Figure S1

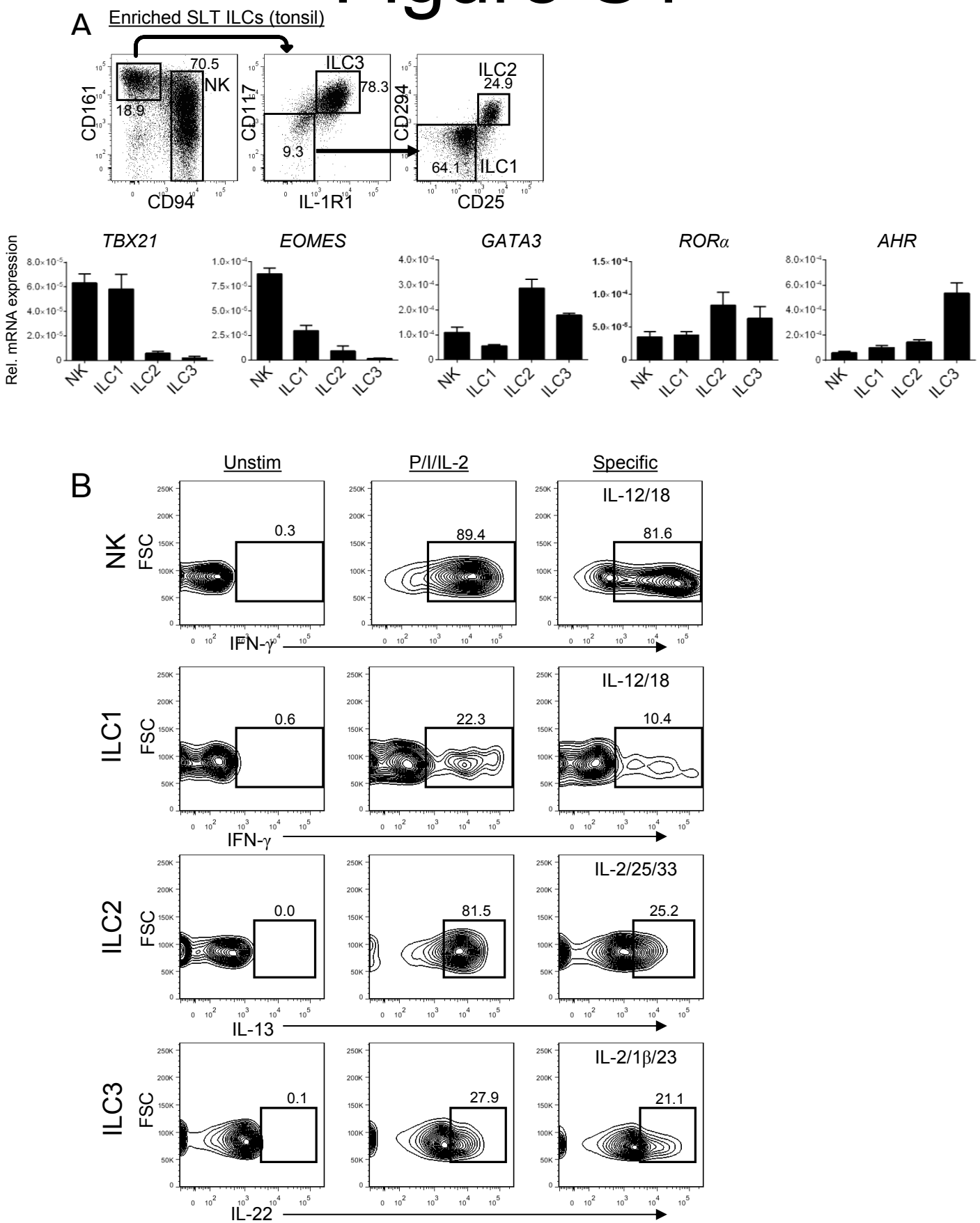

A
Enriched SLT ILCs (tonsil)
CD161
CD94
70.5
NK
18.9
CD117
IL-1R1
ILC3
78.3
9.3
CD294
CD25
ILC2
24.9
64.1
ILC1
Rel. mRNA expression
TBX21
EOMES
GATA3
RORα
AHR
NK
ILC1
ILC2
ILC3
B
Unstim
P/I/IL-2
Specific
NK
ILC1
ILC2
ILC3
FSC
IFN-γ
IL-13
IL-22
0.3
89.4
IL-12/18
81.6
0.6
22.3
IL-12/18
10.4
0.0
81.5
IL-2/25/33
25.2
0.1
27.9
IL-2/1β/23
21.1

Figure S2

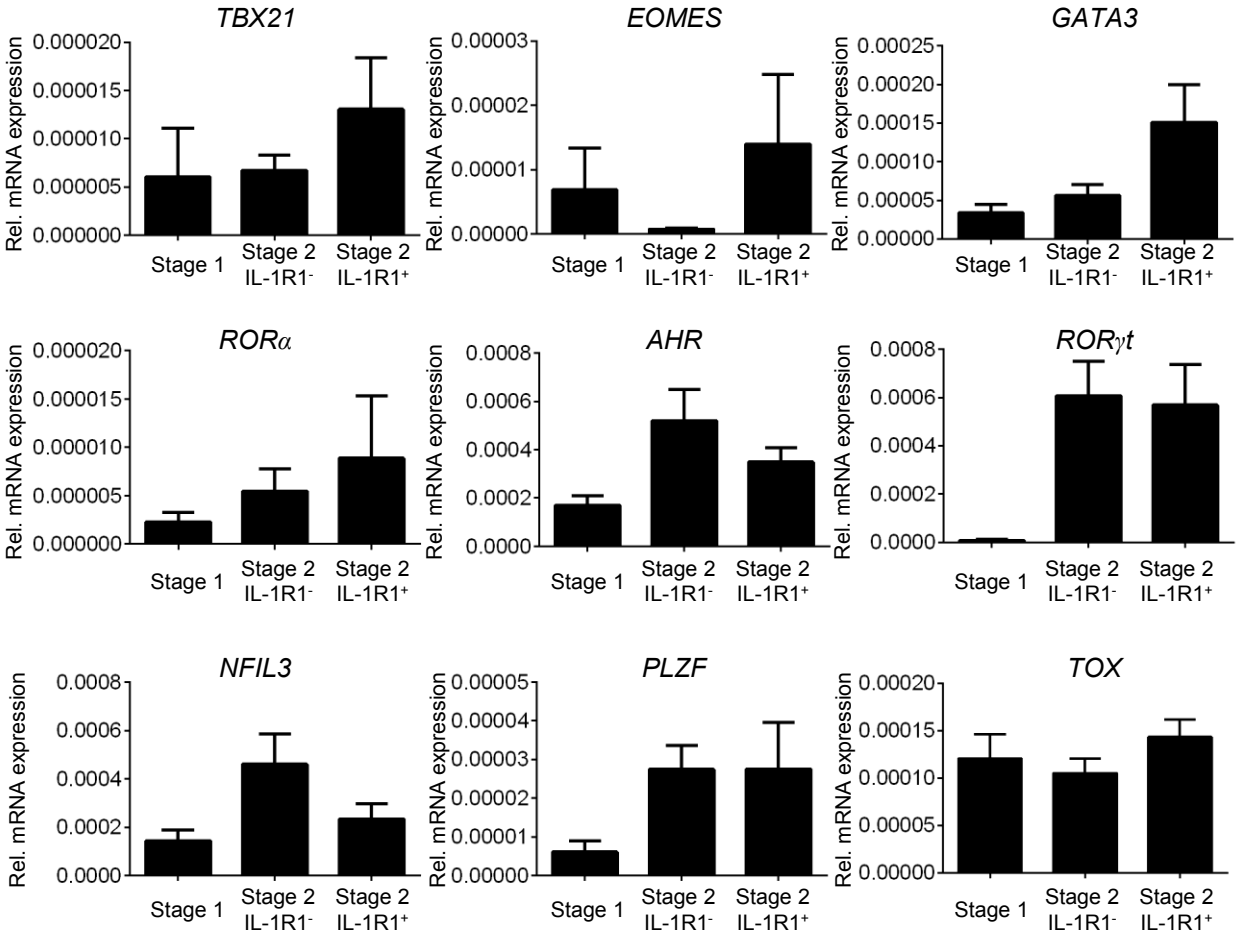
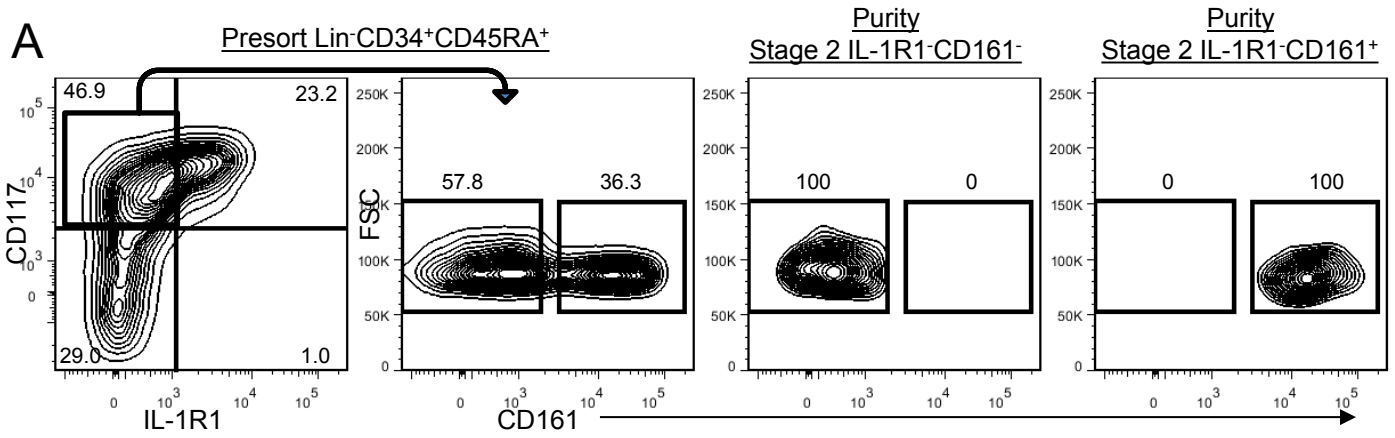

TBX21
EOMES
GATA3
RORα
AHR
RORγt
NFIL3
PLZF
TOX
Rel. mRNA expression
Stage 1
Stage 2 IL-1R1-
Stage 2 IL-1R1+

Figure S3


A
Presort Lin-CD34+CD45RA+
Purity
Stage 2 IL-1R1-CD161-
Purity
Stage 2 IL-1R1-CD161+
46.9
23.2
29.0
1.0
CD117
IL-1R1
FSC
57.8
36.3
CD161
100
0
0
100

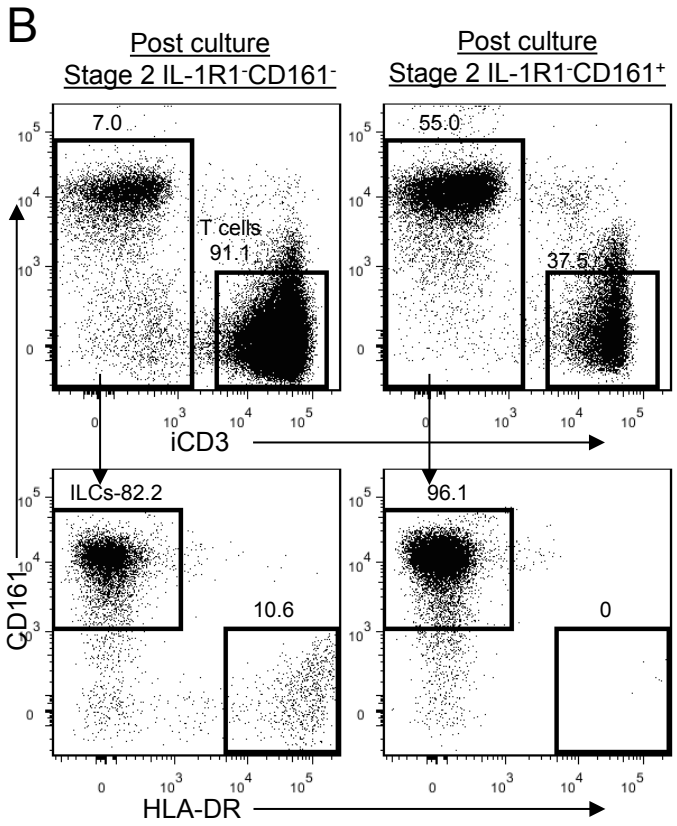

B
Post culture
Stage 2 IL-1R1-CD161-
Post culture
Stage 2 IL-1R1-CD161+
7.0
T cells
91.1
55.0
37.5
iCD3
ILCs-82.2
10.6
96.1
0
CD161
HLA-DR

Figure S4

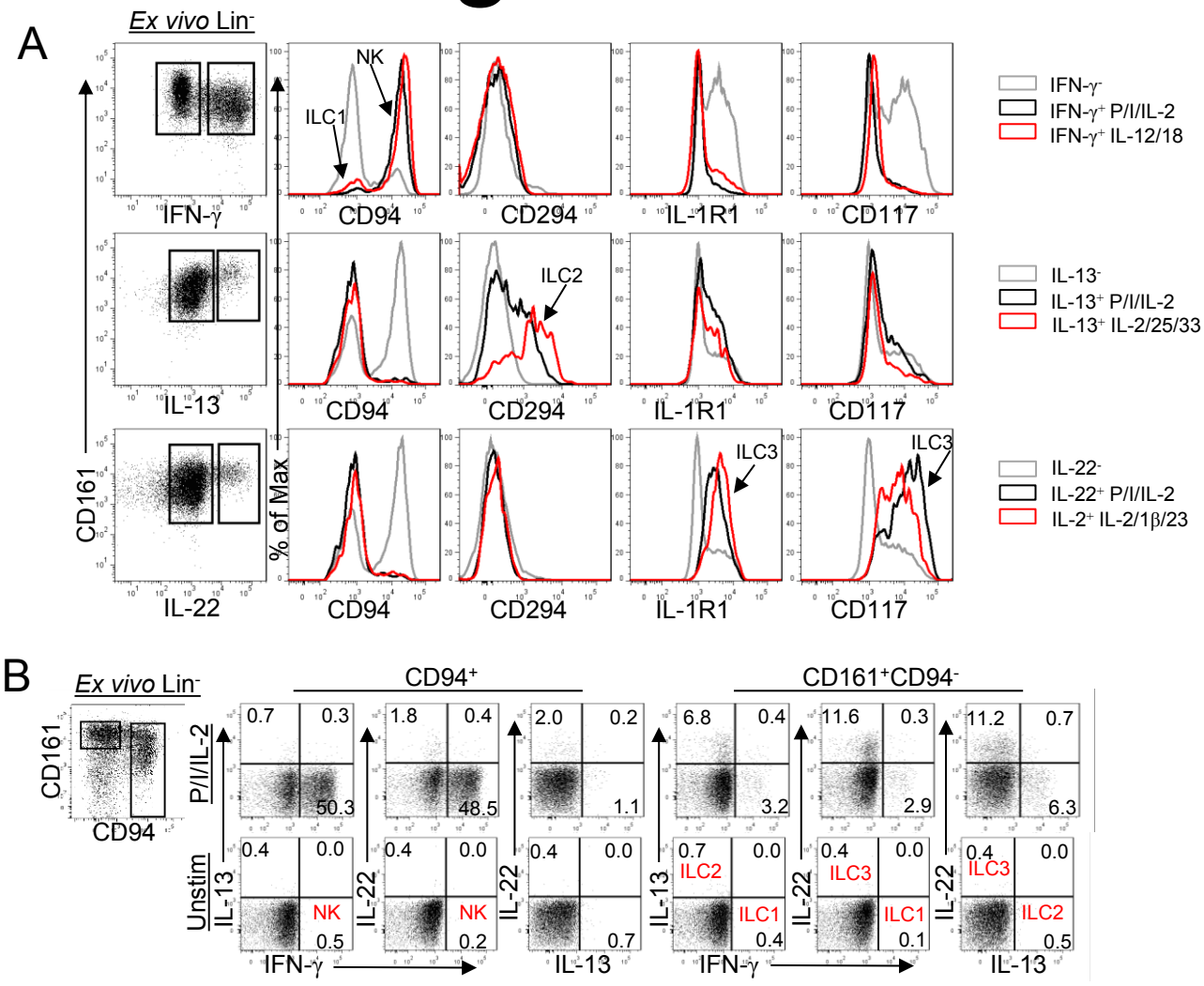

A
Ex vivo Lin-
NK
ILC1
ILC2
ILC3
IFN-γ
IL-13
IL-22
CD161
% of Max
CD94
CD294
IL-1R1
CD117
IFN-γ-
IFN-γ+ P/I/IL-2
IFN-γ+ IL-12/18
IL-13-
IL-13+ P/I/IL-2
IL-13+ IL-2/25/33
IL-22-
IL-22+ P/I/IL-2
IL-2+ IL-2/1β/23
B
Ex vivo Lin-
CD161
CD94
CD94+
CD161+CD94-
P/I/IL-2
Unstim
IL-13
IL-22
IFN-γ
0.7
0.3
50.3
1.8
0.4
48.5
2.0
0.2
1.1
6.8
0.4
3.2
11.6
0.3
2.9
11.2
0.7
6.3
0.4
0.0
NK
0.5
0.2
0.7
ILC2
ILC1
0.4
ILC3
0.1
0.5

Figure S5

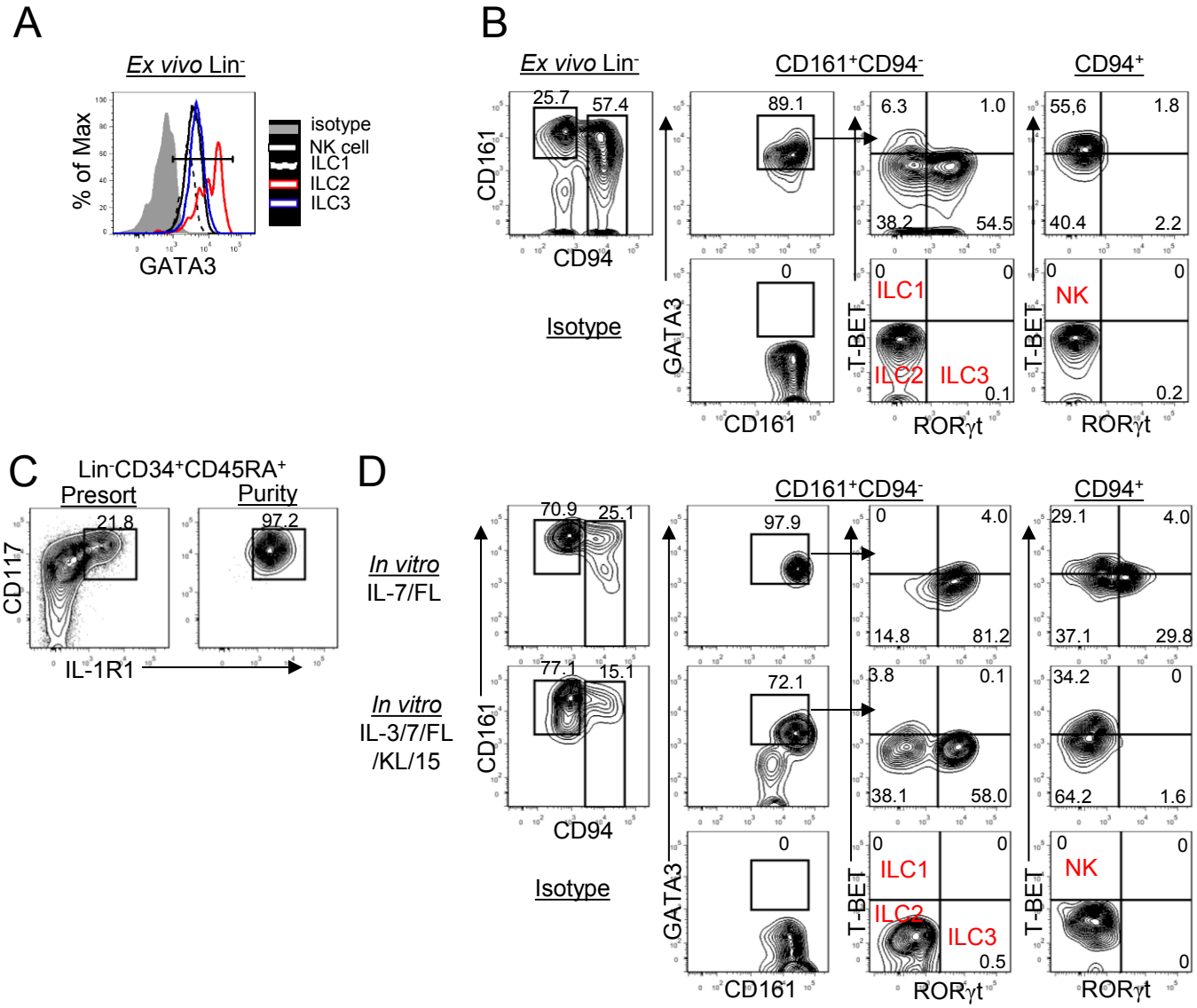
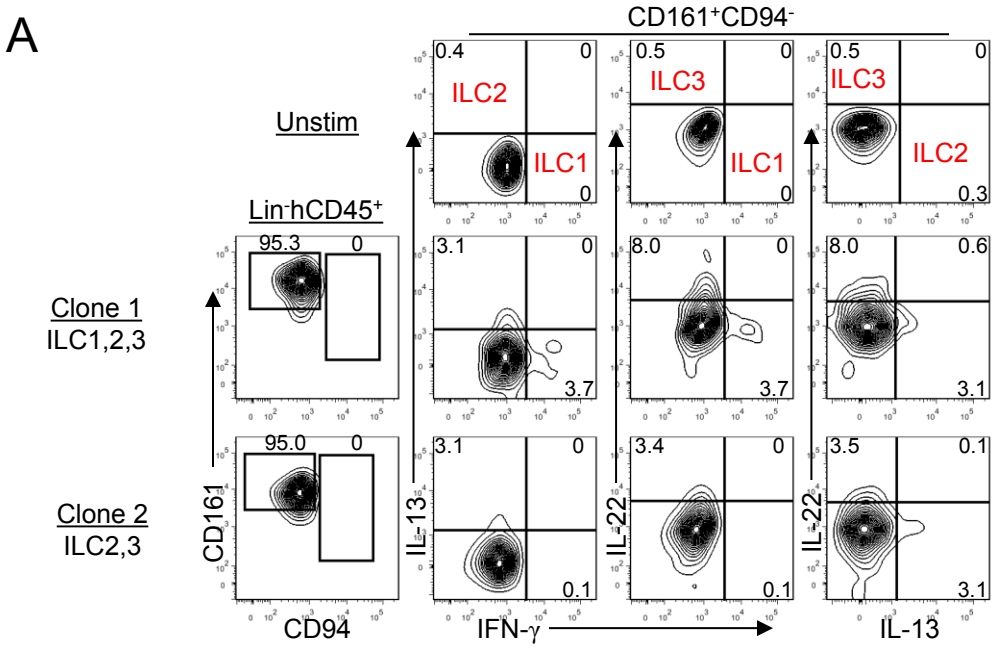
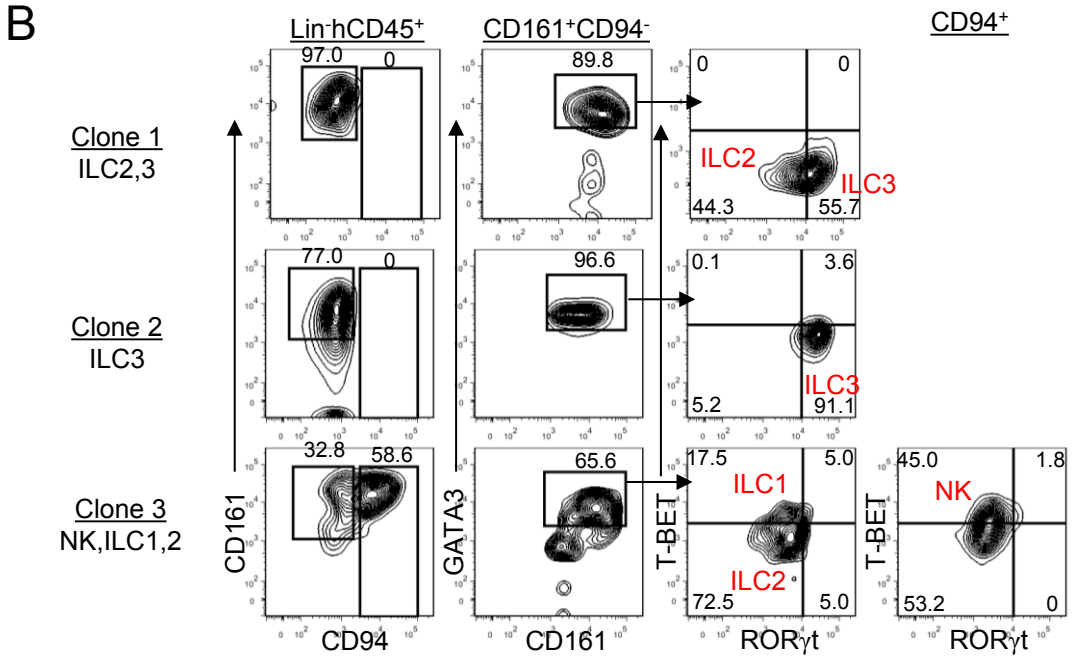

A
Ex vivo Lin-
% of Max
GATA3
isotype
NK cell
ILC1
ILC2
ILC3
B
Ex vivo Lin-
CD161+CD94-
CD94+
CD161
CD94
Isotype
GATA3
CD161
T-BET
RORγt
ILC1
ILC2
ILC3
NK
C
Lin-CD34+CD45RA+
Presort
Purity
CD117
IL-1R1
D
In vitro
IL-7/FL
In vitro
IL-3/7/FL
/KL/15

Figure S6


A
CD161+CD94-
Unstim
Lin-hCD45+
Clone 1
ILC1,2,3
Clone 2
ILC2,3
ILC1
ILC2
ILC3
CD161
CD94
IL-13
IFN-γ
IL-22
IL-13


B
Lin-hCD45+
CD161+CD94-
CD94+
Clone 1
ILC2,3
Clone 2
ILC3
Clone 3
NK,ILC1,2
CD161
CD94
GATA3
CD161
T-BET
RORγt
T-BET
RORγt
ILC2
ILC3
ILC1
NK

Scoville *et al.*, Supplemental figure legends

Figure S1. Transcriptional and functional profiles of FACS-purified SLT ILCs, Related to Figure 1.

(**A**) Using the ILC identification panel based on surface markers (shown at top), ILCs were sorted, RNA was collected, cDNA was prepared, and then qPCR was performed for ILC associated genes *TBX21*, *EOMES, GATA3, RORα*, and *AHR* (n=4, from 2 independent experiments). (**B**) These subsets were similarly sorted to purity and then split equally into 3 tubes (unstim, P/I/IL-2, and a "specific" cytokine cocktail). The P/I/IL-2 stimulation was for 6 hr while specific stimulations (IL-12/18; IL-2/25/33; IL-2/1β/23) were for 24 hr, with Brefeldin A being added 4 hr prior to processing for intracellular flow cytometry (n=4, from 2 independent experiments). Error bars indicate s.e.m.

Figure S2. Transcriptional profile of SLT Lin$^-$CD34$^+$CD45RA$^+$ progenitor subsets, Related to Figure 2.

cDNA from freshly sorted Stage 1, Stage 2 IL-1R1$^-$ and Stage 2 IL-1R1$^+$ progenitors was utilized as the template for qPCR for the indicated ILC-associated genes (n=6, from 2 independent experiments). Error bars indicate s.e.m.

Figure S3. T cell, DC, and ILC differentiation potentials of Stage 2 IL-1R1$^-$CD161$^-$ and Stage 2 IL-1R1$^-$ CD161$^+$ fractions, Related to Figure 3.

(**A**) Stage 2 IL-1R1$^-$ cells were freshly isolated from pediatric tonsils and sorted based on CD161 expression into CD161$^-$ and CD161$^+$ fractions. The sorted cells were then cultured in IL-7/FL on OP9-DL1 stromal feeder cells for 28 days. Cytokines and OP9-DL1 cells were replenished weekly. (**B**) After culture, hCD45$^+$ cells were analyzed with respect to CD161, iCD3 and HLA-DR. Analysis from a representative donor is shown (n=3 independent donors).

Figure S4. Functional identification of *ex vivo* SLT ILCs according to cytokine profile, Related to Figure 5.

(**A**) ILCs were enriched from pediatric tonsils, immediately stimulated *ex vivo* with either P/I/2 (6 hr stimulation, black histograms in the figure) or with the indicated cytokine combinations (24 hr stimulation, red histograms in the figure), and then analyzed by flow cytometry for cytokine production in relation to the expression of the indicated surface antigens. In each row, the four histogram plots were gated on the Lin^- $cytokine^+$ events (black boxes) in the dot plots to the far left (n=6, from 3 independent experiments). (**B**) Enriched tonsil-derived ILCs were stimulated with P/I/2 and stained for the indicated surface and intracellular markers. Unstimulated enriched ILCs were similarly stained for comparison in order to set negative quadrants. The red labels in the bottom plots indicate where the functional ILCs are depicted in the top row (n=4, from two independent experiments).

Figure S5. Transcription factor expression among *ex vivo* and *in vitro*-derived ILCs, Related to Figure 5.

(**A**) Freshly isolated NK cells (Lin^-CD94^+), ILC1s ($Lin^-CD94^-CD161^+CD117^-IL\text{-}1R1^-CD294^-$), ILC2s ($Lin^-CD94^-CD161^+CD117^-IL\text{-}1R1^-CD294^+$), and ILC3s ($Lin^-CD94^-CD161^+CD117^+IL\text{-}1R1^+CD294^-$) obtained from pediatric tonsils were analyzed for expression of GATA3. As shown, all SLT ILCs express GATA3, with the ILC2 population showing the highest levels comparatively (n=4, from 3 independent experiments). (**B**) Representative analysis of GATA3, T-BET, and RORγt expression by $Lin^-CD161^+CD94^-$ and Lin^-CD94^+ SLT ILCs. The figure depicts a representative donor (n=4, from 3 independent experiments). (**C**) Stage 2 $IL\text{-}1R1^+$ cells were sorted to purity and ILCs were derived *in vitro* for 28 days as indicated below. (**D**) $Lin^-CD161^+CD94^{+/-}$ ILCs derived *in vitro* in media supplemented with IL-7/FL (top row) or IL-3/7/FL/KL/15 (middle row) were prepared and ILC populations similarly identified as in (**B**) and compared to an isotype staining control (bottom row). Mature ILC populations are indicated in red (n=6, from two independent experiments).

Figure S6. Clonal analysis of ILCs derived from the Stage 2 $IL\text{-}1R1^+$ population, Related to Figure 6.

Individual cells were sorted from purified Stage 2 $IL\text{-}1R1^+$ cells and cultured on irradiated OP9-DL1 stroma plus IL-3 (first week only) and IL-7/FL/KL/15 for 28 days. The ILC progeny were then analyzed by flow

cytometry for surface and intracellular expression of the indicated antigens. (**A**) Shows two representative clones (from the 55 clones tested) that identified ILCs through a functional readout based on cytokines following stimulation with P/I/2 (similar to **Figure 5A**) (n=10, from 4 independent experiments). (**B**) Shows three representative clones (from 21 clones tested) that identified ILCs by transcription factors (similar to **Figure S5B and S5D**) (n=5, from 2 independent experiments). Overall cloning efficiency was 25.6%. Mature ILC locations (**A**) or populations (**B**) are indicated in red.

Supplemental Experimental Procedures

List of antibodies used

Antigen	Company	Clone
CD56	BD Biosciences (BD)	N901
CD117	BD	YB5.B8
CD294	BD	BV16
CD25	BD	M-A251
IFN-γ	BD	B27
CD34	BD	581
CD45RA	BD	Hl100
CD45	BD	2D1
CD10	BD	Hl10a
HLA-DR	BD	G46-6
CD33	BD	WM53
CD14	BD	M5E2
CD19	BD	HIB19
RORγt	BD	Q21-559
integrinB_7	BD	FIB504
T-BET	BD	04-46
isotype	BD	X40
CD294	BD	BM16
CD94	BD	HP-3D9
CD16	BD	3G8
CD1A	Beckman Coulter	BL6
IL-13	Biolegend	JES10-5A2
EOMES	Ebiosciences (Eb)	WD1928
GATA3	Eb	TWAJ
isotype	Eb	eb149/10H5
isotype	Eb	P3.6.2.8.1
T-BET	Eb	eBio4B10
CD3	Miltenyi (MT)	BW264/56
CD14	MT	Tük4
CD20	MT	LT20
CD161	MT	191B8
CD94	MT	REA113
CD117	MT	A3C6E2
CD127	MT	MB15-18C9
iCD3	MT	BW264/56
CD5	MT	UCHT2
isotype	MT	IS5-21F5
IFN-γ	MT	45-15
isotype	MT	IS6-11E5.11
IL-1R1	R&D	FAB269F
IL-1R1	R&D	FAB269P
IL-22	R&D	142928

List of primers used

Primer name	For/Rev	Sequence (5'-3')	Citation
TBX21	F	CAGAATGCCGAGATTACTCAG	Hughes et al, Cell Reports, 2014
	R	AGGATACTGGTTGGGTAGGA	
EOMES	F	AACAACACCCAGATGATAGTC	Hughes et al, Cell Reports, 2014
	R	TCATAGTTGTCTCTGAAG	
GATA3	F	ACCACAACCACACTCTGGAGGA	Mjosberg et al, Nat Immunology, 2011
	R	TCGGTTTCTGGTCTGGATGCCT	
RORα	F	ACAAGCAGCGGGAGGTGATGT	Mjosberg et al, Nat Immunology, 2011
	R	TGAGAGTCAAAGGCACGGC	
AHR	F	CTTAGGCTCAGCGTCAGTTA	Bernink et al, Nat Immunol, 2013
	R	GTAAGTTCAGGCCTTCTCTG	
RORγt (specific)	F	CTGCTGAGAAGGACAGGGAG	Ratajewski et al, J Immunol, 2012
RORC (specific)	F	CACAGAGACAGCACCGAGC	
RORC (specific)	R	AGTTCTGCTGACGGGTGC	
RORC/γt (common)	taqman	HS01076122_m1	
GAPDH	F	TGCACCACCAACTGCTTAGC	
	R	GGCATGGACTGTGGTCATGAG	
RAG1	taqman	HS00172121_m1	
ID2	F	CGGATATCAGCATCCTGTCC	Cupedo et al, Nat Immunol, 2009
	R	TCATGAACACCGCTTATTCAG	