independent experiments (**b**), or are from three independent experiments (**c**). Error bar = mean ± SEM. *p<0.05. **p<0.01.

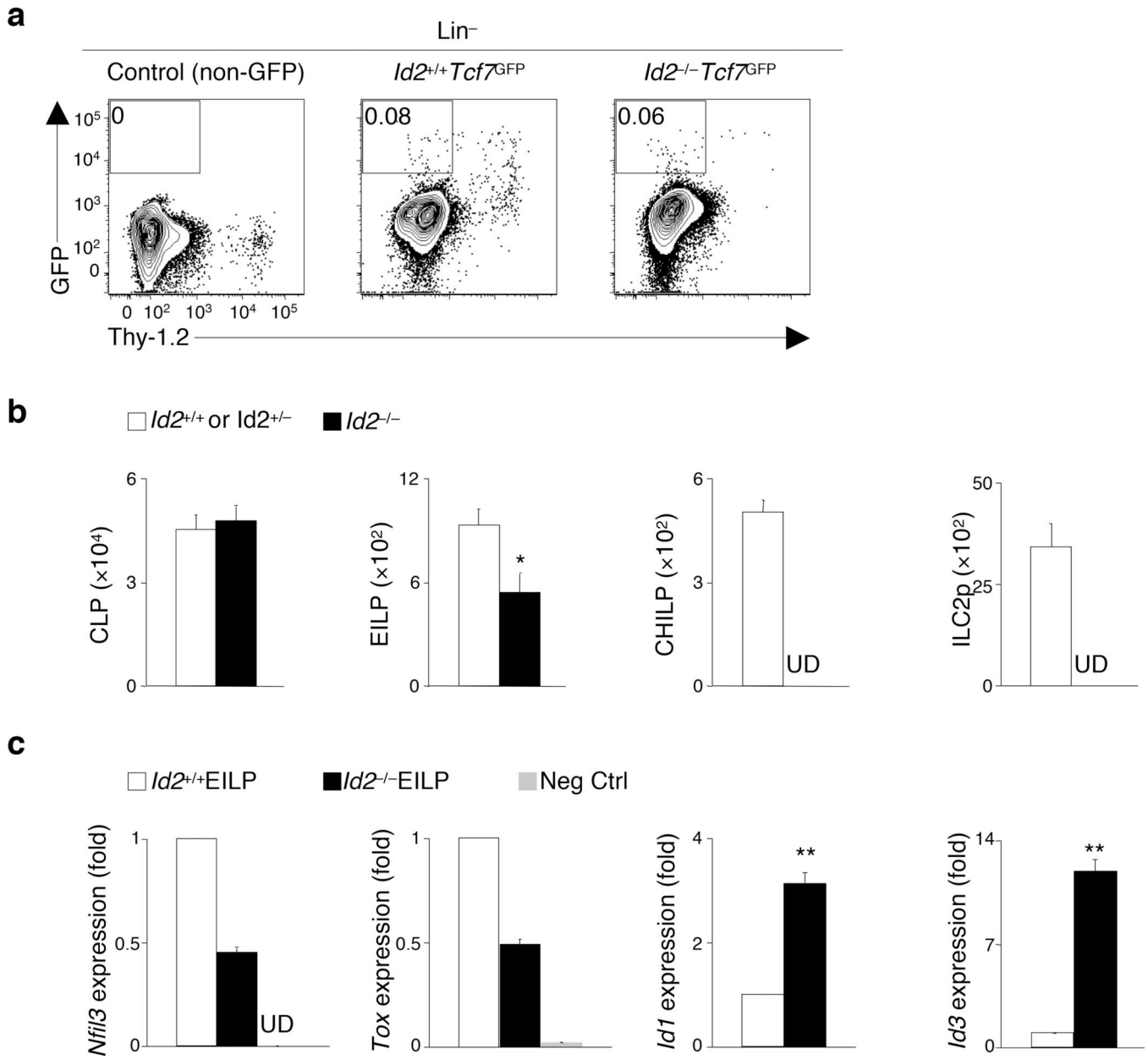

Figure 6. EILP persist in the absence of Id2

(a) Flow cytometry analyzing bone marrow EILP from *Id2*$^{+/+}$ *Tcf7*^{GFP} and *Id2*$^{-/-}$ *Tcf7*^{GFP} mice. Non-GFP wildtype mice were used as gating controls. Plots were pre-gated on BM Lin^{-} cells. (**b**) Quantification of the number of bone marrow CLP, EILP, CHILP and ILC2p from *Id2*$^{+/+}$ *Tcf7*^{GFP} and *Id2*$^{-/-}$ *Tcf7*^{GFP} mice. (**c**) Expression of the indicated genes by EILP from *Id2*$^{+/+}$ *Tcf7*^{GFP} and *Id2*$^{-/-}$ *Tcf7*^{GFP} mice. Results are normalized to those of the control gene *Gapdh*. UD, undetectable. Neg ctrl, negative control. Data are representative of or are pooled from three independent experiments with three mice per group (**a,b**), or are from triplicate samples representative of 2–3 independent experiments (**c**). Error bar = mean ± SEM. *p<0.05. **p<0.01.