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# Different developmental pathways generate functionally distinct populations of natural killer cells

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**Natural killer (NK) cells function by eliminating virus-infected cells or tumor cells. Here, we identified a NK lineage-biased progenitor population, termed early NK progenitors (ENKP), which developed into NK cells independently of common precursors for ILCs (ILCPs). ENKP-derived NK cells (ENKP\_NK cells) and ILCP-derived NK cells (ILCP\_NK cells) were also transcriptionally different. We devised combinations of surface markers that identified highly enriched ENKP\_NK and ILCP\_NK cell populations in wild-**

32 **type mice. Furthermore, Ly49H<sup>+</sup> NK cells that responded to mouse cytomegalovirus**  
33 **(MCMV) infection primarily developed from ENKPs whereas ILCP\_NK cells were better**  
34 **IFN- $\gamma$  producers upon Salmonella and Herpes Simplex Virus (HSV) infections.**  
35 **Interestingly, human CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells were transcriptionally similar to**  
36 **ENKP\_NK cells and ILCP\_NK cells, respectively. Our findings establish the existence of**  
37 **two pathways of NK cell development that generate functionally distinct NK cell subsets in**  
38 **mice, and further suggest these pathways may be conserved in humans.**

39

40 Innate lymphoid cells (ILCs) are transcriptionally and functionally like T cells but lack  
41 rearranged antigen receptors. ILCs are classified into helper ILCs which secrete similar  
42 cytokines as T helper cells and killer ILCs or NK cells which play critical roles in controlling  
43 viral infection and eradicating malignant cells by secreting perforin, granzymes and cytokines  
44 such as IFN- $\gamma$ <sup>1</sup>. ILCs develop from ALPs (all lymphoid precursors). Downstream of ALPs, the  
45 earliest defined ILC-specified progenitor population is early innate lymphoid progenitors  
46 (EILPs), which can differentiate into both helper ILCs and NK cells and gives rise to common  
47 precursors for ILCs (ILCPs), a progenitor population for helper ILCs that still retains NK lineage  
48 potential<sup>2-5</sup>. Because transcription factor PLZF is highly expressed in ILCPs but not expressed in  
49 upstream progenitors or mature ILCs, it has been used for lineage tracing of ILCP-derived cells<sup>3</sup>.  
50 Previous work established that NK cells are labeled at a lower ratio than helper ILCs in PLZF-  
51 Cre, Rosa26-reporter (PLZF lineage-tracing) mice, which suggested an ILCP-independent  
52 pathway for NK development<sup>3</sup>. Previously defined refined NK progenitors (rNKPs) are also  
53 traced by PLZF at intermediate levels, suggesting they are a heterogeneous population and some  
54 of them may develop from ILCPs<sup>6,7</sup>. Thus, developmental pathways between ALPs and mature  
55 NK cells are poorly defined.

56

57 The functional heterogeneity of NK cells has long been studied in both mouse and human<sup>8-12</sup>.  
58 Around 50% of mature NK cells in C57BL/6 mice express NK activating receptor Ly49H that  
59 recognizes the mouse cytomegalovirus (MCMV) antigen m157 and these Ly49H<sup>+</sup> NK cells  
60 preferentially respond to MCMV infection<sup>13,14</sup>. However, it is not known whether functionally  
61 distinct NK subsets develop via different pathways. Here, we used single-cell RNA-sequencing  
62 (scRNA-seq) to assess potential NK progenitor populations within bone marrow (BM). We

63 identified an *Flt3<sup>+</sup>Il2rb* (encoding CD122)<sup>+</sup> cluster that, by trajectory analysis, represented an  
64 ILCP-independent pathway of NK cell development. Consistently, we found that these  
65 *Flt3<sup>+</sup>CD122<sup>+</sup>* progenitors, here referred to as early NK progenitors (ENKPs), were not traced by  
66 PLZF, and were biased to NK cell fates with limited potential for differentiation into helper ILCs  
67 *in vitro* and *in vivo*. In addition, we showed ENKPs but not ILCPs efficiently gave rise to  
68 Ly49H<sup>+</sup> NK cells that were largely untraced by PLZF, and ENKP-derived NK cells expanded  
69 dramatically upon MCMV infection. We also identified NK populations that are enriched for  
70 ENKP-derived or ILCP-derived NK cells in wild-type B6 mice by surface markers. Further,  
71 ILCP\_NK cells were more robust IFN- $\gamma$  producers during Salmonella infection and Herpes  
72 Simplex Virus (HSV) infection. Our data reveal the existence of parallel developmental  
73 pathways that give rise to functionally distinct NK subsets.

74

#### 75 **Characterization of early NK cell development in bone marrow**

76 To identify candidate precursors of NK cells, we performed single-cell RNA sequencing  
77 (scRNA-seq) on cell populations (*Lin<sup>-</sup>CD27<sup>+</sup>2B4<sup>+</sup>CD122<sup>+</sup>*) sorted from bone marrow that we  
78 reasoned were likely to contain NK lineage progenitors, because CD27 and 2B4 are expressed on  
79 ALP, EILP, as well as ILCP populations<sup>15,16</sup>. We also sequenced previously described  
80 progenitors including a population containing ALP (*Lin<sup>-</sup>kit<sup>+</sup>Tcf7<sup>YFP</sup> $\alpha$ 4 $\beta$ 7<sup>-</sup>Flt3<sup>+</sup>IL-7R $\alpha$ <sup>+</sup>*), EILP  
81 (*Lin<sup>-</sup>kit<sup>+</sup>Tcf7<sup>YFP</sup> $\alpha$ 4 $\beta$ 7<sup>+</sup>Thy1.2<sup>-</sup>*), ILCP (*Lin<sup>-</sup>kit<sup>+</sup>Tcf7<sup>YFP</sup> $\alpha$ 4 $\beta$ 7<sup>+</sup>Flt3<sup>-</sup>Thy1.2<sup>+</sup>*), ILC2P (*Lin<sup>-</sup>*  
82 *CD25<sup>+</sup>Sca1<sup>+</sup>*); and separately, group 1 ILCs (*Lin<sup>T</sup>NK1.1<sup>+</sup>CD122<sup>+</sup>*) from bone marrow as  
83 potential upstream and downstream populations of NK progenitors (Fig. 1a, and Extended Data  
84 Fig. 1a,b)<sup>15,16</sup>. ALP, EILP, ILCP, ILC2P, NK and ILC1 clusters were defined based on the  
85 expression pattern of surface markers and transcription factors (Fig. 1b). The UMAP revealed  
86 that the ILCP cluster, which expressed high level of *Zbtb16* (encoding PLZF), overlapped with  
87 some of the cells sorted as candidate NK progenitors (Figure 1a,b and Extended Data Fig. 1b, c),  
88 suggesting overlap between rNKPs (defined as *Lin<sup>-</sup>2B4<sup>+</sup>CD27<sup>+</sup>Flt3<sup>-</sup>CD122<sup>+</sup>* by flow cytometry<sup>6</sup>)  
89 and ILCPs. Consistently, some rNKPs express PLZF<sup>7</sup>, indicating rNKPs overlap with ILCPs. A  
90 cluster was identified as *Il2rb* (encoding CD122)<sup>+</sup>*Flt3<sup>-</sup>Zbtb16<sup>lo</sup>*, which likely is another subset of  
91 rNKPs, thus we named this cluster as rNKP' (Fig. 1a and 1b).

92

93 Interestingly, a cluster within Lin<sup>-</sup>CD27<sup>+</sup>2B4<sup>+</sup>CD122<sup>+</sup> NK progenitors formed close to EILP and  
94 was marked by high expression of both *Il2rb* and *Flt3* (Fig. 1a, b and Extended data Fig. 1b).  
95 *Flt3* is expressed by upstream progenitors such as ALP and EILP during ILC development but  
96 downregulated in downstream progenitors such as ILCP<sup>15</sup>, suggesting this *Il2rb*<sup>+</sup>*Flt3*<sup>+</sup> NK  
97 progenitor cluster could be an early-stage developmental intermediate during NK cell  
98 development. Accordingly, we tentatively named this cluster “Early NK Progenitor” (or ENKP).  
99 Another recently defined NK/ILC1 progenitor population termed aceNKPs expresses  
100 NKG2A/C/E<sup>17</sup>; however the ENKP cluster did not express *Klrc1*, *Klrc2* and *Klrc3* (encoding  
101 NKG2A, NKG2C and NKG2E, respectively) (Extended Data Fig. 1d), suggesting ENKPs  
102 represent a novel population. We predicted the developmental relationships among clusters for  
103 the NK lineage using pseudotime reconstruction. Interestingly, a pathway including the ENKP  
104 but not ILCP cluster showed a sequence of predicted development as “ALP -> EILP -> ENKP ->  
105 rNKP’ -> NK” (lineage 1), which suggested a potential ILCP-independent pathway of NK  
106 development. The known lineage for ILC2 development<sup>15</sup> was also detected as “ALP -> EILP ->  
107 ILCP -> ILC2P” (lineage 2) (Extended Data Fig. 1e). However, we did not detect an ILCP-  
108 dependent pathway of NK cell or ILC1 development, suggesting intermediate populations may  
109 be missing in our analysis, consistent with evidence indicating ILCPs leave the bone marrow to  
110 circulate and differentiate at extramedullary sites<sup>18,19</sup>. Surface markers and transcription factors  
111 critical for NK cell and helper ILC development were displayed as a function of pseudotime  
112 (Extended Data Fig. 1e). In lineage 1, *Flt3* was highly expressed in ALP and EILP clusters and  
113 still maintained in the ENKP cluster but downregulated in rNKP’, whereas *Il2rb* was upregulated  
114 starting from ENKP cluster. Cells in the ENKP cluster expressed high levels of *Nfil3*, *Tcf7* and  
115 *Id2*, but low levels of *Eomes* and *Gata3* (Fig. 1b and Extended Data Fig. 1c). Also, *Zbtb16* was  
116 expressed at low levels in lineage 1. These data suggested the existence of an ILCP-independent  
117 pathway of NK cell development in bone marrow of adult mice, and identified ENKPs as an  
118 interesting candidate population that might represent an early precursor for NK cells.  
119

120 Consistent with the deduced phenotype of the ENKP cluster identified by scRNA-seq, we  
121 identified a Lin<sup>-</sup>NK1.1<sup>-</sup>CD5<sup>-</sup>CD3ε<sup>-</sup>CD27<sup>+</sup>2B4<sup>+</sup>*Flt3*<sup>+</sup>CD122<sup>+</sup> population in bone marrow by flow  
122 cytometry; thus, we named this population identified by flow “ENKP” as well. The frequency of  
123 ENKPs was approximately 0.005% (1 in 20,000 cells, similar to EILP<sup>2</sup>) among total live cells in

124 bone marrow (Fig. 1c). This population is different from rNKP, which was identified as Lin<sup>-</sup>  
125 CD27<sup>+</sup>2B4<sup>+</sup>Flt3<sup>-</sup>CD122<sup>+</sup> cells<sup>6</sup>; thus ENKP is a previously undefined population. Next, we  
126 assessed transcription factor requirements for ENKP development. ENKPs were greatly reduced  
127 in *Nfil3* deficient mice but remained intact in *Eomes*, *Tcf7* and *Gata3* deficient mice (Fig. 1d,e  
128 and Extended Data Fig. 2a,b). NK cell numbers were significantly reduced in *Nfil3*, *Eomes* and  
129 *Tcf7* deficient mice but remained intact in *Gata3* deficient mice, as expected (Fig. 1d,e and  
130 Extended Data Fig. 2a,b)<sup>2,20-22</sup>. Thus, among the transcription factors we assessed, only *Nfil3* is  
131 required for ENKP development. Because ILCPs fail to develop in mice lacking *Tcf7*<sup>16</sup> or  
132 *Gata3*<sup>15,23</sup>, these results also indicate that ENKPs are not downstream of ILCPs. We further  
133 characterized ENKPs by surface markers compared with ALPs, EILPs and ILCPs. ENKPs  
134 expressed high levels of  $\alpha 4\beta 7$  and Kit but low levels of IL-7R $\alpha$ ; they did not express Thy1.2 as  
135 ILCP did, consistent with their representing an early progenitor population (Extended Data Fig.  
136 2c). Approximately 70% of ENKPs had historical expression of *Il7r* (Extended Data Fig. 2d),  
137 which was similar to the ratios in ALPs and EILPs<sup>15</sup>, indicating ENKPs develop from *Il7r*-  
138 expressing lymphoid progenitors, as predicted for NK cells<sup>24</sup>.

139

#### 140 **ENKPs are NK lineage-biased progenitors and developmentally independent of ILCP**

141 To assess the lineage potential of ENKPs *in vitro*, we seeded ENKPs or ILCPs on OP9 stromal  
142 cells with the cytokines stem cell factor (SCF), Flt3L, IL-7 and IL-2. Both ILCPs and ENKPs  
143 gave rise to NK1.1<sup>+</sup> cells, which include both NK cells and ILC1s. However, unlike ILCPs,  
144 which efficiently gave rise to ILC2/ILC3s (ICOS<sup>+</sup>), ENKPs had very limited potential for  
145 ILC2/ILC3s (Fig. 2a), indicating they are an NK-biased progenitor population.

146

147 We next sought to determine the lineage potential of ENKPs at the clonal level *in vitro*. To  
148 distinguish NK from ILC1s *in vitro*, we used *Eomes*<sup>GFP</sup> reporter mice to identify NK cells, and  
149 CD200R to identify ILC1s as CD200R is highly expressed in ILC1s *in vivo*<sup>25</sup>. In pilot  
150 experiments, we cultured mature *Eomes*<sup>GFP-</sup> ILC1s and *Eomes*<sup>GFP+</sup> NK cells on OP9 culture for 7  
151 days; ILC1s remained CD200R<sup>hi</sup> and *Eomes*<sup>-</sup> whereas NK cells were nearly all *Eomes*<sup>+</sup> and did  
152 not express CD200R (Extended Data Fig. 3a). Thus, *Eomes* and CD200R can be used to  
153 distinguish NK cells from ILC1s *in vitro*. Among the ICOS<sup>+</sup> population, we used  $\alpha 4\beta 7$  to further  
154 distinguish ILC3s and ILC2s (Fig. 2b). The majority of ENKP-derived clones (74%) contained

155 NK lineage cells, and 54% of ENKP clones were NK lineage-committed; whereas most ILCP-  
156 derived clones (92%) contained only helper ILC lineages (Fig. 2b and Extended Data Fig. 3b).  
157 Thus, compared with ILCPs, ENKPs are NK lineage-biased progenitors *in vitro*. Compared to  
158 ENKPs and ILCPs, mature NK cells generated negligible numbers of NK cells after *in vitro*  
159 culture (Extended Data Fig. 3c), indicating that the observed results cannot be attributed to  
160 potential NK cell contamination during sorting. Since ENKPs but not ILCPs express Flt3, we  
161 differentiated ENKPs *in vitro* with and without Flt3 ligand (Flt3L) to assess whether their ability  
162 to generate NK cells depends on Flt3. ENKPs cultured without Flt3L generated fewer NK cells  
163 than ENKPs cultured with Flt3L, although the results did not reach statistical significance  
164 (Extended Data Fig. 3d). The data indicate that the Flt3 signal may have an effect on ENKP  
165 expansion, but is not strictly required for the development of ENKP-derived NK cells.

166  
167 To characterize the ILC lineage potential of ENKPs *in vivo*, we transferred ENKPs or ILCPs into  
168 NOD SCID gamma (NSG) immune-deficient recipient mice and assessed frequency of donor-  
169 derived NK cells and ILC1s within group 1 ILCs in liver. ILCPs gave rise to both NK cells and  
170 ILC1s, whereas ENKPs generated primarily NK cells but very few ILC1s (Fig. 2c,d). To  
171 compare the efficiency of ENKPs and ILCPs to generate NK cells *in vivo*, we co-transferred the  
172 same number of ALPs as competitors with ENKPs or ILCPs and found that ENKPs generated  
173 NK cells significantly more efficiently than ILCPs in both spleen and liver (Fig. 2e and Extended  
174 Data Fig. 3e). As expected, ALPs but not ILCPs or ENKPs gave rise to B cells (Fig. 2e; see  
175 Extended Data Fig. 3f-h for examples of sort purity). Thus, ENKPs are NK lineage-biased  
176 progenitors which can give rise to NK cells efficiently. Further, the development of both ENKP-  
177 derived NK cells (ENKP\_NK cells) and ILCP-derived NK cells (ILCP\_NK cells) **but not ILC1s**  
178 depended on Eomes, indicating ILCP\_NK cells are distinct from ILC1s (Extended Data Fig. 3i).

179  
180 Next, we used PLZF lineage tracing mice to assess the developmental relationship between ILCP  
181 and ENKP. As PLZF is also expressed before specification of hematopoietic lineages<sup>3</sup>, we  
182 eliminated the background labeling of PLZF and generated long-term bone marrow chimeras, by  
183 transferring unlabeled YFP<sup>-</sup> LK (Lin<sup>-</sup>kit<sup>+</sup>) cells which are enriched for hematopoietic stem cells  
184 and downstream progenitors from Zbtb16<sup>creGFP</sup>; Rosa26-stop-YFP mice into lethally irradiated  
185 wild-type recipient mice, as previously described<sup>3</sup>. NK cells and ILC1s in liver were labeled at

186 similar ratios to published studies<sup>3,7</sup> (Fig 3a). However, ENKPs were not labeled by PLZF,  
187 indicating they did not develop from ILCPs (Fig 3a). Next, to compare the labeling of  
188 ENKP\_NK cells and ILCP\_NK cells in spleen by PLZF, we transferred YFP<sup>+</sup> ENKPs, ILCPs or  
189 ALPs from *Zbtb16<sup>creGFP</sup>*; *Rosa26-stop-YFP* mice into NSG recipient mice. Unlike ILCP\_NK  
190 cells, which were highly labeled (~70%) by PLZF, ENKP\_NK cells were labeled at a very low  
191 ratio (~10%). ALP-derived NK cells were labeled at a ratio (~25%) similar with total NK cells in  
192 long-term chimeras (Fig 3a,b). Thus, ENKP\_NK cells and ILCP\_NK cells have different  
193 developmental pathways, and it is likely that NK cells are a mixture of ENKP-derived and ILCP-  
194 derived NK cells. In summary, these results showed ENKPs to be developmentally independent  
195 of ILCPs, and further indicate that there are at least two pathways for NK cell development.

196

### 197 **ENKP-derived and ILCP-derived NK cells are transcriptionally distinct**

198 To investigate whether ENKP\_NK cells and ILCP\_NK cells are transcriptionally different, we  
199 adoptively transferred ENKPs, ILCPs, or ALPs as a control, into NSG recipient mice. We  
200 subsequently sorted progenitor-derived NK cells from spleen and performed multiplexed  
201 scRNA-seq for NK cells derived from ENKP (ENKP\_NK cells), ILCP (ILCP\_NK cells) and  
202 ALP (ALP\_NK cells) (Fig. 4a). ENKP\_NK cells and ILCP\_NK cells expressed comparable  
203 levels of NK genes including *Eomes*, *Tbx21* (encoding T-bet) and *Klrl1* (encoding NKG2D)  
204 (Fig. 4c and Extended Data 4b). Genes expressed at higher levels in ENKP\_NK cells included  
205 Ly49 receptors such as *Klra8* and *Klra4*, cytotoxic genes *Gzma*, *Gzmb*, and *Prfl* and  
206 transcription factors *Zeb2*, *Irf8*, and *Klf2*. In contrast, ILCP\_NK cells expressed higher levels of  
207 recognition molecule *Cd226*, chemokine receptors including *Ccr2* and *Cxcr3*, transcription factor  
208 *Tcf7*, the cytokine gene *Ifng* and also *Cd160*, which is critical for IFN- $\gamma$  production<sup>26</sup> (Fig. 4b, c  
209 and Extended Data Fig. 4a, b and c). Gene Ontology analysis showed ENKP\_NK cells were  
210 enriched for cytotoxicity pathways (Extended Data Fig. 4d). ILCP\_NK cells were characterized  
211 by biological processes including cell-cell adhesion, lymphocyte activation and regulation of  
212 IFN- $\gamma$  production (Extended Data Fig. 4d). Heatmap clustering of ALP\_NK cells showed two  
213 major column clusters which have genes expressed higher in ENKP\_NK cells and ILCP\_NK  
214 cells, respectively, with the “ENKP\_NK” column cluster more abundant than “ILCP\_NK”  
215 column cluster, further suggesting the NK population is a mixture of ENKP\_NK cells and  
216 ILCP\_NK cells, with ENKP\_NK cells being more abundant (Extended Data Fig. 4e).



217  
218 UMAP identified 2 clusters; NK cells from all three developmental origins fell into both clusters,  
219 with ENKP\_NK cells enriched for cluster 0 whereas ILCP\_NK cells enriched for cluster 1  
220 (Extended Data Fig 4f). Genes differentially expressed between cluster 0 and cluster 1 were  
221 similar to genes differentially expressed between ENKP\_NK cells and ILCP\_NK cells (Extended  
222 Data Fig. 4a, g), which is consistent with the observation that ENKP\_NK cells and ILCP\_NK  
223 cells were enriched for cluster 0 and cluster 1, respectively (Extended Data Fig 4f). Although  
224 ENKP\_NK cells and ILCP\_NK cells both contributed to cluster 0 and cluster 1, differentially  
225 expressed genes, including *Klra8*, also could be detected within the same cluster when  
226 comparing NK cells derived from ENKPs and ILCPs (Extended Data Fig. 4h).

227  
228 In addition, we utilized our bone marrow data (see Fig. 1a) and published liver data<sup>27</sup> to compare  
229 the transcriptomes of ENKP\_NK cells, ILCP\_NK cells and ILC1s. First, within bone marrow  
230 total NK cells and liver total NK cells, we identified ENKP\_NK cells and ILCP\_NK cells by  
231 expression levels of a list of genes which are differentially expressed between ENKP\_NK cells  
232 and ILCP\_NK cells (see Methods). Then we compared the transcriptome of both NK subsets and  
233 ILC1s in the same tissues, as transcriptome can be influenced by both tissue microenvironment<sup>28</sup>  
234 and batch effects. In both liver and bone marrow, ILCP\_NK cells clustered together with  
235 ENKP\_NK cells rather than with ILC1s (Extended Data Fig. 4i), indicating ILCP\_NK cells are  
236 transcriptionally more similar to ENKP\_NK cells than ILC1s. Interestingly, ILCP\_NK cells  
237 expressed intermediate levels of genes which are differentially expressed between NK cells and  
238 ILC1s (see Methods), suggesting ILCP\_NK cells may exhibit a hybrid phenotype, sharing some  
239 characteristics of both NK cells and ILC1s (Extended Data Fig. 4j).

240  
241 In summary, these data indicate that ENKP\_NK cells and ILCP\_NK cells are transcriptionally  
242 different, with ENKP\_NK cells expressing Ly49 receptors and higher levels of cytotoxic genes,  
243 and ILCP\_NK cells expressing higher levels of genes related with IFN- $\gamma$  production. The results  
244 suggested ENKP\_NK cells and ILCP\_NK cells may be functionally distinct.

245  
246 **Ly49H<sup>+</sup> NK cells primarily develop from ENKPs**

247 Ly49H is a member of the Ly49 family of NK cell receptors and is expressed on approximately  
248 half of the NK cells in C57BL/6 mice<sup>13,14,29</sup>. Ly49H recognizes the viral protein m157 presented  
249 on cells infected by mouse cytomegalovirus (MCMV) and activates NK cells to eliminate virus-  
250 infected cells<sup>14</sup>. Our scRNA-seq analysis showed that *Klra8* (encoding Ly49H) expression in  
251 ENKP\_NK cells was much higher than in ILCP\_NK cells, suggesting Ly49H<sup>+</sup> NK cells may  
252 develop from ENKPs (Fig. 4b, c and Extended Data Fig. 4a-c). To test this hypothesis, we  
253 adoptively transferred ENKPs or ILCPs into NSG mice and assessed frequencies of Ly49H<sup>+</sup> NK  
254 cells developed from these progenitors. Indeed, ILCP\_NK cells were mostly Ly49H<sup>-</sup> whereas  
255 more than half of ENKP\_NK cells expressed Ly49H (Fig. 4d). Adoptive transfer experiments  
256 using ALPs as competitors showed that ENKPs gave rise to Ly49H<sup>+</sup> NK cells much more  
257 efficiently than ILCPs (Extended Data Fig. 5a). Thus, the majority of Ly49H<sup>+</sup> NK cells develop  
258 from ENKPs but not ILCPs. Ly49H was not expressed on ENKPs (Extended Data Fig. 5b),  
259 indicating Ly49H is upregulated at more downstream stages during NK cell development. In  
260 addition, consistent with scRNA-seq data, ENKP\_NK cells also had higher frequencies of cells  
261 expressing Ly49D (encoded by *Klra4*) but comparable frequencies of cells expressing Ly49E/F  
262 (encoded by *Klra5/Klra6*) (Extended Data Fig. 5c) and lower levels of TCF1-YFP (encoded by  
263 *Tcf7<sup>YFP</sup>*) (Extended Data Fig. 5d-e).

264  
265 When we examined the ratio of NK cells that were lineage traced with PLZF and the ratio of NK  
266 cells expressing Ly49H in different organs from PLZF-tracing long-term chimeras, there was an  
267 inverse correlation between PLZF labeling and Ly49H expression (Extended Data Fig. 5f-i).  
268 This is consistent with our finding that Ly49H<sup>+</sup> NK cells mostly derived from ENKPs and  
269 ENKP\_NK cells are labelled by PLZF at lower ratios than ILCP\_NK cells. Indeed, PLZF-tracing  
270 ratios were lower in Ly49H<sup>+</sup> NK cells as compared to Ly49H<sup>-</sup> NK cells (Extended Data Fig. 5f).  
271 Overall, PLZF-tracing ratios were lower in liver, spleen and lung (all ~18%) and higher in  
272 mesenteric lymph node (25%) and thymus (46%) (Extended Data Fig. 5f). NK cells in other  
273 tissues including salivary glands, uterus and bone marrow were also lowly traced by PLZF  
274 (Extended Data Fig. 5h). Because ENKP\_NK cells, ILCP\_NK cells and total NK cells were  
275 marked at different ratios in PLZF lineage-tracing mice (Fig. 3b, Extended Data Fig. 5f), and  
276 assuming that only ENKP\_NK cells and ILCP\_NK cells contributed to the total NK cell  
277 population, we were able to calculate the percentage of ENKP\_NK cells and ILCP\_NK cells (see

278 Methods). These calculations suggested more than 80% of NK cells in spleen, liver, lung,  
279 salivary glands, uterus and bone marrow develop from ENKPs. Consistently, the majority of  
280 ALP\_NK cells recovered from spleen were transcriptionally similar to ENKP\_NK cells  
281 (Extended Fig. 4e), and Ly49H<sup>+</sup> NK cells were labeled by PLZF at ratios significantly lower  
282 than Ly49H<sup>-</sup> NK cells in the tissues we assessed (Extended Data Fig 5f), further indicating that  
283 the majority of Ly49H<sup>+</sup> NK cells develop from ENKPs.

284

### 285 **Human NK subsets resemble mouse ENKP-derived and ILCP-derived NK cells**

286 In humans, NK cells include two major subsets, termed CD56<sup>dim</sup>CD16<sup>+</sup> (CD56<sup>dim</sup>) and  
287 CD56<sup>dim</sup>CD16<sup>-</sup> (CD56<sup>bright</sup>) NK cells. Within the CD56<sup>dim</sup> subset, human NK cells can be further  
288 subdivided into CD57<sup>-</sup> and CD57<sup>+</sup> populations, with the latter representing a more activated or  
289 mature population<sup>30</sup>. The CD56<sup>bright</sup> NK cells are more efficient in producing cytokines including  
290 IFN- $\gamma$ <sup>31</sup>. These observations are reminiscent of the mouse NK cell subsets elucidated in the  
291 present study.

292

293 To compare mouse NK cells with human NK cells, we analyzed previously published scRNA-  
294 seq data of human blood NK cells which includes both CITE-seq and scRNA-seq data<sup>32,33</sup>. This  
295 allowed us to distinguish CD56<sup>bright</sup>, CD56<sup>dim</sup>CD57<sup>-</sup> and CD56<sup>dim</sup>CD57<sup>+</sup> clusters by surface  
296 protein expression of CD56 and CD57 (Extended Data Fig. 6a-b). These clusters were present in  
297 both human cytomegalovirus (HCMV) seropositive and HCMV seronegative individuals, with  
298 an enrichment of CD56<sup>dim</sup>CD57<sup>+</sup> NK cells in HCMV<sup>+</sup> samples (Fig. 4e). Both CD56<sup>dim</sup>CD57<sup>-</sup>  
299 and CD56<sup>dim</sup>CD57<sup>+</sup> NK cells expressed higher levels of genes upregulated in ENKP\_NK cells,  
300 whereas CD56<sup>bright</sup> NK cells expressed higher levels of genes with higher expression in  
301 ILCP\_NK cells (Fig. 4f). Human killer immunoglobulin-like receptors (KIRs) are functionally  
302 similar to mouse Ly49 receptors<sup>34</sup>. NK cells expressing KIRs including *KIR2DL1*, *KIR2DL2* and  
303 *KIR2DL3* expanded upon HCMV infection<sup>35</sup>; these KIRs were mostly expressed in CD56<sup>dim</sup> NK  
304 cells but not CD56<sup>bright</sup> NK cells (Extended Data Fig. 6c), consistent with previous studies<sup>36</sup>.  
305 Importantly, CD56<sup>dim</sup>CD57<sup>-</sup> and CD56<sup>dim</sup>CD57<sup>+</sup> NK cells had higher ENKP\_NK scores whereas  
306 CD56<sup>bright</sup> NK cells had higher ILCP\_NK scores (see Methods for scores), in both the HCMV<sup>+</sup>  
307 and HCMV<sup>-</sup> donors (Fig. 4g), suggesting HCMV status did not affect the transcriptional  
308 similarity between human NK subsets and mouse NK subsets. Furthermore, we also assessed the

309 correlation between each of the mouse NK clusters identified by scRNA-seq (Extended Data Fig.  
310 4f) with each of the human NK clusters by transcriptional similarity. Mouse NK cluster 0 which  
311 was enriched in ENKP\_NK cells, was more correlated with human CD56<sup>dim</sup>CD57<sup>-</sup> and  
312 CD56<sup>dim</sup>CD57<sup>+</sup> NK cells whereas mouse NK cluster 1 which was enriched in ILCP\_NK cells,  
313 was more correlated with human CD56<sup>bright</sup> NK cells (Extended Data Fig. 6d). Consistently, the  
314 total ENKP\_NK cell population was more correlated with human CD56<sup>dim</sup>CD57<sup>-</sup> and  
315 CD56<sup>dim</sup>CD57<sup>+</sup> NK cells whereas total ILCP\_NK population was more correlated with human  
316 CD56<sup>bright</sup> NK cells (Extended Data Fig. 6e). Together, these results indicate that developmental  
317 heterogeneity in NK cells may be evolutionarily conserved between human and mouse.

318

### 319 **ENKP-derived NK cells respond more robustly to MCMV infection than ILCP-derived NK** 320 **cells**

321 Since ENKPs and ILCPs have different lineage potentials for Ly49H<sup>+</sup> NK cells, we challenged  
322 ENKP or ILCP-reconstituted NSG mice with MCMV to compare the function of ENKP\_NK  
323 cells and ILCP\_NK cells. NSG mice were reconstituted with the same number of ENKPs or  
324 ILCPs. The mice were then challenged with MCMV and Ly49H<sup>+</sup> NK cells were subsequently  
325 assessed in spleen and blood (Fig 5a). Upon MCMV infection, the numbers of ILCP\_NK cells in  
326 spleen remained low (Fig 5b-d). However, the numbers of ENKP\_NK cells were approximately  
327 150-fold higher than ILCP\_NK cells in spleen with MCMV infection (Fig. 5d) whereas those  
328 were only 4-fold higher without MCMV infection (Fig. 5c). In addition, ENKP\_NK cells  
329 consisted primarily of Ly49H<sup>+</sup> NK cells following MCMV infection (Fig. 5b and d). These data  
330 indicated ENKP-derived NK cells expanded dramatically. Consistently, Ly49H<sup>+</sup> ENKP\_NK  
331 cells in blood also expanded robustly upon MCMV infection (Extended Data Fig. 7a-c). To  
332 additionally compare ENKP\_NK cells and ILCP\_NK cells, we co-transferred ENKPs and ILCPs  
333 in the same mice. Because ILCPs generated 4-fold fewer NK cells than ENKPs (Fig. 5c), we co-  
334 transferred of ENKPs and ILCPs at a 1:4 ratio to normalize the number of NK cells from each  
335 source prior to MCMV infection. Following infection, ENKP\_NK cells represented the  
336 overwhelming majority compared to ILCP\_NK cells in the same environment (Extended Data  
337 Fig. 7d). In addition, in long-term PLZF lineage-tracing chimeras, the ratios of ILCP\_NK-  
338 enriched PLZF-traced NK cells were significantly decreased after MCMV infection, suggesting  
339 PLZF-untraced (YFP<sup>-</sup>) NK cells expanded to a greater degree than PLZF-traced (YFP<sup>+</sup>) NK cells

340 (Fig. 5e-f), and further demonstrating that ENKP\_NK cells expanded more than ILCP\_NK cells  
341 during MCMV infection. In summary, ENKP\_NK cells expanded more robustly than ILCP\_NK  
342 cells upon MCMV infection.

343

#### 344 **ENKP\_NK cells and ILCP\_NK cells distinguished by surface markers**

345 We identified surface markers differentially expressed between ENKP\_NK cells and ILCP\_NK  
346 cells by scRNA-seq, and verified some of these markers by flow cytometry, including Ly49H,  
347 Ly49D, CD226, Thy1.2 and CXCR3 (Fig. 4d, Extended Data Fig. 5c and 8a). Combinations of  
348 these markers allowed us to identify an ENKP\_NK-enriched cell population (Ly49H<sup>+</sup> or  
349 Ly49D<sup>+</sup>), which was lowly traced by PLZF (~10%); and an ILCP\_NK-enriched cell population  
350 (Ly49H<sup>-</sup>Ly49D<sup>-</sup>CD226<sup>+</sup>Thy1.2<sup>+</sup> or CXCR3<sup>+</sup>), which was highly traced (~50%), similar to the  
351 tracing ratios in ILC1s in this set of experiments (~55%) (Fig. 6a). The ENKP\_NK-enriched cell  
352 population expressed lower levels of TCF1-YFP than the ILCP\_NK-enriched cell population  
353 (Extended Data Fig. 8b), consistent with the results by adoptive transfer (Extended Data Fig. 5d).  
354 Furthermore, the PLZF labeling ratios of the NK subsets identified by these surface markers  
355 remained unchanged after MCMV infection (Extended data Fig. 8c), indicating these markers  
356 identify populations that do not substantially interconvert. The expansion of the ENKP\_NK-  
357 enriched cell population in long-term chimeras and wild-type B6 mice during MCMV infection  
358 was similar to the population expansion of Ly49H<sup>+</sup> NK cells in wild-type mice (Extended Data  
359 Fig. 8d-e), as reported<sup>37</sup>.

360

361 Consistent with the adoptive transfer analysis (Extended Data Fig. 3i), both ENKP\_NK-enriched  
362 and ILCP\_NK-enriched cell populations but not ILC1s were greatly reduced in *Eomes*-deficient  
363 mice (Extended Data Fig. 8f). However, these NK subsets remained intact in *Zbtb16*-deficient  
364 mice (Extended Data Fig. 8g), indicating the developmental divergence between ENKP\_NK  
365 cells and ILCP\_NK cells does not depend on PLZF.

366

367 Downregulation of CD27 and upregulation of CD11b and KLRG1 is correlated with NK cell  
368 maturation<sup>38,39</sup>. However, when CD27<sup>+</sup>CD11b<sup>-</sup> NK cells were adoptively transferred to new  
369 hosts, ~30% of them remained CD11b<sup>-</sup>, suggesting the CD27<sup>+</sup>CD11b<sup>-</sup> NK subset does not only  
370 contain immature NK cells<sup>38</sup>. Interestingly, the CD27<sup>+</sup>CD11b<sup>-</sup> subset was enriched within ILCP\_

371 NK cells compared to ENKP\_NK cells by adoptive transfer of ILCPs or ENKPs (Extended Data  
372 Fig. 9a). Consistently, the CD27<sup>+</sup>CD11b<sup>-</sup> NK subset was traced by PLZF at a higher ratio than  
373 CD27<sup>-</sup>CD11b<sup>+</sup> NK subset (Extended Data Fig. 9b). Similarly, ENKP\_NK cells expressed higher  
374 levels of KLRG1 (Extended Data Fig. 9c) and KLRG1<sup>-</sup>CD11b<sup>-</sup> “immature” NK cells were traced  
375 by PLZF at a higher ratio than KLRG1<sup>+</sup>CD11b<sup>+</sup> NK cells (Extended Data Fig. 9d). These data  
376 indicate that previously defined immature NK populations are likely a mixture of immature NK  
377 cells and mature ILCP\_NK cells. We also assessed recently described Ly49H<sup>+</sup>CD27<sup>+</sup>CD62L<sup>-</sup>  
378 tissue-resident NK cells that are phenotypically similar to ILCP\_NK cells<sup>40</sup>. Although ILCP\_NK  
379 cells expressed lower levels of CD62L than ENKP\_NK cells (Extended Data Fig. 9c),  
380 Ly49H<sup>+</sup>CD27<sup>+</sup>CD62L<sup>-</sup> NK cells were lowly traced by PLZF, indicating they are not derived  
381 from ILCPs but are likely a subset of ENKP\_NK cells (Extended Data Fig. 9e).

382

### 383 **ILCP\_NK cells are better cytokine producers**

384 To assess cytokine production and cytotoxicity by ENKP\_NK cells versus ILCP\_NK cells, we  
385 first separately sorted ILCP\_NK-enriched and ENKP\_NK-enriched cell populations, and  
386 stimulated them *in vitro* to assess their expression of IFN- $\gamma$ , TNF- $\alpha$  and CD107a (Extended Data  
387 Fig. 10a). The ILCP\_NK-enriched cell population contained higher frequencies of cells  
388 expressing IFN- $\gamma$  and TNF- $\alpha$  but comparable frequencies of CD107a-expressing cells when  
389 compared to the ENKP\_NK-enriched cell population (Extended Data Fig. 10b). In addition, we  
390 also assessed *in vitro* killing ability for both ENKP\_NK-enriched and ILCP\_NK-enriched cell  
391 populations by co-culturing them with Yac-1 target cells. We found both NK subsets killed Yac-  
392 1 cells comparably (Extended Data Fig. 10c). *In vitro* differentiated ILCP\_NK cells also had  
393 higher frequencies of cells expressing IFN- $\gamma$  upon stimulation as compared to ENKP\_NK cells  
394 (Extended Data Fig. 10d). These data indicate that ILCP\_NK cells have better cytokine  
395 producing ability than ENKP\_NK cells.

396

### 397 **Function of ILCP\_NK cells and ENKP\_NK cells upon Salmonella infection and Herpes 398 Simplex Virus (HSV) infection**

399 NK cells have been suggested to contribute to immunity against Salmonella infection<sup>41,42</sup>. We  
400 assessed total cell number and IFN- $\gamma$  expression in ILCP\_NK-enriched and ENKP\_NK-enriched  
401 cell populations in cecum lamina propria upon Salmonella infection through oral gavage (Fig.

402 6b). Although both NK subsets increased in number after Salmonella infection (Fig. 6c and  
403 Extended Data Fig. 10e), the ILCP\_NK-enriched cell population expressed higher levels of IFN-  
404  $\gamma$  than ENKP\_NK-enriched subset, indicating that ILCP\_NK cells contribute to the immune  
405 response to Salmonella infection and have better cytokine producing ability than ENKP\_NK  
406 cells (Fig. 6d,e and Extended Data Fig. 10f). Interestingly, the ILC1 population did not expand  
407 and expressed significantly lower levels of IFN- $\gamma$  than the ILCP\_NK-enriched cell population  
408 (Fig. 6d,e and Extended Data Fig. 10f). NK cells also can help control Herpes Simplex Virus  
409 (HSV) infection<sup>43</sup>. We assessed total cell number and IFN- $\gamma$  expression in ILCP\_NK-enriched  
410 and ENKP\_NK-enriched cell populations in ear skin upon HSV infection by intradermal  
411 injection (Fig. 6e). Similar to Salmonella infection, both NK subsets expanded during HSV  
412 infection (Fig. 6f and Extended Data Fig. 10g) and the ILCP\_NK-enriched cell population  
413 contained higher frequencies of cells expressing IFN- $\gamma$  (Fig. 6g-i and Extended Data Fig. 10h),  
414 indicating that ILCP\_NK cells contribute to the immune response to HSV infection and have  
415 better cytokine producing ability than ENKP\_NK cells. Although the ILC1 population also  
416 expanded after HSV infection, the expansion of the ILCP\_NK-enriched cell population was  
417 more robust (34-fold vs 3-fold on day 3, 40-fold vs 11-fold on day 7) (Fig. 6g). Thus these two  
418 additional models of infection each showed that both ILCP\_NK cells and ENKP\_NK cells  
419 contribute to immune responses, but ILCP\_NK cells are more robust cytokine producers during  
420 these infections.

421

## 422 Discussion

423 The early development of NK cells and the relationship between functional heterogeneity of NK  
424 cells and developmental differences are both poorly understood. Here we established the  
425 existence of two separate pathways of NK cell development in mice. We identified ENKPs as a  
426 novel early NK progenitor which was the primary source of Ly49H<sup>+</sup> NK cells, and established  
427 that ENKP\_NK cells expanded dramatically upon MCMV infection. In contrast, ILCP\_NK cells  
428 were heavily lineage-traced by PLZF, were largely depleted for expression of Ly49H, and failed  
429 to robustly respond to MCMV infection. ILCP\_NK cells contained higher frequencies of cells  
430 expressing IFN- $\gamma$  *in vitro* and upon infections with Salmonella or HSV, consistent with previous  
431 work establishing that Ly49H<sup>lo</sup> NK cells possessed greater potential for IFN- $\gamma$  production than  
432 Ly49H<sup>hi</sup> NK cells<sup>44</sup>. Interestingly, mouse ENKP\_NK and ILCP\_NK cell populations were

433 transcriptionally and functionally similar to human CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells,  
434 respectively. However, it is likely that differences of IFN- $\gamma$  expression between ILCP\_NK cells  
435 and ENKP\_NK cells are context-dependent as human CD56<sup>bright</sup> NK cells produce more IFN- $\gamma$  in  
436 response to cytokines<sup>45</sup> but less IFN- $\gamma$  in response to target cells or engagement of activating  
437 receptors compared to CD56<sup>dim</sup> NK cells<sup>46</sup>.

438

439 Previous studies established that ILCPs give rise to NK cells *in vivo*<sup>3,4</sup>, and ILCP\_NK cells are  
440 phenotypically similar to previously described ILC1-like NK cells<sup>47,48</sup>, although the  
441 developmental relationships between ILC1-like NK cells and ILCP\_NK cells remain to be  
442 determined. ILCP\_NK cells shared some phenotypes of ILC1s as well. However, our study  
443 showed that ILCP\_NK cells also express the canonical NK cell transcription factor *Eomes* and  
444 both ILCP\_NK cells and ENKP\_NK cells but not ILC1s depend on *Eomes* for development,  
445 indicating they are distinct from previously described ILC1s<sup>49,50</sup>. We also compared  
446 transcriptomes of ILCP\_NK cells, ENKP\_NK cells and ILC1s, and found that ILCP\_NK cells  
447 were transcriptionally more similar to ENKP\_NK cells than to ILC1s. After *Salmonella*  
448 infection, both ILCP\_NK-enriched and ENKP\_NK-enriched cell populations expanded, but the  
449 ILC1 population did not. Thus, ILCP\_NK cells are transcriptionally and functionally more  
450 similar to ENKP\_NK cells than to ILC1s.

451

452 ILCPs gave rise to NK cells less efficiently than ENKPs, which could be explained by the lower  
453 frequency of precursors that have NK lineage potential within the ILCP population revealed by  
454 our clonal assay (Fig. 2b). Thus, the clonal burst of NK progeny may be equivalent or even  
455 higher from NK-lineage competent cells within ILCPs compared to ENKPs.

456

457 Mouse Ly49H<sup>+</sup> NK cells have been shown to possess adaptive immune features after exposure to  
458 MCMV infection<sup>13,30</sup>. Similarly, human CD56<sup>dim</sup>NKG2C<sup>+</sup> NK cells expand robustly in HCMV-  
459 seropositive individuals<sup>51</sup>, where HCMV-induced “memory” NK cells are enriched within the  
460 CD56<sup>dim</sup> NK population. These similarities between mouse and human suggest that conserved  
461 developmental pathways may underlie the functional heterogeneity in both species. Human  
462 NKG2C and mouse Ly49H are similar in both function and adaptor DAP12 usage<sup>13,51</sup>, and



463 HCMV and MCMV have similar genomic structures<sup>52,53</sup>, suggesting co-evolution of CMV and  
464 NK cells expressing CMV-specific receptors<sup>54</sup>.

465

466 Whether human CD56<sup>bright</sup> NK cells are precursors of CD56<sup>dim</sup> NK cells, or whether these  
467 populations also reflect developmental heterogeneity, is not clearly known<sup>55,56</sup>. However,  
468 transcriptional signatures derived from developmentally distinct mouse NK lineages were  
469 differentially expressed in human blood NK populations, suggesting that developmental  
470 heterogeneity also contributes to the heterogeneous human NK subsets. Indeed, barcoding-based  
471 lineage tracing revealed that CD56<sup>-</sup>CD16<sup>+</sup> and CD56<sup>+</sup>CD16<sup>-</sup> NK cells in non-human primates  
472 are developmentally different<sup>57</sup>, indicating different development pathways of NK cells may  
473 exist and be conserved in multiple vertebrate species. **In future, similar barcoding**  
474 **approaches will provide additional insight into the developmental pathways that generate NK**  
475 **cells in mice.**

476

477 We described surface markers that discriminate NK populations enriched for ENKP\_NK cells  
478 and ILCP\_NK cells in wild-type B6 mice, which will facilitate future studies of the potential  
479 distinct functions of ENKP\_NK cells and ILCP\_NK cells in other models. Recruitment of  
480 conventional type 1 dendritic cells (cDC1) in tumor microenvironment depends on NK cells that  
481 produce the chemoattractants XCL1 and CCL5<sup>58</sup>. Interestingly, *Xcl1* was highly expressed in  
482 ILCP\_NK cells but not ENKP\_NK cells whereas *Ccl5* was expressed more highly in ENKP\_NK  
483 cells (Extended Data Fig. 4d), suggesting that ILCP\_NK cells could be an important source of  
484 XCL1 production in some tumor microenvironments, and ILCP\_NK cells and ENKP\_NK cells  
485 may collaborate in eliminating tumors. Further, *XCL1* is also expressed more highly in human  
486 CD56<sup>bright</sup> NK cells whereas *CCL5* is expressed more highly in CD56<sup>dim</sup> NK cells (Fig. 4e and g);  
487 thus this potential collaboration between NK subsets might be conserved.

488

489 Motifs for ZEB2 and TCF7 were enriched in CD56<sup>dim</sup>CD57<sup>+</sup> NK cells and CD56<sup>bright</sup> NK cells,  
490 respectively<sup>10</sup>, implicating these transcription factors in regulating gene expression in specific  
491 NK subsets. *Zeb2* and *Tcf7* are also relatively highly expressed in ENKP\_NK cells and  
492 ILCP\_NK cells, respectively (Fig. 4c), thus gene regulatory programs in NK cells are predicted  
493 to be conserved between human and mouse. ILCP\_NK cells expressed higher levels of *Cd69*

494 (Fig. 4b) and GO enrichment analysis also revealed ILCP\_NK cells are involved in biological  
495 processes such as cell-cell adhesion, lymphocyte activation (Extended Data Fig. 4e), which  
496 suggests their tissue-resident features, similar to ILC1. We speculate that some specialization for  
497 tissue-residency is established developmentally, which is one direction for future work.

498

499 Our study implicates different developmental pathways as underlying functional heterogeneity of  
500 NK cells and identifies precursors for conventional NK cells expressing Ly49H in mice. We  
501 speculate ILCP\_NK cells evolved initially, and viruses and perhaps specifically CMV drove the  
502 existence of a separate population of NK cells with adaptive features. Thus, although the  
503 activating receptors for response to CMV are different in humans and mice, the underlying logic  
504 of developmental pathways that generate NK cell subsets specialized for the same threat may be  
505 conserved. Further, if these developmental pathways were already present in the common  
506 ancestor of humans and mice, as seems likely, then we predict such developmental pathways are  
507 present in many related vertebrate species.

508

## 509 **Methods**

### 510 **Mice**

511 *Tcf7<sup>YFP</sup>*, *Tcf7<sup>GFP</sup>*, *Zbtb16<sup>GFPcre</sup>*, *Tcf7<sup>-/-</sup>*, *Nfil3<sup>-/-</sup>*, *Il7<sup>cre</sup>*, *Gata3<sup>flox</sup>*, *Eomes<sup>flox</sup>*, *Eomes<sup>GFP</sup>* mice were  
512 described previously<sup>2,3,16,23,24,59-62</sup>. B6-Ly5.2 (CD45.1) and B6.129X1-

513 *Gt(ROSA)26Sor<sup>tm1(EYFP)Cos/J</sup>* (strain #: 006148) mice were obtained from Jackson Laboratory.

514 NOD-scid IL2Rgamma<sup>null</sup> (NSG) mice were obtained from National Cancer Institute. All mice  
515 except NSG mice are maintained on C57BL/6 background. Mice were 6-18 weeks of age and of  
516 either sex. Animal procedures were approved by relevant National Institutes of Health Animal  
517 Care and Use Committees.

518

### 519 **Antibodies and flow cytometry**

520 Bone marrow, spleen and liver samples were incubated with a mix of purified rat, mouse and  
521 hamster immunoglobulin G (IgG) before the addition of specific antibodies, and then stained and  
522 analyzed in FACS buffer (PBS containing 2 mM EDTA and 0.5% FBS). The following anti-  
523 mouse antibodies were used for immunostaining: 2B4 (eBio244F4, PE-Cy7, Thermo Fisher  
524 Scientific, 25-2441-82),  $\alpha 4\beta 7$  (DATK32, BV711, BD biosciences, 740701; DATK32, PerCP-

525 Cy5.5, Thermo Fisher Scientific, 46-5887-82), B220 (RA3-6B2, biotin, Thermo Fisher  
526 Scientific, 13-0452-86), CD11c (N418, biotin, Thermo Fisher Scientific, 13-0114-85), CD122  
527 (TM-b1, PE, Thermo Fisher Scientific, 12-1222-82), CD19 (eBio1D3, biotin, Thermo Fisher  
528 Scientific, 13-0193-85; 6D5, BV605, BioLegend, 115540), CD200R (OX110, APC, Thermo  
529 Fisher Scientific, 17-5201-82), CD25 (BC96, PE, Thermo Fisher Scientific, 12-0259-80), CD27  
530 (LG.7F9, PerCP-Cy5.5, Thermo Fisher Scientific, 46-0271-82), CD3 (17A2, AF700, Thermo  
531 Fisher Scientific, 56-0032-82), CD3e (eBio500A2, biotin, Thermo Fisher Scientific, 13-0033-86;  
532 145-2C11, BV510, BioLegend, 100353; 145-2C11, SB600, Thermo Fisher Scientific, 63-0031-  
533 82), CD4 (GK1.5, biotin, Thermo Fisher Scientific, 13-0041-82; RM4-5, SB600, Thermo Fisher  
534 Scientific, 63-0042-82), CD45.1 (A20, eF450, Thermo Fisher Scientific, 48-0453-82; A20, PE-  
535 eF610, Thermo Fisher Scientific, 61-0453-82), CD45.2 (104, eF450, Thermo Fisher Scientific,  
536 48-0454-82; 104, PerCP-Cy5.5, BioLegend, 109828), CD49a (HM $\alpha$ 1, APC, BioLegend,  
537 142606), CD49b (DX5, AF700, Thermo Fisher Scientific, 56-5971-82; DX5, APC-Cy7, Thermo  
538 Fisher Scientific, 47-5971-82; DX5, PE-eF610, Thermo Fisher Scientific, 61-5971-82), CD5  
539 (53-7.3, BV605, BD biosciences, 563194), CD8 $\alpha$  (53-6.7, biotin, Thermo Fisher Scientific, 13-  
540 0081-85; 53-6.7, SB600, Thermo Fisher Scientific, 63-0081-82), CD8 $\beta$  (CT-CD8b, biotin,  
541 Thermo Fisher Scientific, MA5-17845; eBioH35-17.2, SB600, Thermo Fisher Scientific, 63-  
542 0083-82), Flt3 (A2F10.1, PE-CF594, BD biosciences, 562537), Gr-1 (RB6-8C5, biotin, Thermo  
543 Fisher Scientific, 13-5931-86), ICOS (7E.17G9, PE, Thermo Fisher Scientific, 12-9942-82), IL-  
544 7R $\alpha$  (A7R34, APC, Thermo Fisher Scientific, 17-1271-82), kit (2B8, BV785, BioLegend,  
545 105841; 2B8, PE-Cy7, Thermo Fisher Scientific, 25-1171-82), Ly49H (3D10, PE, BioLegend,  
546 144706; 3D10, FITC, Thermo Fisher Scientific, 11-5886-82), Ly49D (4E5, BV 711, BD  
547 biosciences, 742559), Mac-1 (M1/70, biotin, Thermo Fisher Scientific, 13-0112-86), NK1.1  
548 (PK136, APC-Cy7, Thermo Fisher Scientific, 47-5941-82; PK136, biotin, Thermo Fisher  
549 Scientific, 13-5941-85; PK136, BV785, BioLegend, 108749), NKp46 (29A1.4, PE-Cy7, Thermo  
550 Fisher Scientific, 25-3351-82), streptavidin (eF450, Thermo Fisher Scientific, 48-4317-82; APC-  
551 Cy7, Thermo Fisher Scientific, 47-4317-82), Sca1 (D7, AF700, Thermo Fisher Scientific, 56-  
552 5981-82), TCR $\beta$  (H57-597, biotin, Thermo Fisher Scientific, 13-5961-85; H57-597, SB600,  
553 Thermo Fisher Scientific, 63-5961-82), TCR $\gamma\delta$  (eBioGL3, biotin, Thermo Fisher Scientific, 13-  
554 5711-85; eBioGL3, SB600, Thermo Fisher Scientific, 63-5711-82), Ter119 (TER-119, biotin,  
555 Thermo Fisher Scientific, 13-5921-85), Thy1.2 (53-2.1, AF700, BioLegend, 140324; 53-2.1,

556 BV605, BioLegend, 140318), CD226 (TX42.1, BV650, BioLegend, 133621), CXCR3 (CXCR3-  
557 173, APC, Thermo Fisher Scientific, 17-1831-82). The lineage 'cocktail' (Lin) is a mix of the  
558 following biotin antibodies: anti-Gr-1, NK1.1, CD19, Ter119, CD11c, CD3, CD4, CD8 $\alpha$ , CD8 $\beta$ ,  
559 TCR $\beta$ , TCR $\gamma\delta$ , Ly-6D, B220, Mac-1. T lineage 'cocktail' (Lin<sup>T</sup>) contains anti-CD3, CD4,  
560 CD8 $\alpha$ , CD8 $\beta$ , TCR $\beta$ , TCR $\gamma\delta$ . Non-T/NK lineage 'cocktail' contains anti-Gr-1, CD19, Ter119,  
561 CD11c, Ly-6D, B220.

562

### 563 **BM progenitor definition and isolation**

564 BM progenitors were defined and isolated by flow cytometric sort as follows. ALPs (Lin<sup>-</sup>  
565 Kit<sup>+</sup> $\alpha$ 4 $\beta$ 7<sup>-</sup>Tcf7<sup>-</sup>Flt3<sup>+</sup>IL-7R $\alpha$ <sup>+</sup>), ILCPs (Lin<sup>-</sup>Kit<sup>+</sup> $\alpha$ 4 $\beta$ 7<sup>+</sup>Tcf7<sup>YFP+</sup>Flt3<sup>-</sup>Thy1.2<sup>+</sup> or Lin<sup>-</sup>Kit<sup>+</sup> $\alpha$ 4 $\beta$ 7<sup>+</sup>PD-  
566 1<sup>+</sup>Flt3<sup>-</sup>Thy1.2<sup>+</sup>) and ENKPs (Lin<sup>-</sup>CD27<sup>+</sup>2B4<sup>+</sup>Flt3<sup>+</sup>CD122<sup>+</sup>) were isolated from Tcf7<sup>YFP</sup> mice.  
567 For PLZF tracing (Fig 3b), ILCPs were sorted as Lin<sup>-</sup>Kit<sup>+</sup> $\alpha$ 4 $\beta$ 7<sup>+</sup>Tcf7<sup>GFP+</sup>Flt3<sup>-</sup>Thy1.2<sup>+</sup>. Before  
568 sorting of BM progenitors, bone marrow samples were stained with biotin lineage 'cocktail'  
569 (Lin), and then depleted by streptavidin beads (Biolegend, 480016) according to the  
570 manufacturer's instructions.

571

### 572 **Cell culture**

573 BM progenitors were sorted by flow cytometric cell sorting. BM progenitors were cultured on  
574 irradiated OP9 stromal cells in  $\alpha$ -MEM supplemented with 20% fetal bovine serum, penicillin  
575 and streptomycin. Bulk cultures were supplemented with 30 ng/ml SCF, 30 ng/ml IL-7, 30 ng/ml  
576 Flt3L and 30 ng/ml IL-2, and progenitor differentiation was examined at 7d after seeding. Single  
577 cell cultures were supplemented with 30 ng/ml SCF, 30 ng/ml IL-7, 30 ng/ml Flt3L and 10 ng/ml  
578 IL-2, and progenitor differentiation was examined at 10d after seeding. For single cell assay,  
579 positive well was defined as > 10 total ILCs, and positive lineage was defined as frequency >  
580 10% and number  $\geq$  3. For *in vitro* stimulation, NK cells sorted from bulk culture were  
581 incubated with IL-12 (20 ng/ml), IL-18 (10 ng/ml) or PMA (50 ng/ml), Ionomycin (500 ng/ml)  
582 for 1 hr, then Brefeldin A was added to a final concentration of 5  $\mu$ g/ml for another 2 hrs'  
583 incubation. Cytokines were purchased from PeproTech.

584

### 585 **Adoptive transfer**

586 For ENKP and ILCP transfer, around 200-500 progenitors (CD45.2) were injected into NSG  
587 recipient mice (CD45.1) if not mentioned specifically. For competitive assay, around 300-500  
588 test progenitors (CD45.2) were mixed with same numbers of competitor progenitors (CD45.1)  
589 and injected into NSG recipient mice (CD45.1). Reconstitution of lymphocytes was examined at  
590 6-weeks post-transfer. For PLZF lineage tracing chimeras,  $3 \times 10^5$  YFP<sup>+</sup> LK (Lin<sup>-</sup>Kit<sup>+</sup>)  
591 progenitors (CD45.2) obtained by cell sorting were injected into lethally irradiated (850 rads)  
592 wild-type B6-Ly5.2 recipient mice (CD45.1), or injected into sublethally irradiated (250 rads)  
593 NSG mice. Reconstitution of lymphocytes and progenitors was examined at 12-weeks post-  
594 transfer.

595

### 596 **Calculation of ENKP\_NK cell and ILCP\_NK cell contributions to total NK population**

597 To calculate contribution percentage of ENKP\_NK cells and ILCP\_NK cells, the following  
598 equation set was used:  $10\% * X + 70\% * Y = a\%$ ;  $X + Y = 100\%$ . 10% was the observed PLZF  
599 tracing ratio of ENKP\_NK cells, 70% was the observed PLZF tracing ratio of ILCP\_NK cells,  
600 a% was the observed PLZF tracing ratio of total NK cells in different tissues (for example, in  
601 liver a% was 18%, Extended Fig. 5h), X was the calculated percentage of ENKP\_NK cells and Y  
602 was the calculated percentage of ILCP\_NK cells.

603

### 604 **scRNA-seq and analysis for mouse samples**

605 Cells from bone marrow were isolated by sorting from Tcf7<sup>YFP</sup> mice (Fig. 1). NK cells from  
606 spleen were sorted from NSG mice adoptively transferred with ALPs, ENKPs or ILCPs (Fig 4).  
607 scRNA-seq libraries were prepared using 10X Genomics Single Cell 3' Reagent Kits v3.1,  
608 according to the manufacturer's instructions. The obtained libraries were sequenced with a  
609 NextSeq system. Base calling was performed using RTA 3.9.2 (bone marrow samples) or RTA  
610 3.9.25 (spleen sample), and alignment was performed using cellranger v6.1.1 (STAR 2.7.2a). For  
611 spleen sample, hashtagging antibodies used for the analysis were mHTO01, mHTO02, and  
612 mHTO03 TotalSeqA bought from BioLegend, and demultiplexing was performed using  
613 cellranger v6.1.1 (Bcl2fastq 2.20.0). Cells with low UMI counts were determined by the 10x  
614 Genomics Cell Ranger Algorithm. Single-cell analysis was performed using Seurat (version  
615 4.1.0). Cells with >6% mitochondrial gene content and >8000 genes (bone marrow samples)  
616 or >4000 genes (spleen sample) were excluded from analysis. Mitochondrial content and cell-

617 cycle related genes were regressed out. T cell contaminants were removed from bone marrow  
618 samples. Cells were analyzed mainly by Seurat's functions<sup>63</sup>. Cells were clustered using Seurat's  
619 "FindClusters" function. Pseudotime analysis was performed using "Slingshot" package (version  
620 2.2.1)<sup>64</sup>. Differentially expressed genes (DEGs) were identified using Seurat's "FindMarkers"  
621 function considering those genes expressed in at least 10% of cells in the clusters with a  
622 minimum log<sub>2</sub> fold change threshold of 0.25. Violin plots were made using Seurat's normalized  
623 data. Average gene expression from cells of the same developmental origin was generated using  
624 the Seurat's "AverageExpression" function. Gene ontology pathway analysis of DEGs was  
625 carried out with the "enrichGO" function. Volcano plots were generated using "ggplot2"  
626 package (version 3.4.0).

627 To compare transcriptomes of mouse NK subsets and ILC1s, liver ILC1 and NK cell data were  
628 downloaded from the gene expression omnibus (GSE163452<sup>27</sup>) and loaded into Seurat. In addition,  
629 in house generated bone marrow derived NK and ILC1 scRNAseq (see Figure 1a) was loaded into  
630 Seurat. For published liver data, the hashtags used to label the publicly available data were  
631 demultiplexed using the HTODemux function, following CLR normalization. Cells from Hobit-  
632 KO mice were discarded, and cells were flagged as low quality and removed if they had  $\leq 1000$   
633 or  $\geq 8000$  reads, and  $> 6\%$  mitochondrial reads. All datasets were processed according to a  
634 standard Seurat pipeline. The FindNeighbors and FindClusters functions were used to cluster the  
635 cells and identify NK cells or ILC1s. Gene set scores were calculated using ENKP\_NK gene list  
636 (*Zeb2, Irf8, Klf2, Gzmb, Gzma, Prfl, Itgam, Ccl5, Klra3, Klra4, Klra7, Klra8, Klra9*) and  
637 ILCP\_NK gene list (*Tcf7, Cd160, Cd226, Cd7, Cd27, Ccr2, Cxcr3, Xcl1, Ltb, Ifng, Cd69*). These  
638 scores were fed into the kmeans function, allowing cells to be clustered using these gene scores.  
639 This resulted in the identification of 2 NK cell clusters, allowing them to be annotated as  
640 ENKP\_NK or ILCP\_NK. For each population of NK cells and ILC1s the average gene expression  
641 was calculated using the AverageExpression function, and Euclidean distance was calculated using  
642 the dist function. The averaged data was used to examine expression of genes previously  
643 determined to be differentially expressed between NK cells and ILC1s<sup>17</sup> that also were  
644 significantly different between NK cells and ILC1s in both liver and bone marrow, with a fold  
645 change  $\geq 0.25$  and FDR of  $\leq 0.05$ .

646

647 **scRNA-seq analysis of human NK cell**

648 The human NK scRNAseq data was obtained from GSE130430<sup>32</sup> and GSE197037<sup>33</sup> was  
649 analysed using a standard Seurat analysis. Batch correction was performed using the  
650 RunHarmony function from the “harmony” package to correct batch effects. The CITEseq data  
651 was normalized by applying a centered log ratio normalization. Clusters identified using the  
652 RNA modality were annotated based on the protein expression of CD56 and CD57. The  
653 annotation was extended using the glmnet and cv.glmnet function to all datasets. Cross species  
654 comparisons were done by identifying homologs between mouse and human using the  
655 homologene package; and by turning mouse genes to uppercase. Differentially expressed gene  
656 (DEG)s were identified by applying the “FindMarkers” function from Seurat within the  
657 populations or samples of one species. The DEG shared between species were retained and for  
658 each population or sample the counts of individual cells were summed using the  
659 “AggregateExpression” function and log transformed, generating pseudobulk data. The genes of  
660 the pseudobulk data were centered by dividing by their average gene expression. Spearman  
661 correlation was done on these matrices using the “cor” function, allowing correlation of the  
662 populations between different species. Scores were calculated by averaging the expression of  
663 gene sets identified in the mouse scRNA-seq data (ENKP\_NK score: ZEB2, IRF8, KLF2,  
664 GZMB, GZMA, PRF1, ITGAM and CCL5; ILCP\_NK score: TCF7, CD160, CD226, CD7,  
665 CCR2, CXCR3, XCL1, LTB, IFNG and CD69) and subtracting their average by the average  
666 gene expression of 100 randomly sampled genes. Plots were generated using either the “Seurat”  
667 or “ggplot2” packages.

668

### 669 **MCMV infection**

670 MCMV (Smith strain) was prepared as described previously<sup>44</sup> and virus titer was 2200 PFU/ml.  
671 MCMV stock was 1:400 diluted in 1XPBS and 200 µl of MCMV diluent was intraperitoneally  
672 injected into each mouse. Blood and spleen samples were analyzed by flow cytometry 7 days  
673 after infection.

674

### 675 **Salmonella infection and cecal immune cell isolation**

676 *Salmonella Typhimurium* strain IR715, a nalidixic acid resistant strain, was used for all  
677 experiments. Bacteria was cultured in Luria broth (LB) at 37°C shaking for 18 hours. Cells were  
678 washed in PBS and adjusted to final concentration of 5x10<sup>8</sup> CFU/mL in PBS. C57BL6 mice, 8–

679 10-week-old, were gavaged with 20mg streptomycin, and fasted overnight. 24 hours later mice  
680 were gavaged with  $10^8$  CFU *S. Typhimurium* in 200  $\mu$ L PBS. Infection was confirmed by  
681 measuring CFU at 24 hours by homogenizing fecal pellets in PBS and plating on LB agar with  
682 50 $\mu$ g/mL nalidixic acid. Cecal contents was also plated at takedown to quantify *S. Typhimurium*  
683 CFU. Mice were sacrificed at 1, 3 or 5 days post infection to analyze gut immune cells in the  
684 cecum. Immune cells from the cecum of *S. Typhimurium* infected mice were isolated as  
685 previously described with some modifications<sup>65</sup>. Briefly, ceca were extracted and contents  
686 removed into sterile PBS for CFU quantification. Ceca were then cut open longitudinally,  
687 washed and incubated in complete medium (RPMI 1640 plus 3% fetal bovine serum, 1 mM  
688 pyruvate, 0.1 mM nonessential amino acids, 2 mM glutamine, 20 mM HEPES, 100 U/ml  
689 penicillin, 0.1 mg/ml streptomycin, and 50  $\mu$ M  $\beta$ -mercaptoethanol) with 5mM EDTA and 1mM  
690 dithiothreitol for 15 minutes at 37°C stirring. Ceca were shaken 3 times in 10mL RPMI + 2mM  
691 EDTA to remove epithelial cells, then cut into small pieces for digestion in RPMI media  
692 containing 0.5 mg/ml Collagenase Type 4 (Worthington Biochemical), 8% FBS and 50 mM  
693 CaCl<sub>2</sub>. After a 30-minute digestion at room temperature with stirring, cells were passed through a  
694 70 $\mu$ m filter and washed with 10mL complete media. Finally, cells were pelleted, resuspended in  
695 37.5% Percoll, spun at 600g for 5 minutes, and cell pellets were resuspended in complete media  
696 for flow cytometry analysis.

697

### 698 **Herpes simplex virus-1 (HSV-1) infection and ear digestion**

699 Female mice were injected intradermally with 15 $\mu$ L of HSV-1 ( $1 \times 10^6$  PFU in sterile tissue-  
700 culture-grade PBS) in the ear pinnae using a 29G needle. Control mice were injected with same  
701 amount of sterile tissue-culture-grade PBS. Immune responses were analyzed at day 3 or 7 post  
702 infection. At day 3 or 7 post infection, mice were euthanized and ears were cut and the ventral  
703 and dorsal sheets of ears were separated and digested in the media with DNase and Liberase  
704 (RPMI 1640 media with 2 mM L-glutamine, 1 mM sodium pyruvate and nonessential amino  
705 acids, 55 mM  $\beta$ -mercaptoethanol, 20 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin,  
706 0.5 mg/ml DNase and 0.025 mg/ml Liberase TL purified enzyme blend) at 37°C for 1 hour and  
707 45min. Digested ears were homogenized and filtered through 70- $\mu$ m cell strainer.

708

### 709 ***In vitro* cytotoxicity assay**



710 Freshly sorted spleen NK cells were co-cultured with Yac-1 mouse lymphoma cells at 4:1, 2:1  
711 and 1:1 ratios in IMDM media (supplemented with 20% fetal bovine serum, nonessential amino  
712 acids, L-glutamine, 2-mercaptoethanol, penicillin and streptomycin) for 4 hours. Yac-1 cells  
713 were traced by CFSE to distinguish from NK cells. Specific lysis was calculated by the formula:  
714  $(a - b) / a \times 100\%$ ; a is the number of Yac-1 cells without NK cell co-culture; b is the number of  
715 Yac-1 cells with NK cell co-culture.

716

### 717 **Statistical analysis**

718 Statistical significance was performed with GraphPad Prism. Differences between groups were  
719 determined by a two-tailed unpaired or paired Student's t test and were considered to be  
720 statistically significant when P values were less than 0.05. P value < 0.05 (\*), P value < 0.01(\*\*),  
721 P value < 0.001 (\*\*\*)).

722

### 723 **Data availability**

724 Single cell RNA sequencing data will be deposited on GEO and made available.

725

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733

### 734 **Author contributions**

735 Conceptualization: A.B., Y.D.; Methodology: Y.D., M.L., S.G., V.B., L.C., S.C.S, J.M.;  
736 Investigation: Y.D., M.L., S.G., V.B., L.C., A.D., S.D., C.H., S.C.S., J.M., D.P., Y.Z., J.Z.,  
737 Y.B.,J.C.S.; Visualization: Y.D., M.L.; Supervision: A.B.; Writing: Y.D., M.L., V.B., L.C., A.B.

738

### 739 **Competing interests**

740 Authors declare that they have no competing interests.

741

742

743 **References**

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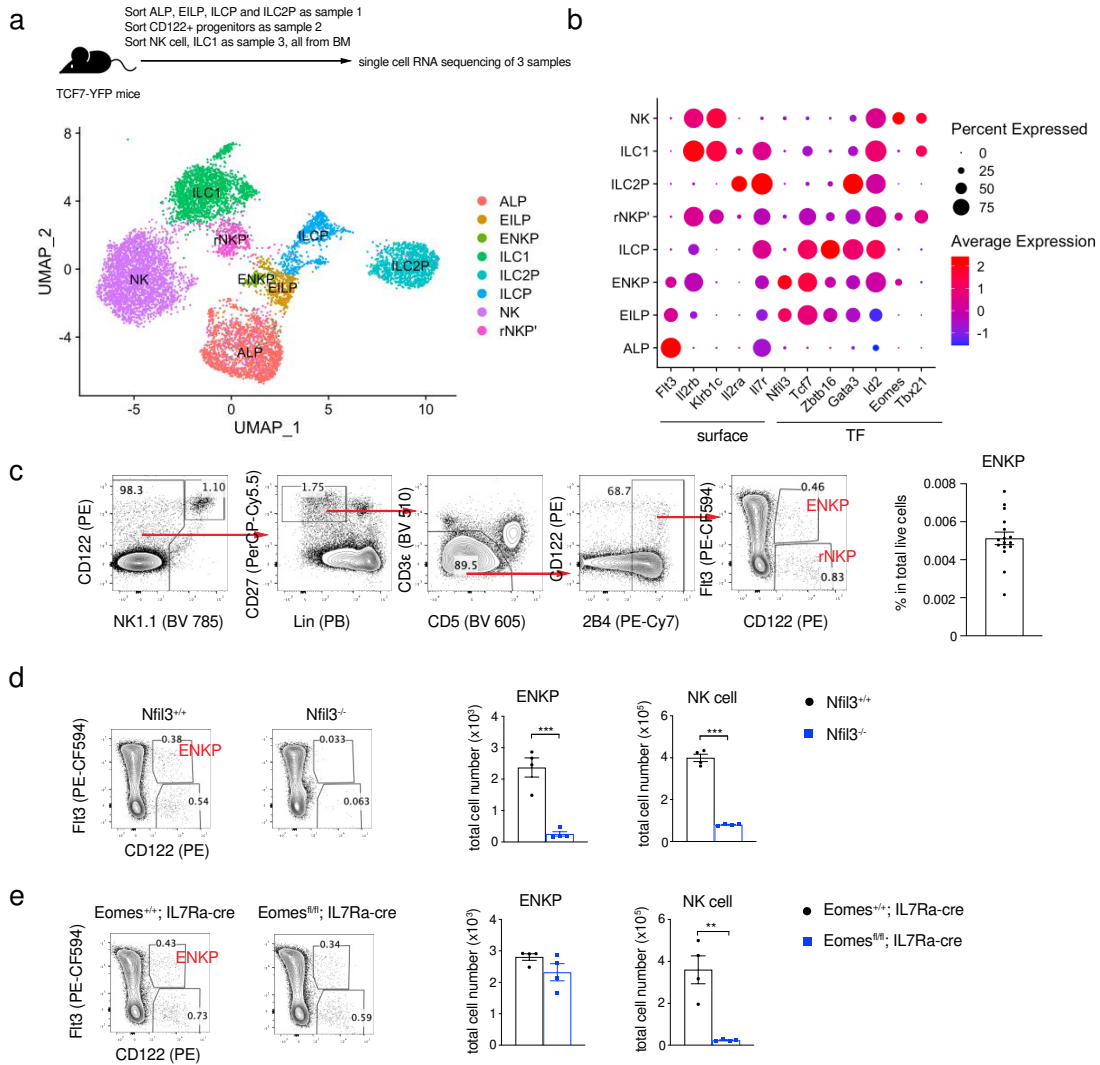
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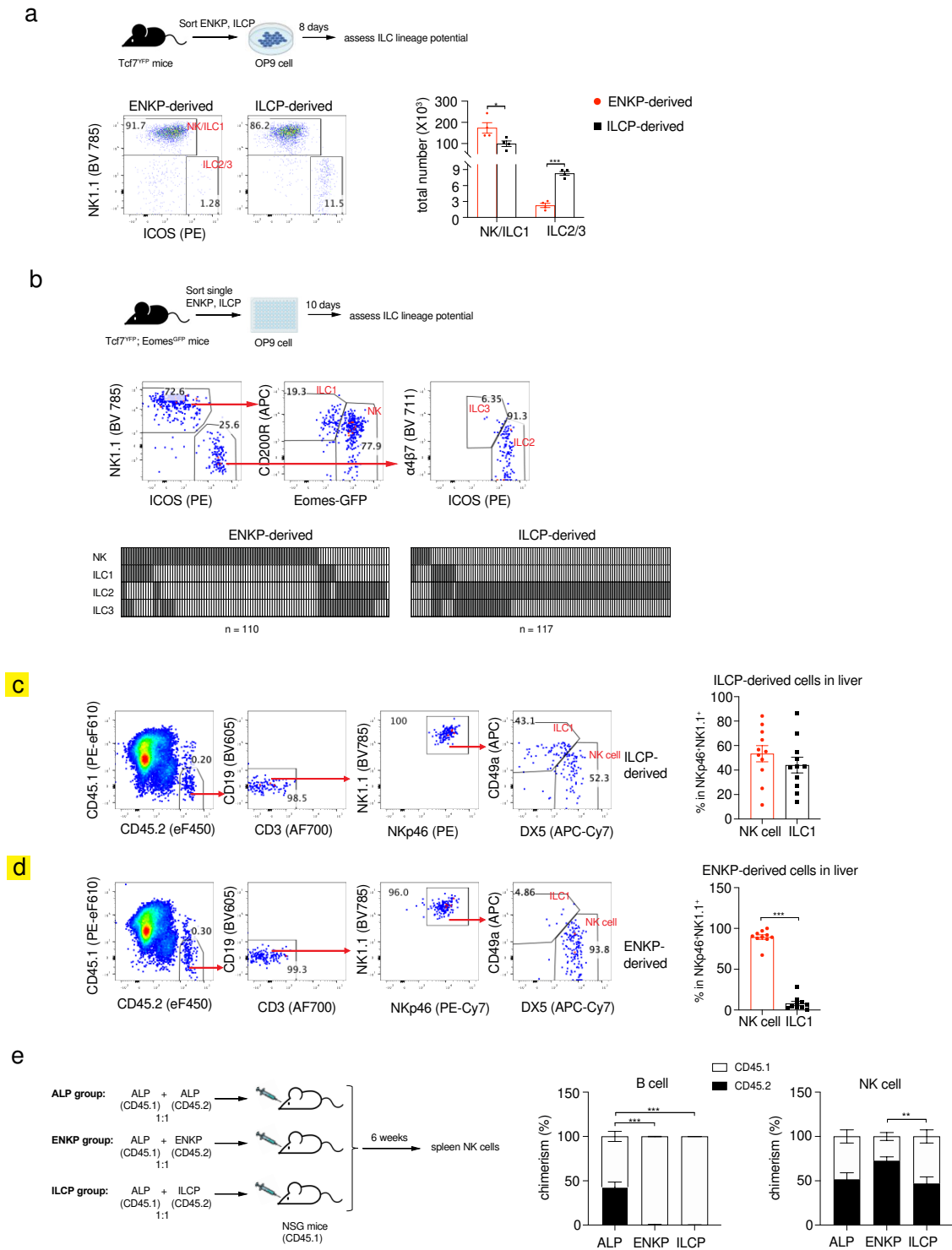
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Figure 1 Characterization of early NK cell development in bone marrow



**Fig.1 Characterization of early NK cell development in bone marrow.** **a - b**, scRNA-seq of bone marrow cells: Experimental schematic and UMAP (**a**), and dotplots (**b**) of expression of surface genes and transcription factors grouped by clusters in **a**. **c**, Gating strategy and frequency of ENKPs combined from 3 independent experiments. **d**, Representative flow plots of ENKPs and quantifications of ENKPs and NK cells in bone marrow of *Nfil3*-deficient mice,  $n = 4$ . Combined data from 2 independent experiments. **e**, Representative flow plots of ENKPs and quantifications of ENKPs and NK cells in bone marrow of *Eomes*-deficient mice,  $n = 4$ . Combined data from 2 independent experiments. For **c**, **d** and **e**, data are mean  $\pm$  s.e.m, and for **d** and **e**, statistical analysis was performed using two-tailed unpaired Student's t-tests.

**Figure 2 ENKPs are early NK-lineage biased progenitors**

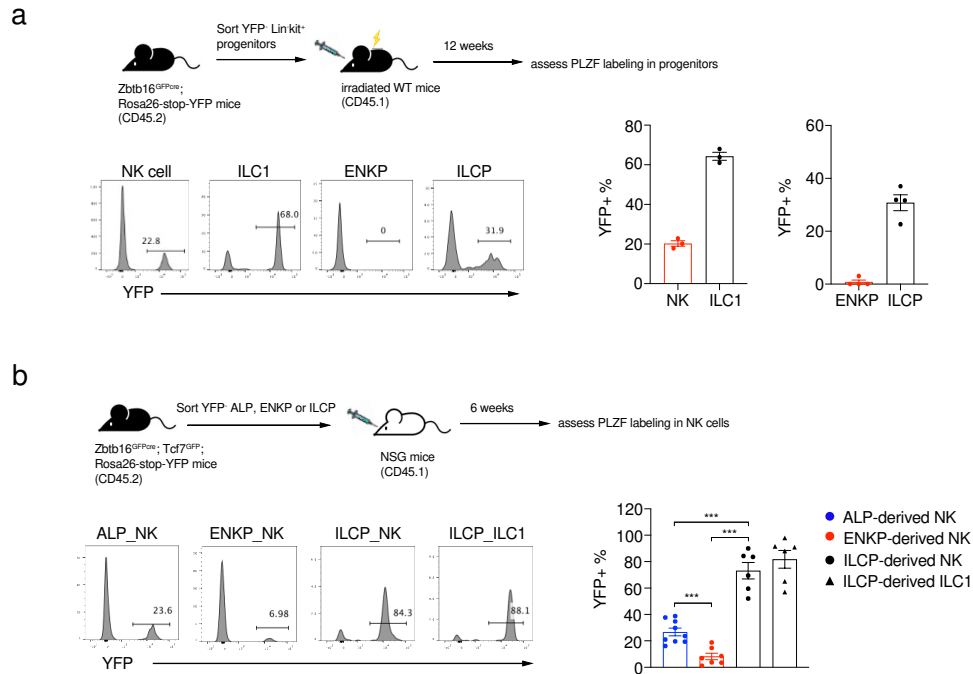


**Fig. 2 ENKPs are early NK-lineage biased progenitors. a**, Strategy of in vitro bulk assay for ILC lineage potential, representative flow plots and quantification of in vitro differentiated ILCs, n = 4. Representative data from



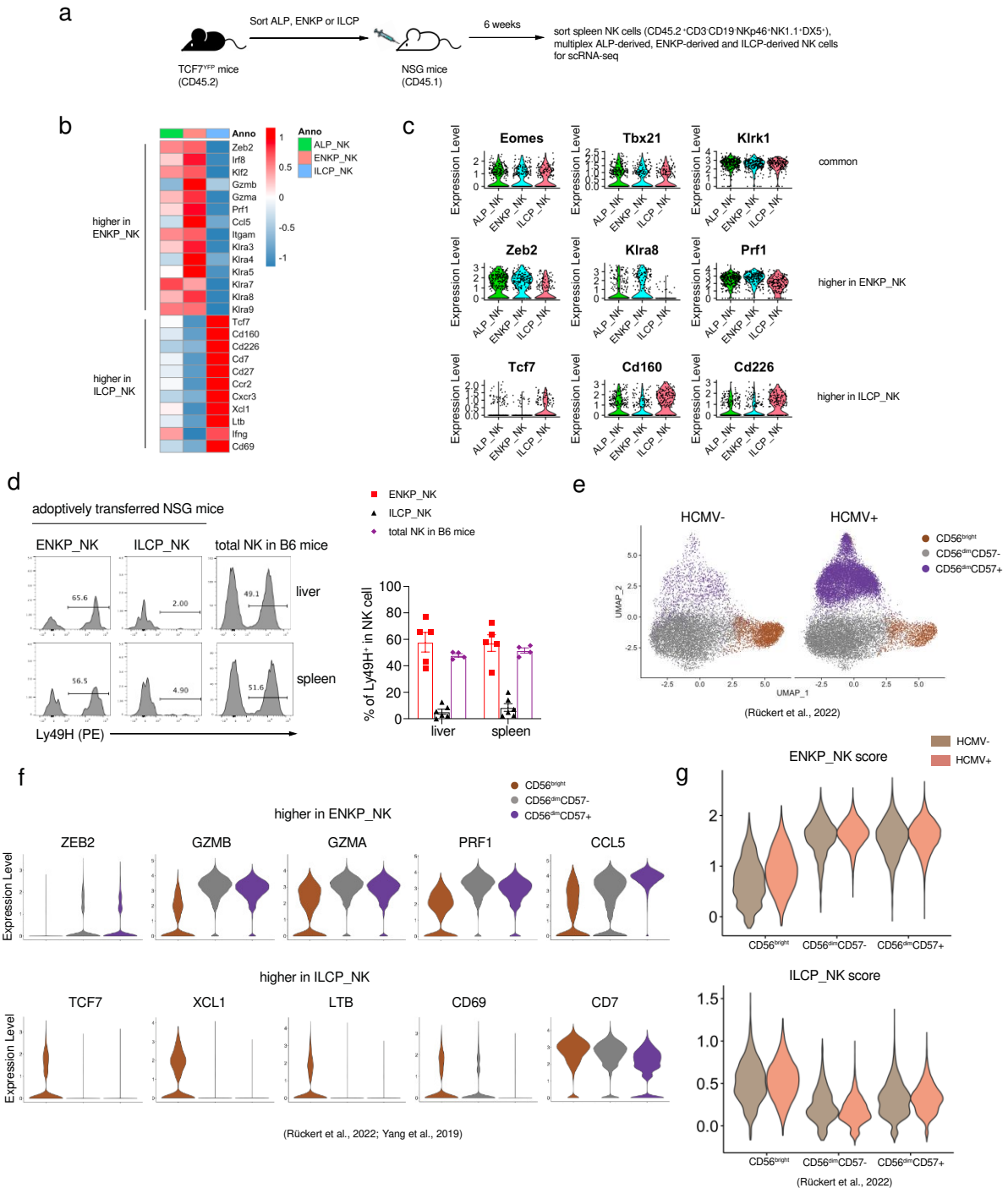
3 independent experiments. **b**, Strategy of in vitro clonal assay for ILC lineage potential, gating strategy of ILCs, and result of individual positive clones combined from 2 independent experiments. Each column represents one well, with detected ILC lineages in grey. **c-d**, Flow plots and quantification of group 1 ILCs in liver from ILCP (n = 10) (**c**) or ENKP-transferred (n = 10) (**d**) NSG mice combined from 3 independent experiments. **e**, Strategy of competitive assay and quantification of chimerism in spleen combined from 2 independent experiments. ALP group, n = 8; ENKP group, n = 9; ILCP group, n = 8. For **a**, **c-e**, data are mean  $\pm$  s.e.m, and statistical analysis was performed using two-tailed unpaired Student's t-tests.

Figure 3 ENKPs develop independent of ILCPs



**Fig. 3 ENKPs develop independent of ILCPs.** **a**, Strategy of generating long-term chimeras for PLZF lineage tracing, representative histograms and quantification of group 1 ILCs (n = 3) in liver and PLZF-labeling ratios of progenitors (n = 4) in bone marrow combined from 2 independent experiments. **b**, Strategy of assessing PLZF lineage tracing in progenitor-derived NK cells, representative histograms and quantification of PLZF-labeling ratios of progenitor-derived NK cells in spleen and ILC1s in liver combined from 3 independent experiments. ALP-derived NK, n = 9; ENKP-derived NK, n = 7; ILCP-derived NK, n = 6; ILCP-derived ILC1, n = 6. Data are mean  $\pm$  s.e.m, and statistical analysis was performed using two-tailed unpaired Student's t-test.

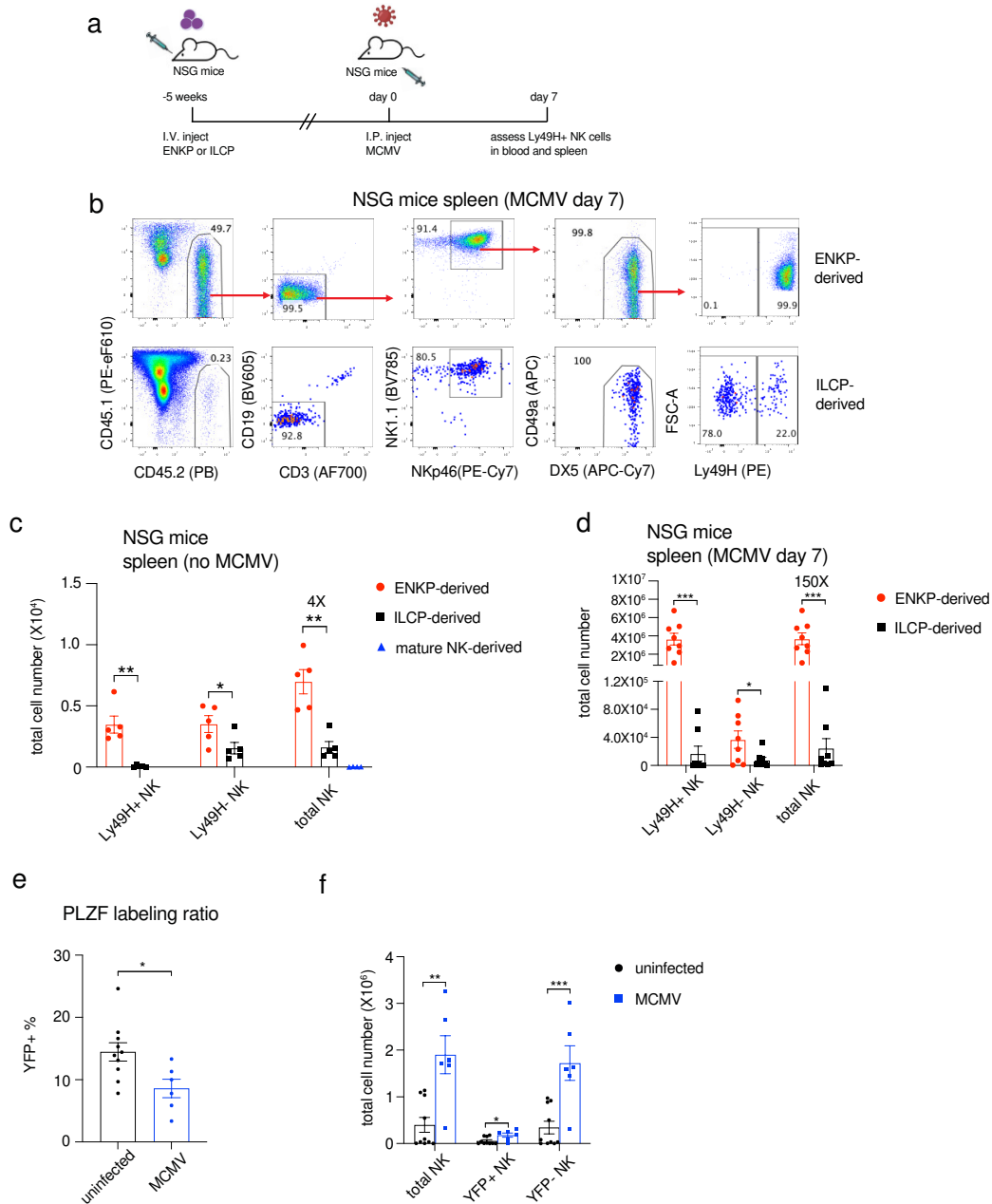
Figure 4 ENKP and ILCP give rise to distinct NK cells



**Fig. 4 ENKP and ILCP give rise to distinct NK cells.** **a**, Strategy of scRNA-seq of NK cells derived from different progenitors. **b**, Heatmap of average expression levels of representative genes differentially expressed in ENKP\_NK and ILCP\_NK from scRNA-seq. Each column is one developmental origin. Data are shown as z-score, with high expression in red and low expression in blue. **c**, Violin plots grouped by developmental origins show representative

genes expressed similarly (upper) or differently (middle, higher in ENKP\_NK; lower, higher in ILCP\_NK) between ENKP\_NK and ILCP\_NK. **d**, Representative histograms, and quantification of Ly49H<sup>+</sup> ratios in NK cells derived from ENKPs or ILCPs combined from 2 independent experiments. “Total NK” group is total NK cells from wild-type C57BL/6 mice. ENKP\_NK, n = 5; ILCP\_NK, n = 6; total NK, n = 4. Data are mean ± s.e.m. **e**, UMAP of human blood NK cells split by HCMV status analyzed by scRNA-seq. Annotation defined by surface protein expression measured by CITE-seq. **f**, Violin plots of human blood NK cells visualizing homologous genes identified to be upregulated in either mouse ENKP\_NK or ILCP\_NK cells, grouped according to the classification in Extended Data Fig. 6b. **g**, Violin plots of gene set scores calculated in the human single cell blood NK data using the human homologues of the genes upregulated in either ENKP or ILCP mouse NKs Plots split by HCMV status. For **d**, data are mean ± s.e.m. For **e**, **g**, data are from dataset of *Rückert et al., 2022*. For **f**, data are integrated from datasets of *Rückert et al., 2022* and *Yang et al., 2019*.

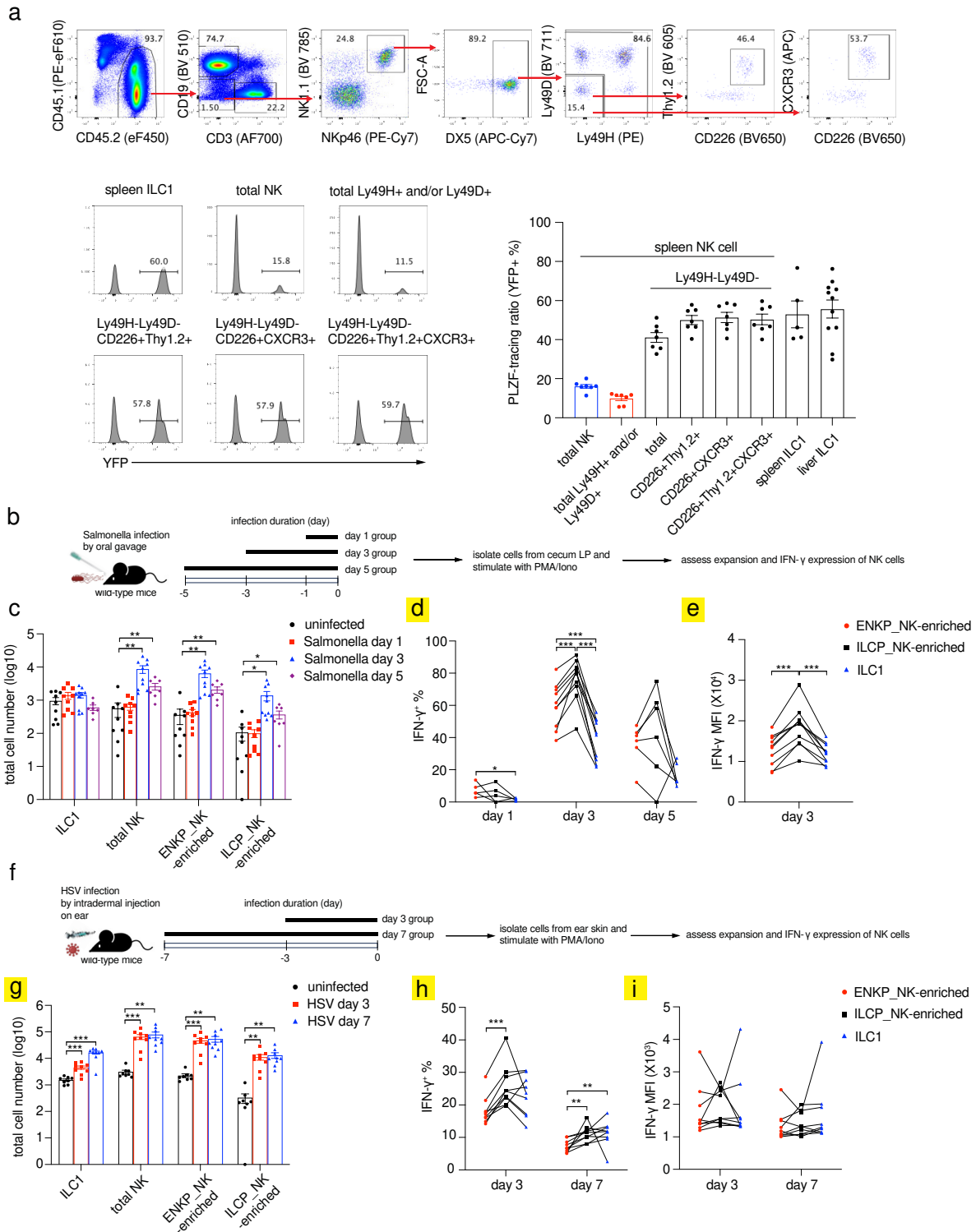
Figure 5 ENKP-derived NK cells respond to MCMV infection



**Fig. 5 ENKP-derived NK cells respond to MCMV infection.** **a**, Strategy of assessing ENKP\_NK cells and ILCP\_NK cells for response to MCMV. **b**, **d**, Analysis of ENKP\_NK cells and ILCP\_NK cells from spleen on day 7 after MCMV infection. Representative flow plots (**b**), quantification of total cell numbers ( $n = 8$ ) (**d**) of ENKP\_NK cells and ILCP\_NK cells combined from 2 independent experiments. 300 ENKPs or ILCPs were transferred into each NSG recipient mouse. **c**, Quantification of total numbers of ENKP\_NK cells and ILCP\_NK cells from NSG mice without MCMV infection,  $n = 5$ . Total numbers were normalized to 300 ENKPs, ILCPs or mature NK cells. Combined data from 2 independent experiments. **e**, Quantification of total NK cells analyzed in PLZF tracing long-

term chimeras on day 7 after MCMV infection combined from 2 independent experiments. control, n = 9; MCMV, n = 6. **f**, Quantification of NK subsets analyzed in PLZF tracing long-term chimeras on day 7 after MCMV infection combined from 2 independent experiments. control, n = 10; MCMV, n = 6. For **c - f**, data are mean  $\pm$  s.e.m, and statistical analysis was performed using two-tailed unpaired Student's t-tests.

Figure 6 Characterization of functions of NK subsets

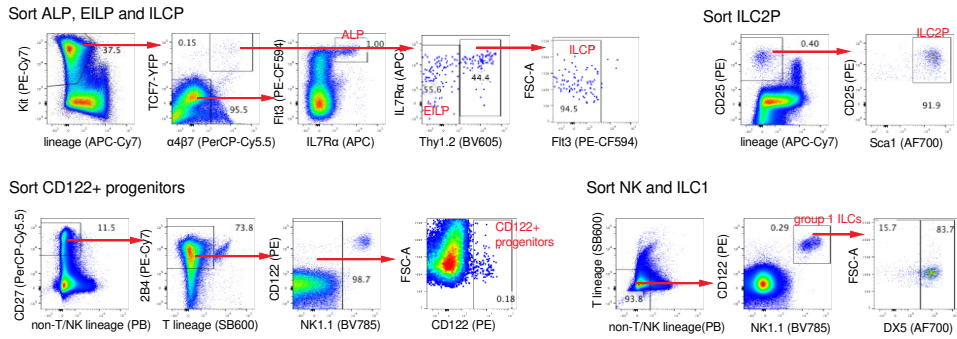


**Fig. 6 Characterization of functions of NK subsets.** **a**, Representative flow plots and histograms, and quantification of PLZF-tracing ratios in spleen NK subsets analyzed from irradiated NSG mice transferred with Lin<sup>-</sup>Kit<sup>+</sup> progenitors (NSG PLZF-tracing long-term chimera) combined from 3 independent experiments. spleen NK cells, n = 7; spleen ILC1, n = 5; liver ILC1, n = 11. **b**, Strategy of assessing NK subsets for response to Salmonella infection. **c**, Quantification of number of NK cells and ILC1s in cecum LP after Salmonella infection combined from 2 independent experiments. control, n = 9; day 1, n = 9; day 3, n = 10; day 5, n = 7. **d - e**, Quantification of IFN- $\gamma$ <sup>+</sup> ratios (**d**) and IFN- $\gamma$  MFI (**e**) in NK subsets and ILC1s after Salmonella infection combined from 2 independent experiments. day 1, n = 4; day 3, n = 9; day 5, n = 6. **f**, Strategy of assessing NK subsets for response to HSV infection. **g**, Quantification of number of NK cells in ear skin after HSV infection combined from 2 independent experiments. control, n = 8; day 3, n = 9; day 5, n = 9. **h - i**, Quantification of IFN- $\gamma$ <sup>+</sup> ratios (**h**) and IFN- $\gamma$  MFI (**i**) in NK subsets and ILC1s after HSV infection combined from 2 independent experiments. day 3, n = 9; day 5, n = 9. For **c - e, g - i**, ENKP<sub>NK</sub>-enriched population, Ly49H<sup>+</sup> and/or Ly49D<sup>+</sup>; ILCP<sub>NK</sub>-enriched population, Ly49H<sup>-</sup>Ly49D<sup>-</sup>CD226<sup>+</sup>Thy1.2<sup>+</sup>. For **a, c - e, g - i**, data are mean  $\pm$  s.e.m; for **c, g**, statistical analysis was performed using two-tailed unpaired Student's t-tests; for **d, e, h, i**, statistical analysis was performed using two-tailed paired Student's t-tests.

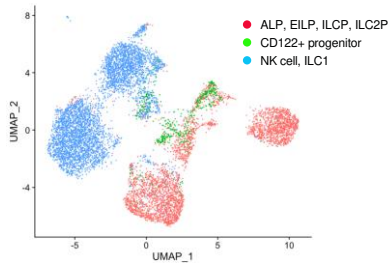


Extended Data Fig. 1 Characterization of early NK cell development in bone marrow

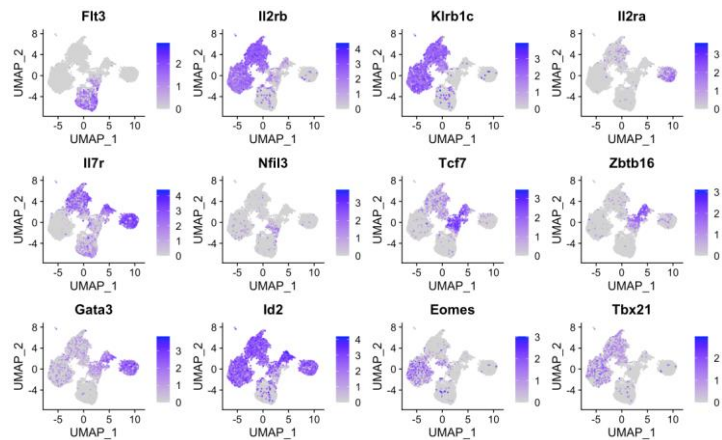
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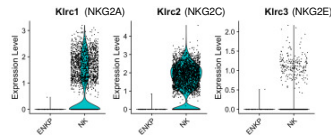
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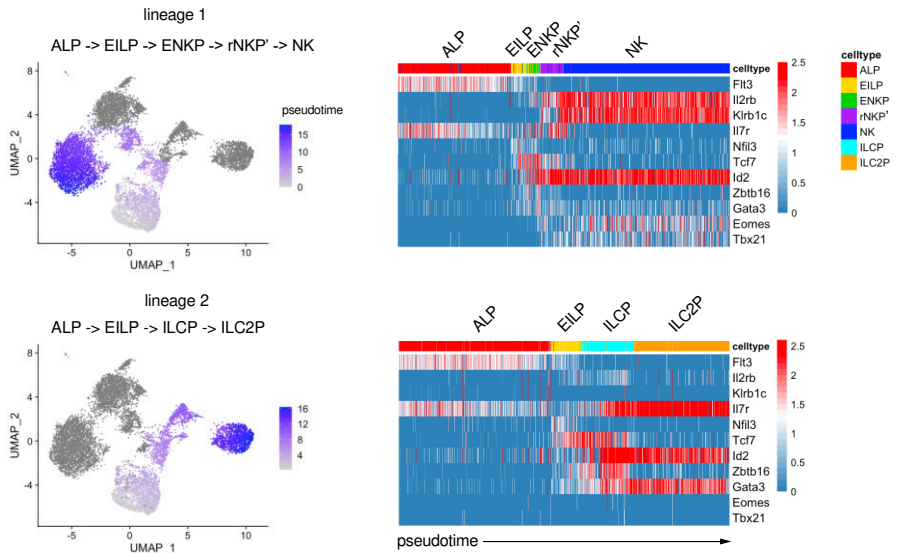
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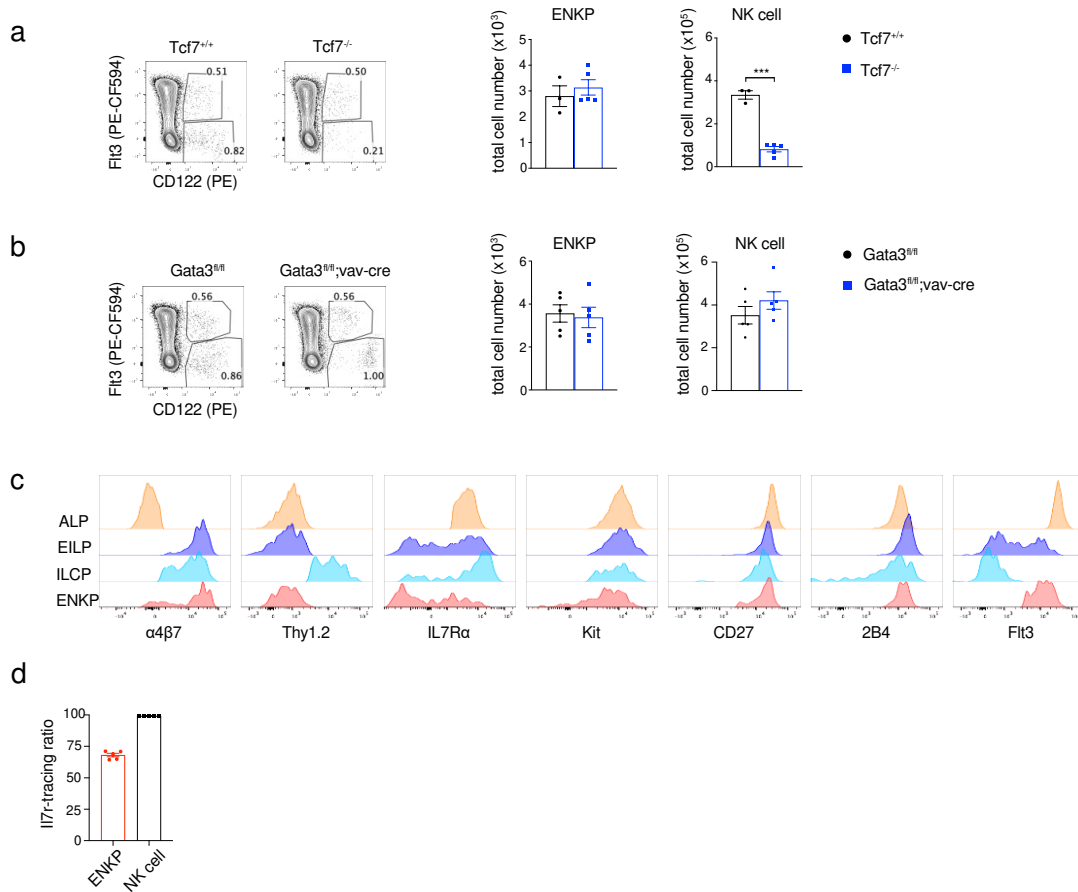
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Extended Data Fig. 1 Characterization of early NK cell development in bone marrow. a, Gating strategy for sorting ALP, EILP, ILCP, ILC2P, CD122<sup>+</sup> progenitor, NK cell and ILC1 from bone marrow for scRNA-seq. b,

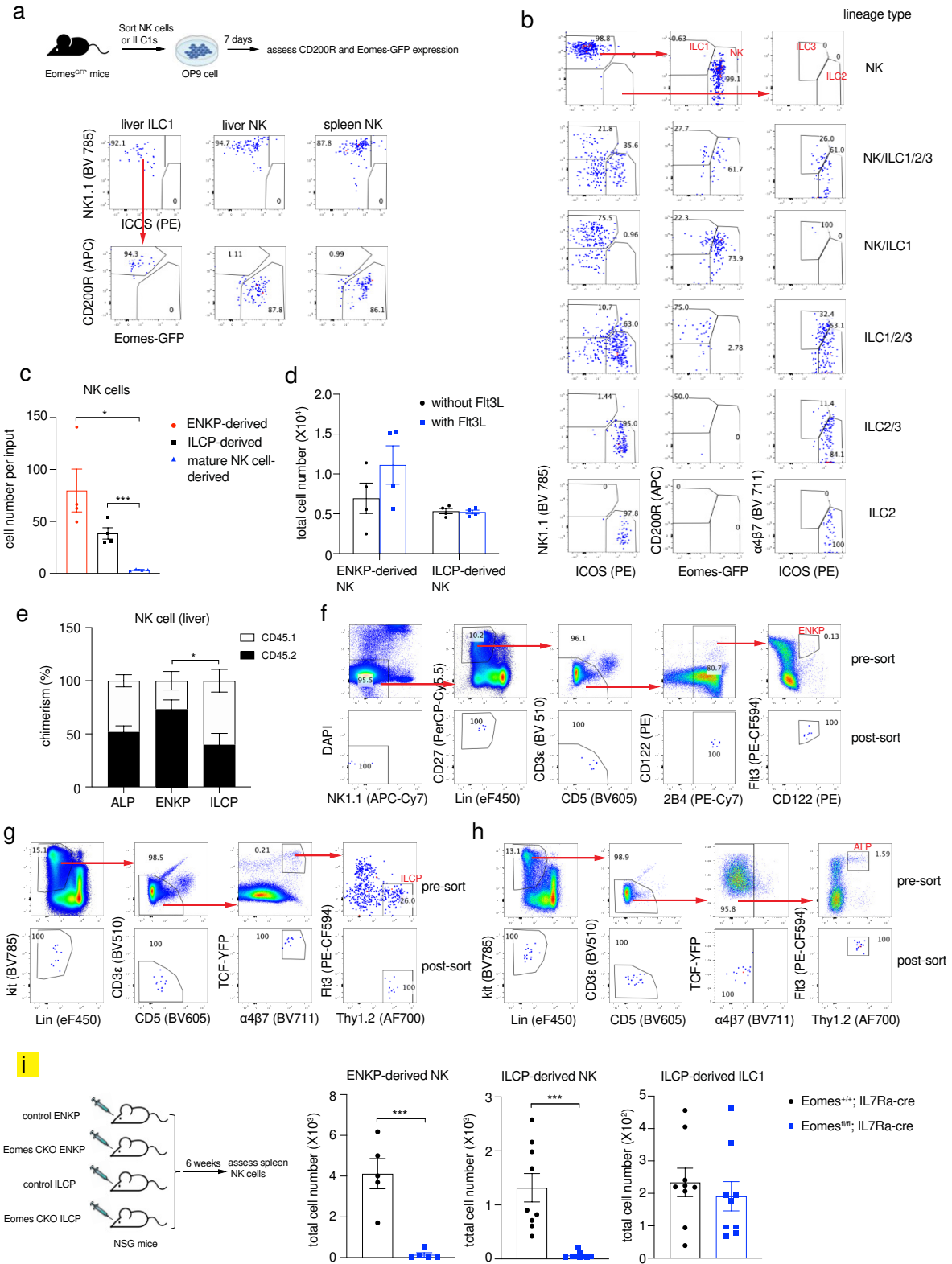
UMAP of scRNA-seq samples grouped by original idents. **c**, Feature plots of genes critical for identifying UMAP clusters. **d**, Violin plots of ENKP and NK cluster. **e**, Slingshot pseudotime and heatmap of gene expression ordered by pseudotime.

Extended Data Fig.2 Characterization of ENKP



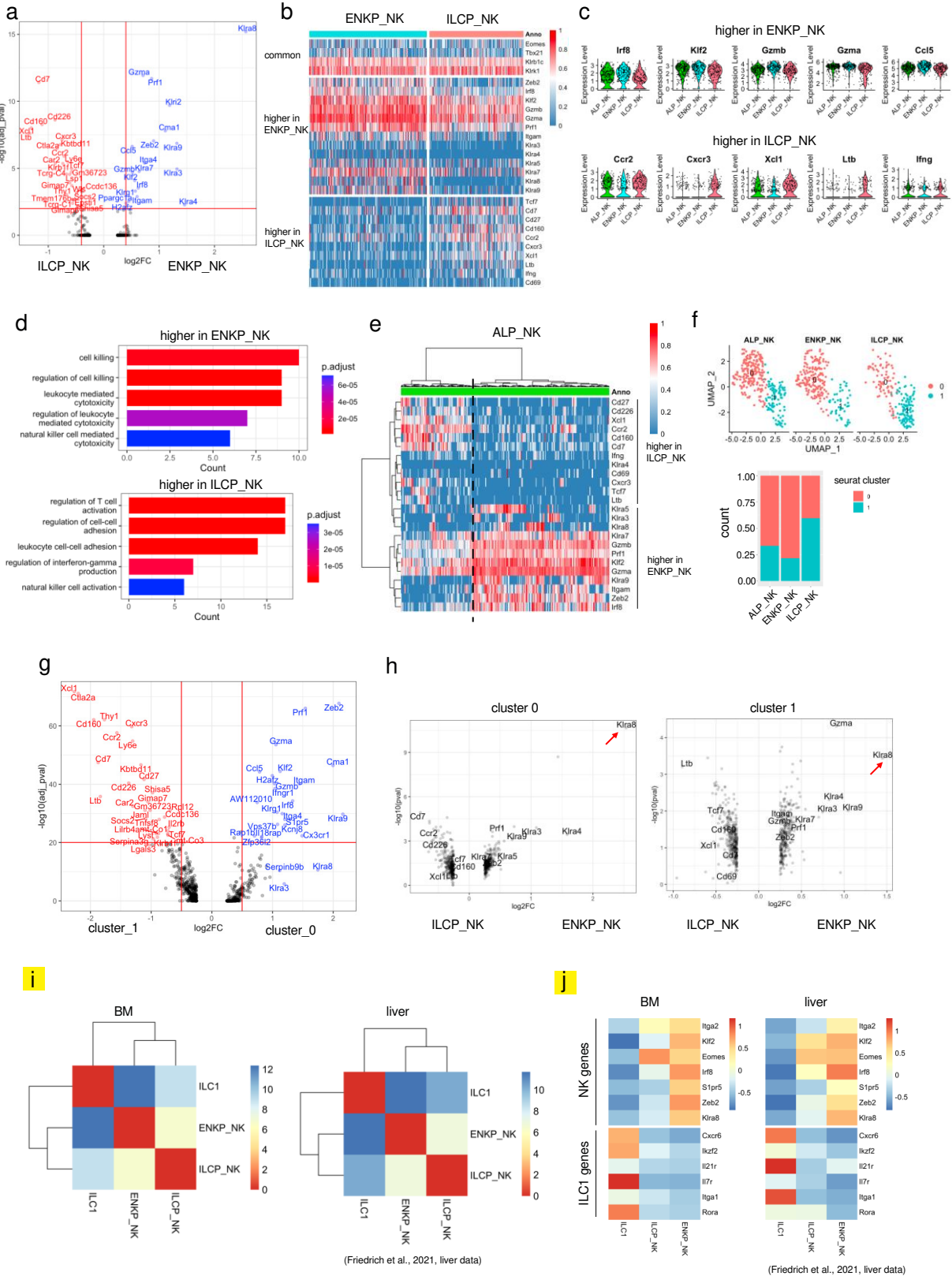
**Extended Data Fig. 2 Characterization of ENKPs.** **a**, Representative flow plots of ENKPs and quantifications of ENKPs and NK cells in bone marrow of *Tcf7*-deficient mice (n = 3) and control mice (n = 5). Combined data from 2 independent experiments. **b**, Representative flow plots of ENKPs and quantifications of ENKPs and NK cells in bone marrow of *Gata3*-deficient mice, n = 5. Combined data from 2 independent experiments. **c**, Representative histograms of surface marker expression by different progenitors from 2 independent experiments. **d**, Quantifications of *Il7r*-lineage tracing ratios in ENKPs and NK cells in bone marrow, n = 5. Representative data from 3 independent experiments. For **a**, **b** and **d**, data are mean  $\pm$  s.e.m. For **a** and **b**, statistical analysis was performed using two-tailed unpaired Student's t-tests.

### Extended Data Fig. 3 Assessment of lineage potential of ENKP



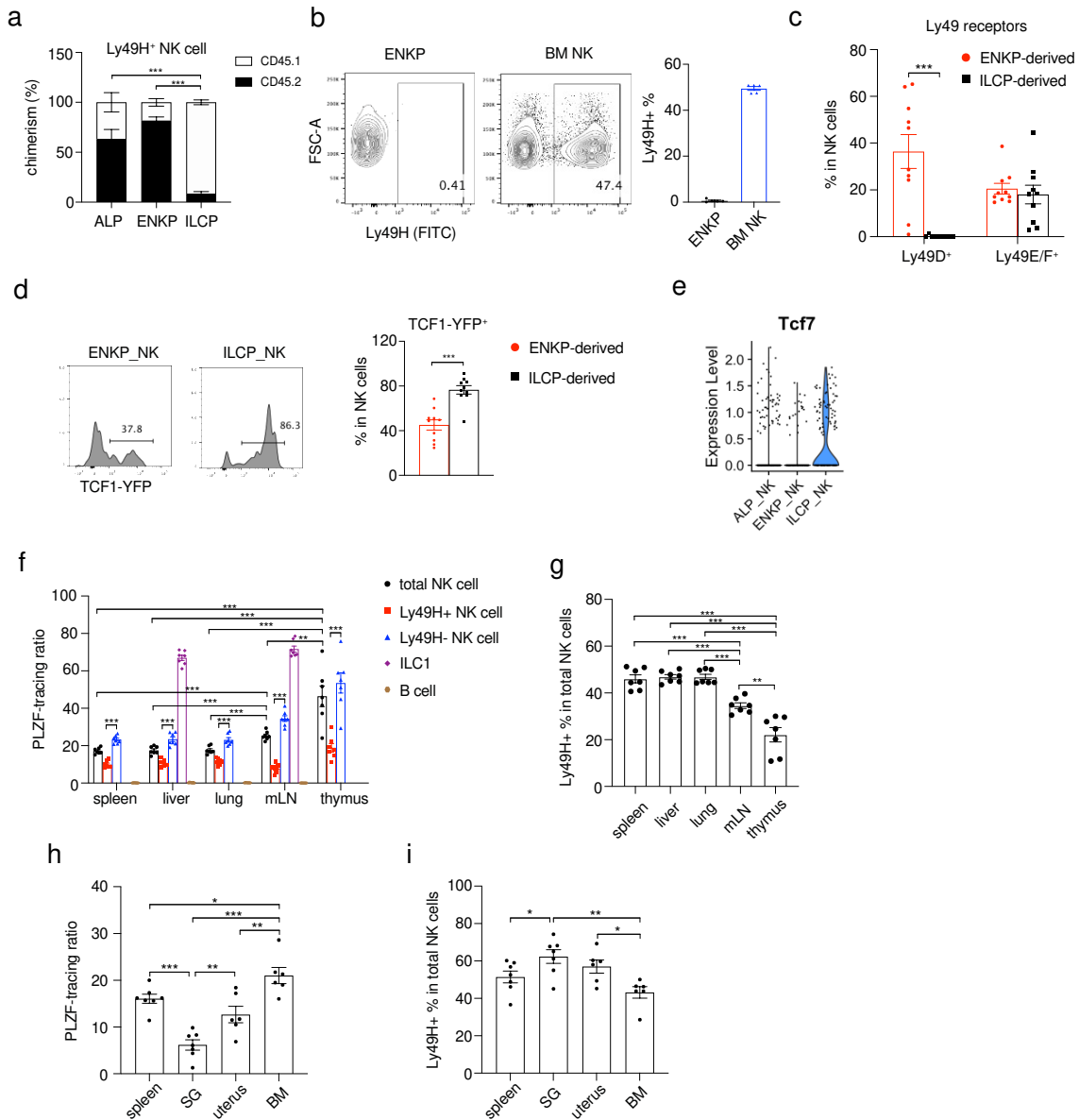
**Extended Data Fig. 3 Assessment of lineage potential of ENKPs.** **a**, Strategy and flow plots of in vitro cultured NK cells ( $CD3^{-}CD19^{-}NKp46^{+}NK1.1^{+}CD49a^{-}DX5^{+}Eomes^{GFP^{+}}$ ) and ILC1s ( $CD3^{-}CD19^{-}NKp46^{+}NK1.1^{+}CD49a^{+}DX5^{-}Eomes^{GFP^{-}}$ ) sorted from liver of  $Eomes^{GFP}$  mice. **b**, Representative flow plots of in vitro clonal assay. **c**, Quantification of in vitro differentiated NK cells, comparing progenitors and mature NK cells,  $n = 4$ . NK cells were defined as  $NK1.1^{+}Eomes^{GFP^{+}}CD220R^{-}$ . Representative data from 2 independent experiments. **d**, Quantification of in vitro differentiated NK cells, comparing with and without Flt3L,  $n = 4$ . NK cells were defined as  $NK1.1^{+}Eomes^{GFP^{+}}CD220R^{-}$ . Representative data from 2 independent experiments. **e**, Quantification of chimerism after competitive assay in liver combined from 2 independent experiments. ALP group,  $n = 5$ ; ENKP group,  $n = 5$ ; ILCP group,  $n = 4$ . **f - h**, Representative flow plots of post-sort purity check for ENKP (**f**), ILCP (**g**) and ALP (**h**). **i**, Strategy and quantification of total spleen NK cells and ILC1s derived from control or  $Eomes$ -deficient progenitors combined from 2 independent experiments. ENKP-derived NK,  $n = 5$ ; ILCP-derived NK,  $n = 9$ . For **c**, **d**, **e**, **i**, data are mean  $\pm$  s.e.m., and statistical analysis was performed using two-tailed unpaired Student's t-tests.

Extended Data Fig. 4 ENKP\_NK and ILCP\_NK are transcriptionally different



**Extended Data Fig. 4 ENKP\_NK and ILCP\_NK are transcriptionally different.** **a**, Volcano plot of differentially expressed genes between ENKP\_NK cells and ILCP\_NK cells. **b**, Heatmap of genes equally expressed (upper) and differentially expressed between ENKP\_NK and ILCP\_NK (middle and lower). Each column represents one cell. **c**, Violin plots of genes expressed higher in ENKP\_NK (upper) and higher in ILCP\_NK (lower) grouped by developmental origins. **d**, GO enrichment analysis using genes expressed higher in ENKP\_NK cells (upper) or ILCP\_NK cells (lower). **e**, Hierarchically clustered heatmap of gene expression levels of genes differentially expressed between ENKP\_NK cells and ILCP\_NK cells in ALP\_NK cells. Each column represents one cell. Dashed line indicates separation between two major column clusters. **f**, UMAP and bar plot of scRNA-seq of progenitor-derived NK cells grouped by developmental origins. **g**, Volcano plot of differentially expressed genes between cluster 0 and cluster 1. **h**, Volcano plot of differentially expressed genes between ENKP\_NK and ILCP\_NK in cluster 0 (left) and in cluster 1 (right). Arrow highlights the gene “*Klra8*”. **i**, Hierarchically clustered Euclidean distance matrix of transcriptomes of NK cells and ILC1s in bone marrow (left) and liver (right). **j**, Heatmap of select genes (see Methods) differentially expressed between NK cells and ILC1s in ENKP\_NK cells, ILCP\_NK cells and ILC1s in bone marrow (left) and liver (right).

Extended Data Fig. 5 ENKP and ILCP give rise to distinct NK cells

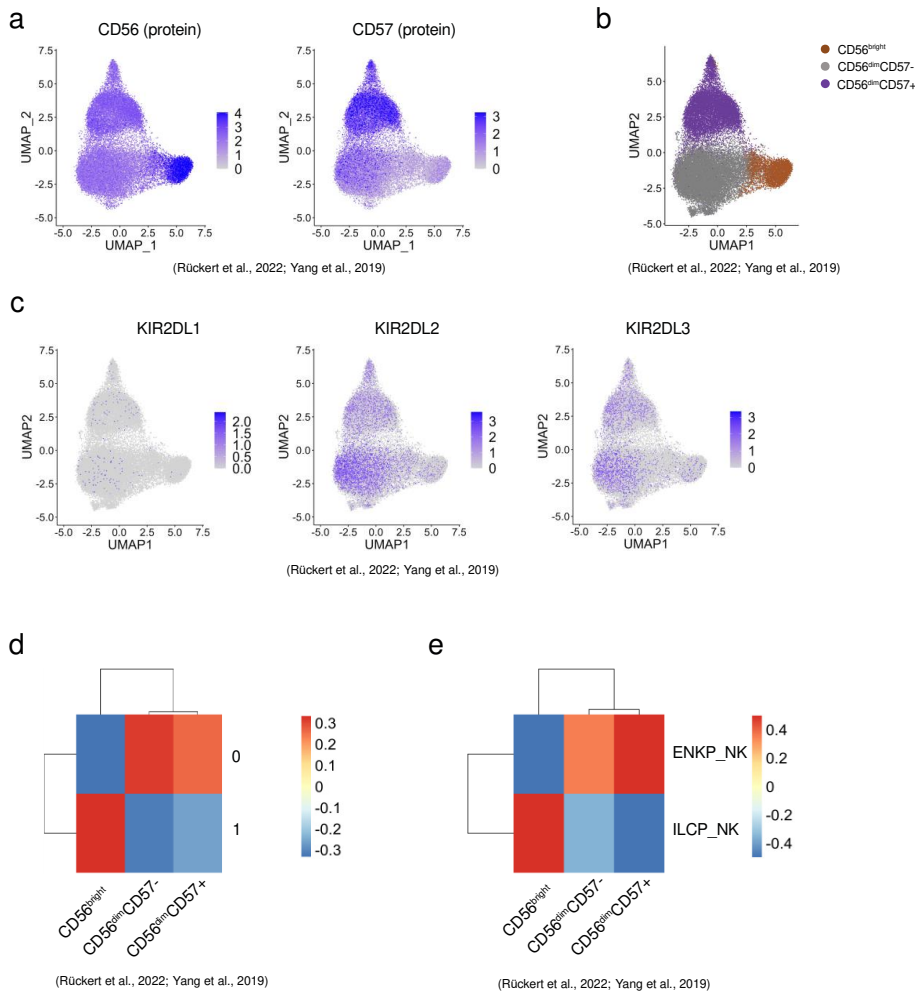


**Extended Data Fig. 5 ENKPs and ILCPs give rise to distinct NK cells.** **a**, Quantification of chimerism in competitive assay (same strategy as in **Fig. 2e**) from 2 independent experiments. ALP group, n = 8; ENKP group, n = 9; ILCP group, n = 8. **b**, Representative flow plots, and quantification of Ly49H<sup>+</sup> ratios in ENKPs and NK cells in bone marrow combined from 2 independent experiments, n = 5. **c**, Quantification of Ly49 receptor positive ratios in ENKP\_NK cells and ILCP\_NK cells from NSG mice transferred with ENKPs or ILCPs, n = 10 combined from 2 independent experiments. **d**, Representative histogram of TCF1-YFP expression and quantification of TCF1-YFP<sup>+</sup> ratios in ENKP\_NK cells and ILCP\_NK cells from NSG mice transferred with ENKPs or ILCPs, n = 10 combined



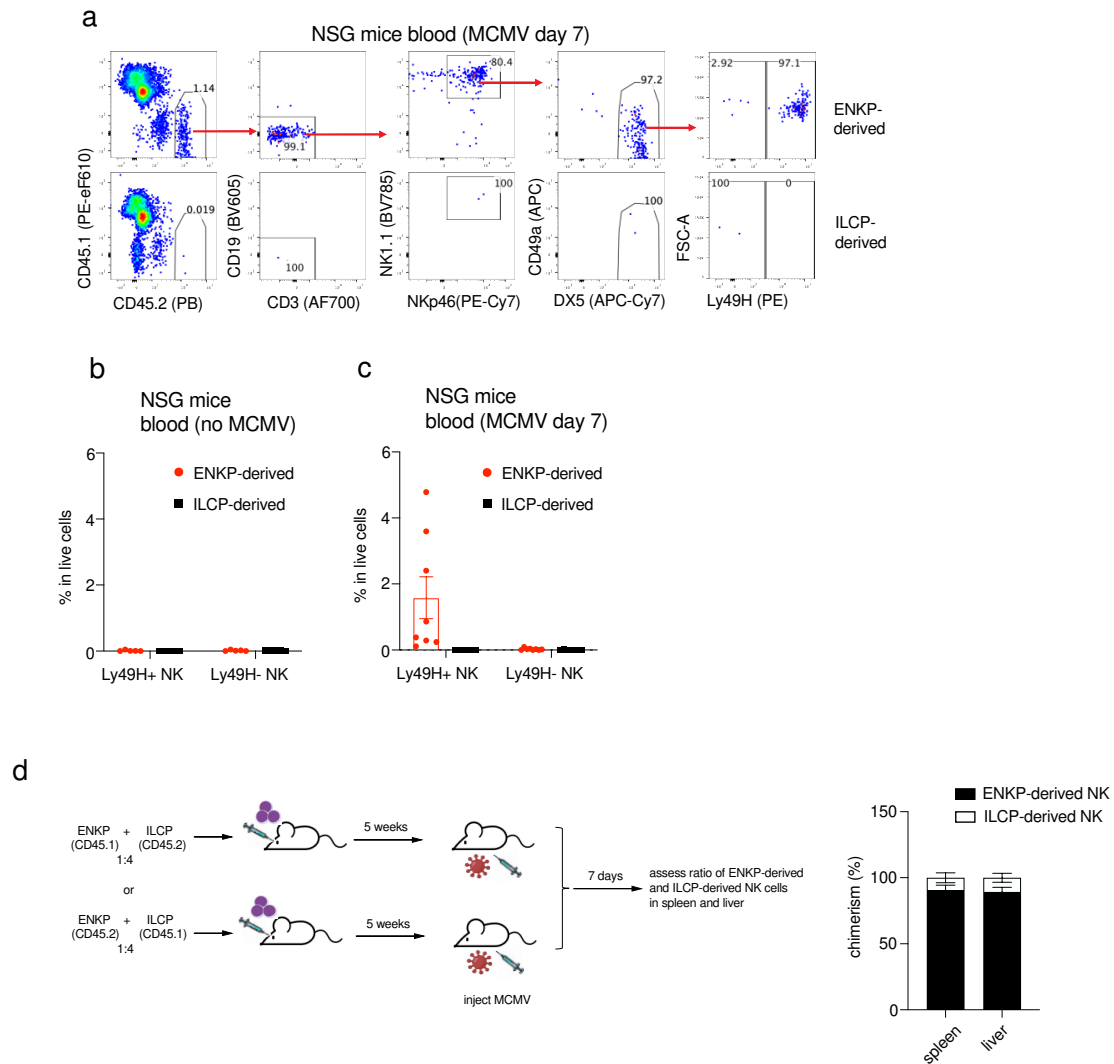
from 2 independent experiments. **e**, Violin plots of *Tcf7* from scRNA-seq grouped by developmental origins. **f**, Quantification of PLZF lineage tracing ratios in different tissues from irradiated wild-type mice transferred with Lin<sup>-</sup>Kit<sup>+</sup> progenitors (PLZF-tracing long-term chimera), n = 7 combined from 2 independent experiments. B cells were used as negative control for PLZF labeling. **g**, Quantification of Ly49H<sup>+</sup> ratios in NK cells in different tissues from PLZF-tracing long-term chimeras, n = 7 combined from 2 independent experiments. **h - i**, Quantification of PLZF lineage tracing ratios (**h**) and Ly49H<sup>+</sup> ratios in NK cells (**i**) in different tissues from irradiated NSG mice transferred with Lin<sup>-</sup>Kit<sup>+</sup> progenitors (NSG PLZF-tracing long-term chimera) combined from 3 independent experiments. spleen and salivary glands (SG), n = 7; uterus and bone marrow (BM), n = 6. For **a-d, f - i**, data are mean ± s.e.m. For **a,c,d, f - i**, statistical analysis was performed using two-tailed unpaired Student's t-tests.

Extended Data Fig. 6 ENKP-derived and ILCP-derived NK cells are similar to human CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells, respectively



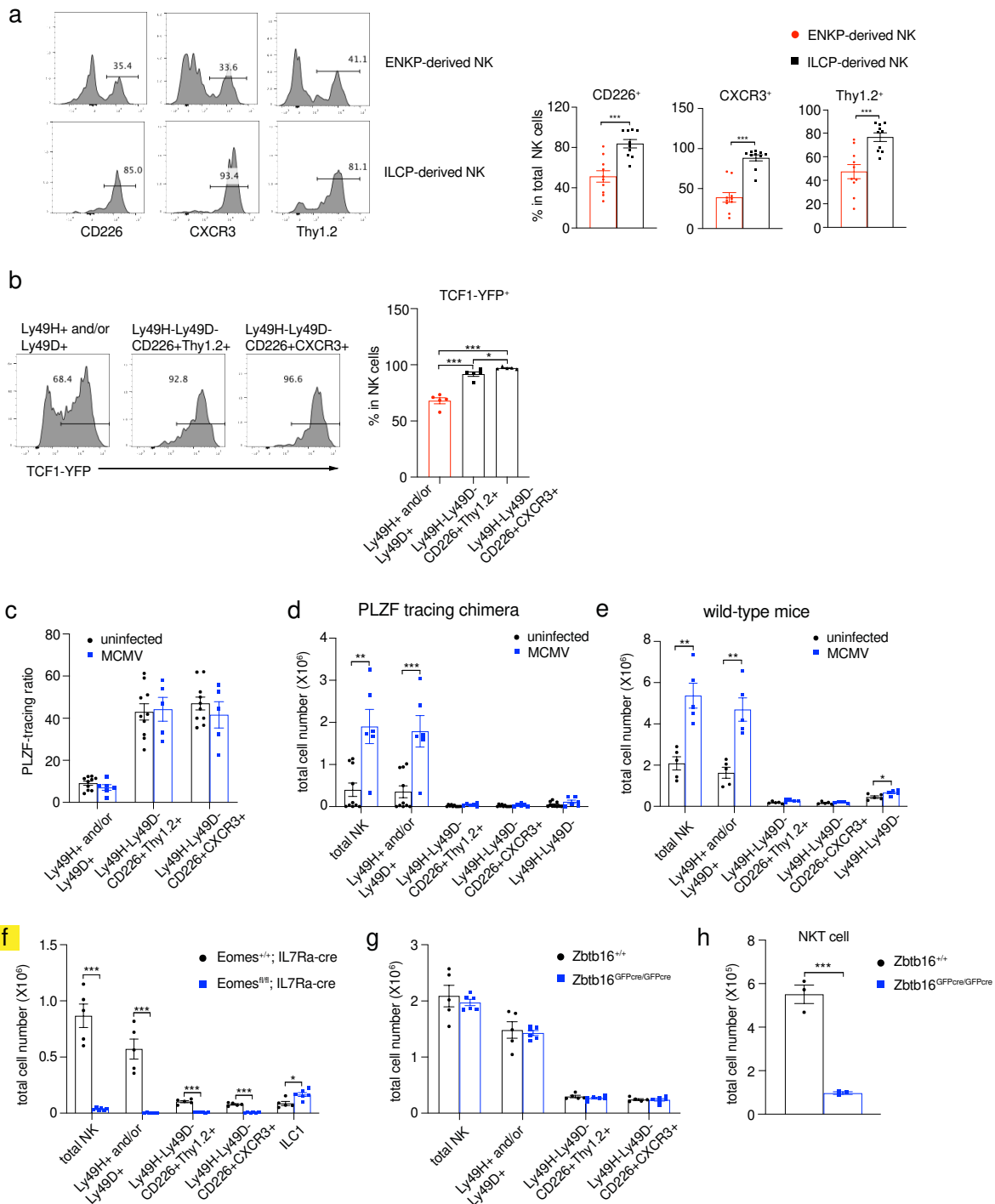
**Extended Data Fig. 6 ENKP-derived and ILCP-derived NK cells are similar to human CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells, respectively.** **a.** Feature plots of surface protein expression in human blood NK measured by CITE-seq. **b.** UMAP of human blood NK cells analyzed by scRNA-seq. Annotation defined by surface protein expression measured by CITE-seq. **c.** Feature plots of KIR genes in human blood NK cells analyzed by scRNA-seq. **d.** Heatmap of Spearman correlation analysis between mouse NK clusters and human NK clusters. **e.** Heatmap of Spearman correlation analysis between mouse NK developmental originals and human NK clusters. For **a - e**, data were integrated from datasets of Rückert *et al.*, 2022 and Yang *et al.*, 2019.

Extended Data Fig. 7 ENKP-derived NK cells respond to MCMV infection



**Extended Data Fig. 7 ENKP-derived NK cells respond to MCMV infection.** **a-b**, Analysis of ENKP\_NK cells and ILCP\_NK cells in peripheral blood on day 7 after MCMV infection. Representative flow plots (**a**) and quantification of frequencies (**c**) of ENKP\_NK cells and ILCP\_NK cells,  $n = 8$  combined from 2 independent experiments. **b**, Quantification of frequencies of ENKP\_NK and ILCP\_NK cells in blood without MCMV infection,  $n = 5$  combined from 2 independent experiments. For **b** and **c**, 300 ENKPs or ILCPs were transferred into each NSG mice. **d**, Strategy and quantification of ratios of ENKP and ILCP-derived NK cells day 7 after MCMV infection combined from 2 independent experiments.  $n = 9$ . For **b - d**, data are mean  $\pm$  s.e.m.

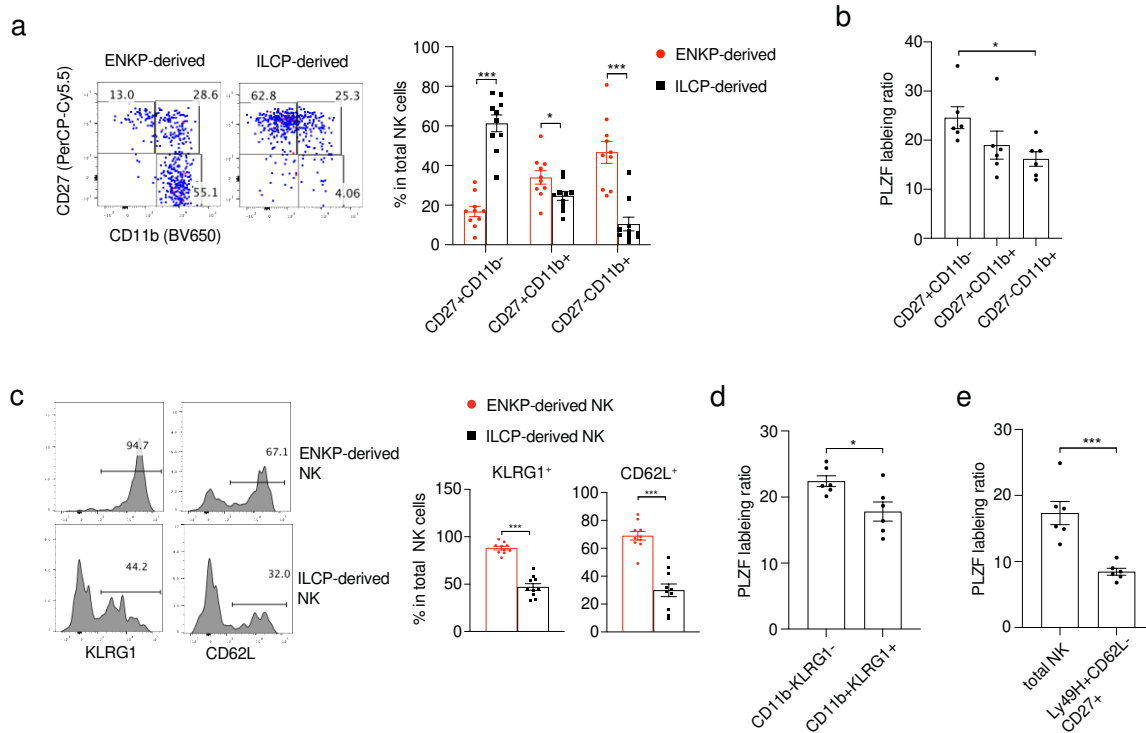
Extended Data Fig. 8 Verification of ENKP\_NK and ILCP\_NK enriched populations



**Extended Data Fig. 8 Verification of ENKP\_NK and ILCP\_NK-enriched cell population. a,** Representative histograms, and quantification of frequencies of cells expressing specific surface markers in spleen NK cells derived from ENKPs or ILCPs combined from 2 independent experiments. n = 10. **b,** Representative histograms and

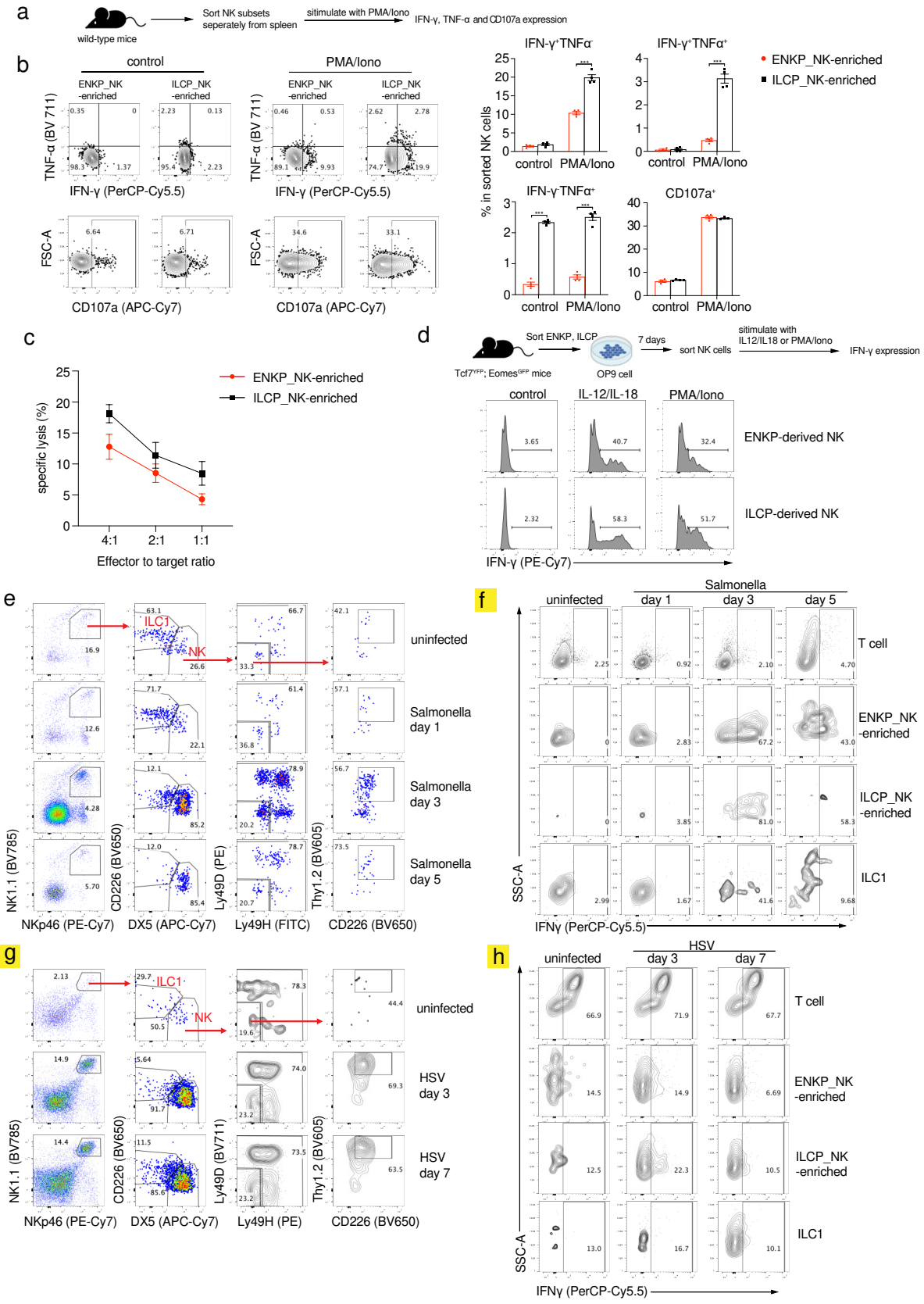
quantification of frequencies of cells expressing TCF1-YFP in spleen NK subsets from TCF1-YFP reporter mice combined from 2 independent experiments. n = 5. **c - d**, Quantification of PLZF traced ratios (**c**) and NK cell numbers (**d**) in spleen NK subsets from NSG PLZF-tracing long-term chimera day 7 after MCMV infection combined from 2 independent experiments. control, n = 10; MCMV, n = 5. **e**, Quantification of NK cell numbers in spleen NK subsets in wild-type B6 mice on day 7 after MCMV infection combined from 2 independent experiments. n = 5. **f**, Quantification of cell number in spleen of total NK cells, NK subsets and ILC1s in *Eomes*-deficient mice combined from 2 independent experiments. *Eomes*<sup>+/+</sup>; *IL7Ra-cre*, n = 5; *Eomes*<sup>fl/fl</sup>; *IL7Ra-cre*, n = 6. **g**, Quantification of cell number in spleen of total NK cells and NK subsets in *Zbtb16*-deficient mice combined from 2 independent experiments. *Zbtb16*<sup>+/+</sup>, n = 5; *Zbtb16*<sup>GFPcre/GFPcre</sup>, n = 6. **h**, Quantification of cell numbers of spleen NKT cells in *Zbtb16*-deficient mice combined 2 independent experiments. n = 3. For **a - h**, data are mean ± s.e.m., and statistical analysis was performed using two-tailed unpaired Student's t-tests.

Extended Data Fig. 9 Distinguish ENKP and ILCP-derived NK cells by surface markers



**Extended Data Fig. 9 Distinguish ENKP\_NK cells and ILCP\_NK cells by surface markers.** **a**, Representative flow plots, and quantification of frequencies of NK subsets in spleen NK cells derived from ENKPs or ILCPs combined from 2 independent experiments. n = 10. **b**, Quantification of PLZF-tracing ratios in spleen NK subsets from NSG PLZF-tracing long-term chimeras combined from 2 independent experiments. n = 6. **c**, Representative histograms, and quantification of frequencies of cells expressing specific surface markers in spleen NK cells derived from ENKPs or ILCPs combined from 2 independent experiments. n = 10. **d**, Quantification of PLZF-tracing ratios in spleen NK subsets from NSG PLZF-tracing long-term chimeras from 2 independent experiments. n = 6. **e**, Quantification of PLZF-tracing ratios in spleen NK subsets from NSG PLZF-tracing long-term chimeras combined from 2 independent experiments. n = 6. For **a - e**, data are mean  $\pm$  s.e.m., and statistical analysis was performed using two-tailed unpaired Student's t-tests.

# Extended Data Fig.10 Characterization of functions of NK subsets



**Extended Data Fig. 10 Characterization of functions of NK subsets.** **a**, Strategy of assessing cytokine expression in sorted NK subsets. **b**, Representative flow plots, and quantification of frequencies of cells expressing cytokines in NK subsets, representative data from 2 independent experiments.  $n = 4$ . **c**, Quantification of specific lysis ratios of Yac-1 cells co-cultured with NK subsets, representative data from 2 independent experiments.  $n = 4$ . **d**, Strategy of assessing IFN- $\gamma$  expression of ENKP\_NK and ILCP\_NK after stimulation in vitro, and representative histograms of IFN- $\gamma$  expression levels from 2 independent experiments. **e - f**, Representative flow plots of gating (**e**) and IFN- $\gamma$  expression (**f**) of NK subsets and ILC1s from cecum lamina propria (LP) after Salmonella infection. **g - h**, Representative flow plots of gating (**g**) and IFN- $\gamma$  expression (**h**) of NK subsets and ILC1s from ear skin after HSV infection. For **b, c**, ENKP\_NK-enriched population, Ly49H<sup>+</sup> and/or Ly49D<sup>+</sup>; ILCP\_NK-enriched population, Ly49H<sup>+</sup>Ly49D<sup>-</sup>CD226<sup>+</sup>(Thy1.2<sup>+</sup> and/or CXCR3<sup>+</sup>). For **f, h**, ENKP\_NK-enriched population, Ly49H<sup>+</sup> and/or Ly49D<sup>+</sup>; ILCP\_NK-enriched population, Ly49H<sup>+</sup>Ly49D<sup>-</sup>CD226<sup>+</sup>Thy1.2<sup>+</sup>. For **b - c**, data are mean  $\pm$  s.e.m, and statistical analysis was performed using two-tailed unpaired Student's t-tests.