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A progenitor cell expressing transcription factor RORγt generates all human innate lymphoid cell subsets

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Summary

The current model of murine innate lymphoid cell (ILC) development holds that mouse ILCs are derived downstream of the common lymphoid progenitor through lineage-restricted progenitors. However, corresponding lineage-restricted progenitors in humans have yet to be discovered. Here we identified a progenitor population in human secondary lymphoid tissues (SLTs) that expressed

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Author Contributions

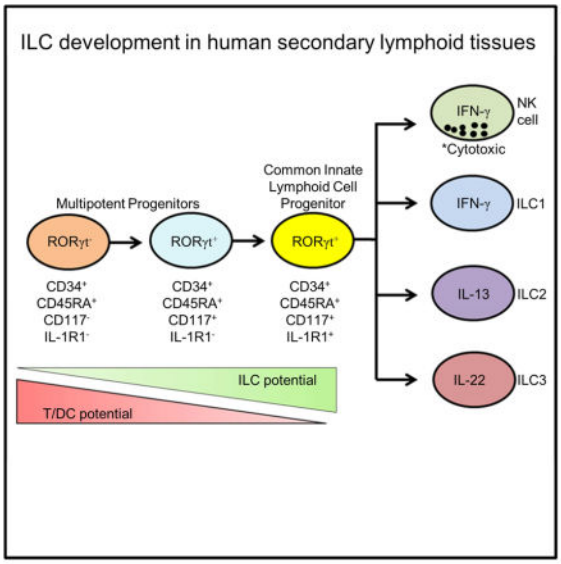
S.D.S., B.L.M., M.A.C. and A.G.F., designed experiments, analyzed the data, and wrote the manuscript. M.H.Z., Li.C., Lu C., K.A.K., T.H., S.C., S.M.B., S.M., and J.Y. performed experiments and analyzed data. X.L.Z. helped with statistical design and analysis. H.C.M. helped with flow cytometry design and analysis. W.E.C. III procured lymph node samples and participated in the preparation of the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

the transcription factor, RORγt, and was unique in its ability to generate all known ILC subsets, including natural killer (NK) cells, but not other leukocyte populations. In contrast to murine fate-mapping data, which indicate that only ILC3 cells express Rorγt, these human progenitor cells as well as human peripheral blood NK cells and all mature ILC populations expressed RORγt. Thus, all human ILCs can be generated through an RORγt^{+} developmental pathway from a common progenitor in SLTs. These findings help establish the developmental signals and pathways involved in human ILC development.

Graphical Abstract



Introduction

Innate lymphoid cells (ILCs) are closely related effector lymphocyte populations distinct from T cells and B cells and thought to derive from the common lymphoid progenitor (CLP) (Cherrier et al., 2012). ILCs have been categorized primarily into three functionally distinct groups: Group 1, consisting of natural killer (NK) cells and ILC1s; Group 2, consisting of ILC2s; and Group 3, consisting of ILC3s and lymphoid tissue inducer cells (Spits et al., 2013). These ILC subsets are naturally enriched in various immune cell rich compartments of the body, such as secondary lymphoid tissues (SLT), and they are primarily defined by distinct cell surface marker, transcription factor, and cytokine expression profiles (Hazenberg and Spits, 2014).

NK cells were the first ILC population discovered for their ability to mediate natural cytotoxicity against certain types of cancer cells (Herberman et al., 1975) and to produce cytokines, most notably interferon gamma (IFN-γ). Among SLT cells lacking lineage antigens (CD3, CD4, CD14, CD19, CD20, and CD123), henceforth referred to as Lin^{-}, CD94 is defined as a specific marker of NK cells (Hazenberg and Spits, 2014). While many transcription factors are important for NK cell maturation and function, the T-box related T-BET (encoded by *TBX21*) and Eomesodermin (EOMES) transcription factors are critical for

end-stage NK cell maturation and function, as mice lacking *Tbx21* and *Eomes* lack mature NK cells (Gordon et al., 2012; Townsend et al., 2004). ILC1s comprise the other major Group 1 ILC population and are characterized as non-cytolytic, $Lin^{-}CD161^{+}CD127^{+}CD94^{-}$ cells that produce IFN-γ in response to monokine stimulation (Bernink et al., 2015; Bernink et al., 2013; Fuchs et al., 2013). Like NK cells, murine ILC1s also require T-bet for their development but are not dependent on Eomes (Klose et al., 2014). ILC2s can be identified in fresh human tissues by their $Lin^{-}CD161^{+}CD127^{+}CD294^{+}CD25^{+}$ immunophenotype (Mjösberg et al., 2011). ILC2s are dependent on the transcription factors GATA3 and the retinoic-acid related orphan receptor (ROR)α (Hoyler et al., 2012; Wong et al., 2012), and they respond to interleukin 25 (IL-25) and IL-33 stimulation to produce IL-5 and IL-13 (Moro et al., 2010). These cells are important immune mediators in the settings of anti-helminth immunity, airway repair and inflammation, allergic reactions, and metabolic regulation of adipose tissue (Kim and Artis, 2015). ILC3s are defined as $Lin^{-}CD161^{+}CD127^{+}CD94^{-}$ cells that express CD117 (also known as c-kit) and IL-1 receptor 1 (IL-1R1) and also variably express the NK cell associated markers, CD56, NKp30, NKp44, and NKp46 (Cella et al., 2009; Cupedo et al., 2009; Hazenberg and Spits, 2014). ILC3s are further characterized by their relatively high expression of the transcription factors RORγt and aryl hydrocarbon receptor (AHR). In addition, ILC3s respond to IL-1β and IL-23 to produce IL-22 and IL-17 that promote the release of anti-microbial peptides from adjacent mucosal associated epithelial cells (Cella et al., 2009; Cupedo et al., 2009; Hughes et al., 2010; Hughes et al., 2009).

A comprehensive model describing how human ILCs develop has yet to be reported. We previously provided evidence for a five-stage model of human NK cell development in SLTs (Freud and Caligiuri, 2006). Among these five stages, the Stage 1 and Stage 2 populations are immature $CD34^{+}CD45RA^{+}$ progenitor cells, while Stages 3–5 are $CD34^{-}$ and include more mature developmental intermediates (Freud et al., 2006). Stage 1 cells are defined as $Lin^{-}CD34^{+}CD45RA^{+}CD117^{-}CD94^{-}$ while Stage 2 cells express CD117 and are thus defined as $Lin^{-}CD34^{+}CD45RA^{+}CD117^{+}CD94^{-}$. Both Stage 1 and Stage 2 progenitors are selectively enriched in human SLT, such as tonsils, and both are capable of giving rise to $CD94^{+}$ NK cells, T cells, and dendritic cells (DCs) *in vitro* but not to B cells or myeloid cells (Freud et al., 2006). Thus, these populations were originally described as multipotent progenitors involved in, but not restricted to, NK cell development. As this work was prior to the identification of other ILCs a comprehensive analysis of the ILC lineage of these populations was not included in the former study.

Data in mice strongly support a divergent model of ILC development between NK cells and non-NK ILCs. For example, a $Lin^{-}\alpha_4\beta_7{}^{+}Id2^{+}$ common helper ILC progenitor (CHILP) was discovered in mice and shown to have ILC1, ILC2, and ILC3 potential without the capacity for NK cell development (Klose et al., 2014). In addition, studies using mice fate-mapped for *Rorγt* demonstrated that murine NK cells are *Rorγt* fate-map negative, meaning they do not have any history or current expression of *Rorγt* (even at the transcript level) (Satoh-Takayama et al., 2010; Vonarbourg et al., 2010). In light of these findings, it is noteworthy that both the human Stage 2 and Stage 3 cell populations originally described by Freud *et al.* (Freud et al., 2006) express RORγt (Cupedo et al., 2009; Montaldo et al., 2014). Thus, the potential role of these two human $ROR\gamma t^{+}$ populations as physiologic NK cell precursors is

unclear given the fate-mapping data in mice. Nonetheless, a comprehensive evaluation of RORγt and its fidelity in the human system remains to be established.

Here we identified a $Lin^{-}CD34^{+}CD45RA^{+}CD117^{+}IL\text{-}1R1^{+}ROR\gamma t^{+}$ progenitor population that expressed *ID2* and gave rise to all ILCs, including NK cells, *in vitro*, yet it did not give rise to other leukocyte lineages. The progenitor was exclusively found in SLTs including tonsils, lymph nodes, and spleen, but it was not found in other hematopoietic tissues such as peripheral blood (PB), bone marrow (BM), umbilical cord blood (UCB), or thymus. We also demonstrated that these human SLT progenitor cells, PB $CD56^{bright}$ NK cells, SLT resident NK cells, and all other non-NK ILCs expressed *RORγt*. These findings identify a human common ILC progenitor cell and suggest a model whereby all human ILCs can share an $ROR\gamma t^{+}$ developmental pathway in SLTs.

Results

Human PB $CD56^{bright}$ NK cells and SLT resident ILC populations express *RORγt*

We hypothesized that if the human NK cell developmental system is similar to that in mice then mature NK cells should completely lack *RORγt* expression. To test this hypothesis, we sorted $Lin^{-}CD56^{bright}CD94^{hi}$ (referred to as $CD56^{bright}$) and $Lin^{-}CD56^{dim}CD94^{lo}$ (referred to as $CD56^{dim}$) NK cell subsets from healthy adult PB samples and performed RT-PCR using primers specific for the *RORγt* isoform (Ratajewski et al., 2012). As shown in Figure 1A, we reproducibly observed that freshly purified human PB $CD56^{bright}$ NK cells expressed *RORγt*. We did not detect *RORγt* expression in PB $CD56^{dim}$ NK cells, yet both NK cell subsets expressed detectable *EOMES* (Figure 1A).

We next wanted to determine the lineage specificity of *RORγt* expression within other mature ILC subsets. To this end, we purified SLT ILC subsets in accordance with published surface marker expression patterns (Hazenberg and Spits, 2014) and then performed RT-PCR as well as quantitative RT-PCR (qPCR) for *RORγt* (Figure 1B). The identities of the ILCs were confirmed both by qPCR analysis of other ILC-associated transcription factors (T-BET, EOMES, GATA3, RORα, and AHR) (Figure S1A) as well as functionally by demonstrating that Group 1, 2, and 3 ILCs specifically responded to both PMA/ionomycin/IL-2 (P/I/IL-2) as well as established cytokine stimuli (IL-12/18; IL-2/25/33; and IL-2/1β/23, respectively) to produce their characteristic subset-defining cytokines (IFN-γ, IL-13, and IL-22, respectively; Figure S1B) (Hazenberg and Spits, 2014). Consistent with our findings in PB (Figure 1A) we found that all mature ILC subsets, including SLT-resident NK cells, expressed *RORγt* transcript, with ILC3s having the most as expected (Figure 1B) (Klose et al., 2014). In addition, RORγt protein was detected by intracellular flow cytometry in each of the SLT ILC subsets compared to an isotype control, with ILC3s having the highest percentage and intensity of signal comparatively (Figure 1C). Thus, in contrast to the reported patterns of Rorγt expression by mouse ILCs, we observed that human PB $CD56^{bright}$ NK cells and all ILCs in human SLTs expressed RORγt.

Identification of a Lin$^-$CD34$^+$CD45RA$^+$CD117$^+$IL-1R1$^+$RORγt$^+$ subset in SLTs

Given the detection of RORγt in all ILC subsets in human SLTs, we hypothesized that they may derive from an RORγt$^+$ progenitor. Previous reports (Cupedo et al., 2009; Montaldo et al., 2014) showed that human SLT Lin$^-$CD34$^+$CD45RA$^+$CD117$^+$ Stage 2 multipotent progenitor cells express RORγt. We observed that the SLT Stage 2 population may be divided into two distinct subsets according to the surface expression of IL-1R1 and relative intracellular expression of RORγt, with the IL-1R1$^+$ subset showing the highest expression of RORγt (Figures 2A, B). The two Stage 2 subsets showed some similar features, including uniform positivity for integrin β_7 and at least partial expression of CD161 (Figure 2C). Moreover, both largely lacked CD10 in comparison to Stage 1 cells (Lin$^-$CD34$^+$CD45RA$^+$CD117$^-$IL-1R1$^-$). However, the Stage 2 IL-1R1$^+$ subset expressed the highest amount of CD127 and appeared to be the only subset expressing CD56. We also observed that the IL-1R1$^+$ Stage 2 subset was present in pediatric tonsils, adult spleen, and adult axillary lymph nodes, but it was essentially undetectable in PB, UCB, thymus, and BM. Thus it was naturally restricted to SLTs (Figure 2D). In comparison, the Stage 1 population was present in each tissue, while the Stage 2 IL-1R1$^-$ subset appeared to be enriched in certain tissues such as SLT, PB, and UCB, but it was absent from thymus (Figure 2D and data not shown).

We also evaluated each SLT Lin$^-$CD34$^+$CD45RA$^+$ subset by qPCR for expression of *RAG1* and *ID2* (Figure 2E), as these transcription factors are associated with early T cell and ILC development, respectively (Blom and Spits, 2006; Galy et al., 1995; Guy-Grand et al., 1992; Wilson et al., 1994). ID2 in particular has been shown to repress the E protein transcription factors necessary for T and B cell development, and it is thus critical for promoting all ILC differentiation (Hoyler et al., 2012; Kee, 2009). At the transcript level, we found that Stage 1 and Stage 2 IL-1R1$^-$ cells expressed some *RAG1*, but it was undetectable within Stage 2 IL-1R1$^+$ cells. In contrast, Stage 2 IL-1R1$^-$ and Stage 2 IL-1R1$^+$ cells had significantly higher *ID2* expression when compared to Stage 1 cells. These differences in surface antigen expression and *RAG1* and *ID2* expression, along with the expression patterns of other ILC-related transcription factors (Figure S2), suggested that each subset had unique developmental potential.

SLT Stage 2 IL-1R1$^+$ cells are ILC lineage restricted

SLT Stage 1 and unfractionated Stage 2 cells were previously shown to have *ex vivo* potential for T cell, DC, and NK cell differentiation; however, they were incapable of giving rise to B cells and myeloid cells under the conditions tested (Freud et al., 2006). As Stage 2 IL-1R1$^-$ and Stage 2 IL-1R1$^+$ cells both expressed *ID2*, yet only the Stage 2 IL-1R1$^-$ expressed *RAG1* (Figure 2E), we next wanted to determine the lineage differentiation potentials of these newly described Stage 2 subsets. Therefore, we sorted Stage 1, Stage 2 IL-1R1$^-$, and Stage 2 IL-1R1$^+$ cells to ≥98% purity and co-cultured them with OP9-DL1 murine stromal cells, human FLT3 ligand (FL), and human IL-7, which allows for multilineage differentiation, including that of T cells, *in vitro* (Freud et al., 2006; McClory et al., 2012; Schmitt and Zúñiga-Pflücker, 2002). As shown in Figure 3A (top panel), cells of relatively large size and high side scatter were produced by Stage 1 and Stage 2 IL-1R1$^-$ cells yet were distinctly lacking from cultures initiated with Stage 2 IL-1R1$^+$ cells. These

large cells were phenotypically consistent with myeloid DCs as they expressed CD1c, CD33, CD141, CLEC9A, and HLA-DR yet lacked CD14 and CD303 expression (Figure 3B and data not shown). In addition, each cultured SLT Lin$^-$CD34$^+$CD45RA$^+$ subset gave rise to lymphocytes with low forward and side scatter characteristics [Figure 3A, (top panel)]. Among the derived human (h)CD45$^+$ lymphocyte populations, CD161$^-$ intracellular (i)CD3$^+$CD5$^+$CD1a$^+$ T cells were detected from Stage 1 and Stage 2 IL-1R1$^-$ initiated cultures, whereas essentially no T cells were detected from Stage 2 IL-1R1$^+$ initiated cultures [Figures 3A (middle panel) and 3C–D]. The Stage 2 IL-1R1$^-$ population showed an intermediate capacity for T cell and DC developmental potential compared to the Stage 1 and the Stage 2 IL-1R1$^+$ populations (Figure 3A,D). To determine if lineage potential within the Stage 2 IL-1R1$^-$ population was associated with CD161 expression, which is bimodal in this population (Figure 2C), we purified and similarly cultured the CD161$^-$ and CD161$^+$ fractions (Figure S3A). Both fractions differentiated into T cells; however, essentially no HLA-DR$^+$ DCs were produced from the Stage 2 IL-1R1$^-$CD161$^+$ fraction (Figure S3B).

Among the hCD45$^+$CD161$^+$iCD3$^-$ cells generated under these conditions from each of the three SLT Lin$^-$CD34$^+$CD45RA$^+$ subsets, both Lin$^-$CD161$^+$CD94$^-$ non-NK ILCs and Lin$^-$CD161$^+$CD94$^+$ NK cells were produced [Figure 3A (bottom panel) and 3D]. The development of NK cells from the RORγt$^+$ Stage 2 IL-1R1$^+$ subset is consistent with the original report showing that Stage 2 cells are NK cell precursors (Freud et al., 2006). However, our findings diverge from a recent study showing that SLT resident RORγt$^+$ progenitors are specified for ILC3 development (Montaldo et al., 2014). Therefore, to determine the NK cell potential of the Stage 2 IL-1R1$^+$ subset under *in vivo* conditions we sorted this subset and injected it into Non-obese diabetic *Prkdc*^{scid}*Il2rg*^{tm1}(NSG) immune-deficient mice supplemented with hIL-15 (Figure 4A). After five weeks among the hCD45$^+$ events we detected a Lin$^-$CD161$^+$CD94$^+$ NK cell population including some CD94$^+$CD16$^+$ mature Stage 5 NK cells present in each tissue tested (Figure 4B). No T cells, B cells, or DCs were detected under these NK-promoting conditions (data not shown). A similar *in vivo* experiment conducted with administration of hIL-7 instead of hIL-15 showed that no hCD45$^+$ cells were detectable after 5 weeks (not shown). Collectively, these data demonstrate that under the *in vivo* and *in vitro* conditions used here, the SLT Stage 2 IL-1R1$^+$ population is ILC lineage restricted and capable of NK cell differentiation, consistent with our findings that mature human NK cells express *RORγt* (Figure 1).

All four ILC subsets differentiate from the Stage 2 IL-1R1$^+$ population

We next wanted to determine the ILC subset differentiation potential of each SLT-derived Lin$^-$CD34$^+$CD45RA$^+$ progenitor population, particularly the Stage 2 IL-1R1$^+$ population that we observed to lack T and DC lineage developmental potential (Figure 3). Initially we attempted to identify *in vitro*-derived ILC subsets using surface marker expression similar to how we identified these ILC subsets *ex vivo* (Figure 1 and Figure S1); however, consistent with our and others' prior observations, we noted that surface antigen expression following *in vitro* differentiation was not perfectly concordant with what is observed when evaluating SLT ILCs *ex vivo* (Ahn et al., 2013; Freud et al., 2005; Freud et al., 2006). For instance, while all non-NK ILCs from SLT expressed CD161 and CD127 *ex vivo* (Hazenberg and Spits, 2014), we only detected CD161 but not CD127 expression following culture of SLT

Lin$^-$CD34$^+$CD45RA$^+$ progenitor cells (Figure 3B and data not shown), consistent with previous reports (Ahn et al., 2013; Ghazawi et al., 2013; Vranjkovic et al., 2007). In addition, we rarely detected or did not detect other ILC subset-associated surface antigens, including CD294 and ST2 (Mjösberg et al., 2011), among Lin$^-$CD161$^+$ ILCs derived *in vitro* (data not shown).

Given the aforementioned differences in surface antigen expression between Lin$^-$CD161$^+$ ILC subsets freshly obtained from SLT and *in vitro*-generated Lin$^-$CD161$^+$ ILCs, coupled with our demonstration that staining for intracellular cytokine expression specifically identifies functional ILC subsets *ex vivo* (Figure S1 and Figure S4), we sought to provide functional evidence for the generation of all four major ILC subsets following *in vitro* culture of Lin$^-$CD34$^+$CD45RA$^+$ progenitor cells by way of assessment of cytokine production. To this end, we stimulated the *in vitro* progeny derived from Stage 1, Stage 2 IL-1R1$^-$, and Stage 2 IL-1R1$^+$ cells with P/I/2 and evaluated them for surface Lin-associated antigens as well as CD161 and CD94 expression in conjunction with intracellular staining for IFN-γ, IL-13, and IL-22. For these experiments, the progenitor cells were cultured under two distinct conditions to determine if the culture conditions themselves had an impact on the generation of ILC subsets: OP9-DL1 stroma plus FL and IL-7 *or* OP9-DL1 stroma plus IL-3 (added only once at the beginning of culture), FL, c-kit ligand (KL), IL-7 and IL-15. As shown in Figure 5A, we reproducibly observed distinct IFN-γ$^+$, IL-13$^+$, and IL-22$^+$ populations (all Lin$^-$CD161$^+$) following P/I/2 stimulation of the progeny of SLT Stage 2 IL-1R1$^+$ progenitor cells derived in both conditions. Similar populations were also detected from culture of Stage 1 and Stage 2 IL-1R1$^-$ populations (data not shown). The IFN-γ$^+$, IL-13$^+$, and IL-22$^+$ populations were largely mutually exclusive (Figure 5A) similar to what was observed following P/I/2 stimulation of enriched SLT ILCs *ex vivo* (Figure S4B), supporting the conclusion that functionally distinct Group 1, 2, and 3 ILCs, respectively, were generated *in vitro*. Additional studies evaluating intracellular protein expression of the GATA3, T-BET, and RORγt ILC-associated transcription factors among *ex vivo* and *in vitro*-derived Lin$^-$CD161$^+$CD94$^{+/-}$ ILCs showed evidence of distinct GATA3$^+$T-BET^{hi}RORγt^{lo}, GATA3$^+$T-BET^{lo}RORγt^{lo}, and GATA3$^+$T-BET^{lo}RORγt^{hi} populations (Figure S5), further supporting our conclusion based on the functional readout that Group 1 ILC, ILC2, and ILC3 subsets, respectively, were produced *in vitro*.

In agreement with our *ex vivo* data, we also observed that CD94 was critical to distinguish *in vitro*-derived NK cells (Lin$^-$CD94$^+$IFN-γ$^+$) from ILC1s (Lin$^-$CD161$^+$CD94$^-$IFN-γ$^+$) (Figure 5B). Indeed the expression of CD94 among Lin$^-$ ILCs specifically correlated with the expression of EOMES and CD16 (Figure 5C) as well as with cytotoxic potential against K562 target cells (Figure 5D), all properties found exclusively among NK cells. These data support the designation of CD94 as an NK-specific marker among SLT- and *in vitro*-derived ILCs, consistent with the current human ILC classification (Hazenberg and Spits, 2014). In contrast to the Lin$^-$CD94$^+$IFN-γ$^+$ NK cells, the Lin$^-$CD161$^+$CD94$^-$IFN-γ$^+$ cells expressed T-BET but not EOMES or CD16 (Figure 5C), consistent with ILC1s (Klose et al., 2014).

When comparing the *in vitro*-derived progeny generated in the two culture conditions, we noted differences in the abilities to generate T cells, NK cells, and ILC1s from the SLT progenitor populations (Figure 6A and 6B). Specifically, T cell development from the Stage

1 and Stage 2 IL-1R1^{-} populations was only detected in the OP9-DL1/FL/IL-7 condition. In contrast, NK cell and ILC1 generation were more prominent when the progenitor cells were cultured in the OP9-DL1/FL/KL/IL-3/IL-7/IL-15 condition (Figure 6B). ILC2s and ILC3s were similarly derived in both culture conditions.

Lastly, we performed a clonal analysis of single cells sorted from purified Stage 2 IL-1R1^{+} cells using the *in vitro* condition that best supported robust generation of all four ILC subsets (i.e. OP9-DL1/FL/KL/IL-3/IL-7/IL-15) (Figure 6C). The *in vitro*-derived progeny were evaluated for functional ILC subsets as outlined in Figure 5. Consistent with our "bulk" culture data, we observed that individually sorted and cultured Stage 2 IL-1R1^{+} cells gave rise to both non-NK ILCs as well as NK cells in multiple different combinations (Figure 6C and S6A). Indeed of 55 total clones tested, approximately two-thirds gave rise to at least two functionally distinct ILC subsets, with few clones giving rise to all four ILC subsets. As described above, these functional data were further validated by the demonstration of distinct ILC subsets according to transcription factor expression analyses of other Stage 2 IL-1R1^{+}-derived clones (Figure S6B). Clones from the Stage 1 and Stage 2 IL-1R1^{-} populations were also generated and analyzed in parallel, and as expected individually sorted and cultured cells from these populations gave rise to all four ILC subsets, with marked heterogeneity in terms of ILC subset generation (data not shown). Collectively, these data support the conclusion that each of the three SLT Lin^{-}CD34^{+}CD45RA^{+} populations has potential to differentiate into each of the four major ILC subsets, including NK cells, and even at the clonal level (Figure 5 and Figure 6). Moreover, the Stage 2 IL-1R1^{+} population, which was naturally restricted to SLT (Figure 2), was unique in its ability to give rise to all four ILC subsets, but it was not able to also give rise to T cells and DCs under the conditions tested (Figure 3 and Figure 6). Thus, the Stage 2 IL-1R1^{+} population represents an ILC lineage-restricted progenitor.

Discussion

Here we have identified a human Lin^{-}CD34^{+}CD45RA^{+}CD117^{+}IL-1R1^{+}RORγt^{+} population that appears to be found only in SLT and is capable of differentiating into all major ILC populations, including NK cells, as determined using both highly specific functional as well as sensitive transcription factor methods of ILC identification. In contrast to Stage 1 cells and Stage 2 IL-1R1^{-} cells, this newly characterized Stage 2 IL-1R1^{+} population lacks expression of *RAG1* and the ability to differentiate into T cells or DCs under established *in vitro* conditions. Therefore, it appears to be a lineage-restricted common ILC progenitor (CIP) and, to the best of our knowledge, represents a novel population identified in humans.

There is still much unknown concerning the early developmental intermediates and pathways involved in human lymphocyte development. Compelling evidence exists in mice that all lymphocytes, including T cells, B cells, NK cells and other ILCs, are ultimately derived from a common lymphoid progenitor (CLP) population (Klose et al., 2014). Recently, a Lin$^{-}\alpha_4\beta_7{}^{+}$Id2^{+} common helper ILC progenitor (CHILP) was discovered in mice and was shown to be restricted to non-NK ILC development as it was only able to give rise to ILC1, ILC2, and ILC3s (Constantinides et al., 2014; Klose et al., 2014). The CHILP likely exists downstream of the CLP (Diefenbach et al., 2014). However, as the CHILP is

unable to give rise to NK cells that also share a dependence on Id2 for differentiation, it has been proposed that global ILC development may proceed through a CIP that exists as a developmental intermediate between the CLP and the CHILP (Diefenbach et al., 2014; Hazenberg and Spits, 2014). This CIP, as proposed from evidence in the murine system including a recent report (Yang et al., 2015), could in turn differentiate into an NK cell specific progenitor as well as the CHILP, which has the potential to differentiate into all other non-NK ILC populations.

Our data demonstrated the existence of a human ILC lineage-restricted progenitor cell, the $Lin^{-}CD34^{+}CD45RA^{+}CD117^{+}IL\text{-}1R1^{+}$ or Stage 2 $IL\text{-}1R1^{+}$ population, that is found only in SLTs. At the clonal level we observed that Stage 2 $IL\text{-}1R1^{+}$ cells had the capacity to differentiate into each major ILC population, including NK cells, indicating that the human Stage 2 $IL\text{-}1R1^{+}$ subset is distinct from the CHILP identified in mice that can only give rise to non-NK ILCs. Thus the Stage 2 $IL\text{-}1R1^{+}$ population may in fact represent the CIP population in humans similar to the population recently described in mice (Yang et al., 2015). It is important that these data should not be interpreted to mean that this is the only pathway for ILC development. Alternative pathways for human ILC differentiation likely exist, especially in other tissues that have not yet been evaluated.

We also showed that Stage 2 $IL\text{-}1R1^{+}$ cells lacked detectable expression of *RAG1*, and despite being cultured in established *in vitro* T cell-promoting conditions (McClory et al., 2012; Schmitt and Zúñiga-Pflücker, 2002), they did not give rise to T cells (or DCs), further distinguishing this population from the other two major SLT resident $Lin^{-}CD34^{+}CD45RA^{+}$ populations that did express *RAG1* (i.e. Stage 1 and Stage 2 $IL\text{-}1R1^{-}$ cells). Indeed, in concordance with *RAG1* and *ID2* expression levels, T cell generation was lost with an accompanying increase in ILC generation during progression from Stage 1 (both T and ILC potential) to Stage 2 $IL\text{-}1R1^{-}$ (intermediate potential for both T and ILC subsets) to Stage 2 $IL\text{-}1R1^{+}$ that lacked T cell potential but maintained ILC potential. We also observed that CD161 expression within the Stage 2 $IL\text{-}1R1^{-}$ population was associated with the loss of DC differentiation potential. Collectively, the gene expression and culture data we present indicate that these human SLT $Lin^{-}CD34^{+}CD45RA^{+}$ progenitor populations are biologically distinct from one another and may represent serial stages of lymphoid differentiation towards ILCs.

In a recent study working with unfractionated human tonsil Stage 2 cells (referred to as $ROR\gamma t^{+}CD34^{dim}$ and purified as $CD34^{+}CD117^{+}$), Montaldo *et al.*, showed that when cultured in FL, KL, IL-7, and IL-15, these cells preferentially developed into ILC3s, thus concluding that they are lineage specified ILC3 precursors (Montaldo et al., 2014). This conclusion is in contradiction to prior findings (Freud et al., 2006) as well as those reported here, showing that unfractionated Stage 2 cells are capable of differentiation that extends beyond ILC3s. As Montaldo *et al.* did not report testing for other lineages including ILC1s, ILC2s, T cells, or DCs, it is possible that further experimentation utilizing additional culture conditions and/or analytic measures would clarify these discrepancies. Our data indicate that a dissection of the Stage 2 population based on IL-1R1 expression, along with testing using established *in vitro* conditions and *in vivo* transplantation into NSG mice, reveals less restricted differentiation potentials for the two Stage 2 subpopulations, with only the

IL-1R1^{+} subset lacking T cell and DC potential yet showing pan-ILC lineage differentiation potential.

Our demonstration that all human SLT ILC populations as well as PB CD56^{bright} NK cells express *RORγt* does not definitively prove that all human ILCs arise from an RORγt^{+} precursor. Indeed ethical considerations preclude performing genetic fate-map studies in humans, and it is certainly possible if not likely that other pathways of human ILC differentiation exist *in vivo*. Nonetheless, given the results of our *in vitro* clonal assays and *in vivo* experiments exploring the potential of Stage 2 IL-1R1^{+}RORγt^{+} progenitors, the data support the notion that in humans all ILC subsets can derive from RORγt^{+} progenitor cells. These findings now raise a question regarding the physiologic relevance and importance of RORγt expression in some NK cells and other ILC subsets. Although it is not currently known whether there is sufficient RORγt protein in each mature ILC subset to impart some functional effect, this will be important to address in future studies.

Previous studies that identified mouse conventional NK cells as *Rorγt*$^{-}$, both currently and ancestrally with the use of the fate-mapping model, established a clear rationale for empirically excluding any human RORγt^{+} cell as having NK cell developmental potential (Klose et al., 2014; Satoh-Takayama et al., 2010; Vonarbourg et al., 2010). However, the evidence provided to date in human experimental systems has not always supported the translation of these fate-map data (Freud and Caligiuri, 2006; Freud et al., 2006; Hughes et al., 2014; Mjösberg et al., 2011). In light of our demonstration here that *RORγt* expression is detectable in freshly isolated human PB CD56^{bright} NK cells, it is worth noting that a previous report found that human ILC2s express RORγt (Mjösberg et al., 2011), despite the fact that mouse ILC2s are also fate-map negative in the murine model (Mjösberg et al., 2011; Moro et al., 2010). These data indicate that RORγt may be more promiscuous in humans than in mice. This mouse-human discrepancy is not without precedent. The transcription factor Foxp3 was originally shown to be specific to regulatory T cells in mice, but later FOXP3 was shown to have a more promiscuous expression profile in humans (Walker et al., 2003; Ziegler, 2007). The data presented here suggest that RORγt expression represents a similar scenario, and the observation that Stage 2 IL-1R^{+}RORγt^{+} progenitors can differentiate into CD16^{+} NK cells *in vivo* in NSG mice further supports this notion.

Multiple lines of evidence currently support a linear developmental relationship between human CD56^{bright} and CD56^{dim} NK cells (Dulphy et al., 2008; Huntington et al., 2009; Lopez-Verges et al., 2010; Romagnani et al., 2007; Yu et al., 2010). Thus, the lack of detectable *RORγt* transcript in the CD56^{dim} subset could be the result of further differentiation. Nonetheless, given our observation of constitutive *RORγt* expression in some PB CD56^{bright} NK cells but not CD56^{dim} NK cells, an alternative possibility is that these subsets represent two separate lineages, a scenario which would be more in keeping with the mouse *Rorγt* fate-mapping data (Vonarbourg et al., 2010). In support of this alternative possibility, Wu *et al* generated a system of lineage-traced progenitors in Rhesus Macaques using transplanted, autologous, retrovirally-infected, and genetically bar-coded CD34^{+} progenitors. In this system, they showed that CD56^{bright}-like and CD56^{dim}-like PB NK cells from these animals might derive through separate developmental pathways (Wu et al., 2014).

Further studies are warranted to address these two intriguing possibilities regarding human PB NK cell subsets.

Prior demonstration of the natural enrichment of ILCs in SLTs (Hazenberg and Spits, 2014) and now of the Stage 2 IL-1R1$^+$ subset in SLTs suggests the presence of a specific microenvironmental niche supporting ILC development in these tissues. It has been well established that other lymphocytes require tissue-specific signals for their development, such as those provided for immature B and T cells in the BM and thymus, respectively. Thus, deciphering the roles of other cells and their produced factors within SLT and potentially within discrete microenvironments in these tissues will likely prove to be insightful in furthering our overall understanding of ILC development. For example, in our studies we observed robust alterations of lineage potential of most noticeably the Stage 1 and Stage 2 IL-1R1$^-$ populations depending on the cytokines present in the culture media. The addition of IL-3, KL, and IL-15 completely abrogated T cell development from these progenitors, and in fact the addition of IL-15 alone nearly completely abrogated T cell differentiation from both of these populations as well (data not shown). These data indicate that the microenvironment critically influences differentiation potential.

Given that Stage 2 IL-1R1$^+$ cells were found exclusively within human SLTs but not within the BM or thymus, one possibility is that their putative upstream precursors (i.e. Stage 1 and/or Stage 2 IL-1R1$^-$ cells) derive in the BM and then traffic to various SLT where in response to local signals acquire the Stage 2 IL-1R1$^+$ phenotype and become restricted in their potential for ILC differentiation. Such a model is supported by the previous demonstration that Stage 2 IL-1R1$^-$ progenitor cells are relatively enriched in PB compared to the BM and that in the PB they show high expression of cell adhesion molecules, including integrin $\alpha_4\beta_7$ and CD62L, that facilitate homing and entrance into SLTs (Freud et al., 2005). Although we propose that SLTs can support the development of all ILCs, our data do not exclude the possibility that other pathways of human ILC differentiation occur in other anatomical locations. For instance, ILC2 progenitor cells were discovered in mouse BM (Hoyler et al., 2012). As such, future studies are warranted to further establish the relationship(s) between BM and SLT progenitors and to determine if alternative pathways of ILC development occur in non-SLT sites. Indeed the potential translational impact of identifying therapeutic targets that could influence ILC development in human patients warrants further investigation given the accumulating evidence that ILCs are impactful in multiple disease settings (Munneke et al., 2014; Ruggeri et al., 2002).

In conclusion, we have identified a lineage-restricted human ILC progenitor with the capacity to differentiate into each of the major ILC subsets known to date. The identification of RORγt within this human subset as well as within a fraction of PB NK cells and SLT-resident mature ILCs also raises many new and interesting questions regarding human ILC development.

Online Methods

Tissue collection—All tissues were collected under protocols approved by The Ohio State University (OSU) Institutional Review Board (IRB) and performed in accordance with approved guidelines. Donor consent was obtained where applicable under accordance with

our approved OSU IRB protocol. Human PB was obtained from the American Red Cross (Columbus, Ohio). Fresh human pediatric tonsils, thymus, and adult lymph nodes were obtained through the NCI approved Cooperative Human Tissue Network (CHTN) from Nationwide Children's Hospital, Columbus, Ohio as performed previously (Hughes et al., 2014). Human UCB and BM specimens were obtained through the Translational Cell Processing Core at Cincinnati Children's Hospital Medical Center.

Cell isolation—Human tissues were processed as recently described (Scoville et al., 2015). When appropriate, $CD34^+$ selection using the indirect $CD34^+$ isolation kit (Miltenyi) was performed from enriched ILC populations following manufacturer's instructions with MS columns (Miltenyi). Populations were sorted to ≥98% purity with a FACSAriaII sorter (BD Biosciences).

Cell culture—OP9-DL1 cells were maintained in MEM-α supplemented with 20% fetal bovine serum and 1% antibiotic/antimycotic (Life Technologies). Differentiation assays utilized media containing DMEM/F12 (2:1), 1% antibiotic/antimycotic (Life Technologies), 24uM 2-mercaptoethanol, 20 ug/mL ascorbic acid, 0.05 ug/mL sodium selenite (Sigma), and 10% heat inactivated human AB serum (Valley Biomedical, Inc) (Cichocki and Miller, 2010). Sorted $CD34^+$ progenitor cells were plated on non-irradiated OP9-DL1 feeder cells in media supplemented with 10 ng/mL human FL and 20 ng/mL human IL-7 with or without the following additional human cytokines: 5 ng/mL IL-3 (first week only), 10 ng/mL IL-15 (Miltenyi), and 20 ng/mL KL (Amgen) (Cichocki and Miller, 2010). For clonal assays, individual progenitor cells were sorted directly into culture wells at a concentration of one cell per well following an initial round of sorting (i.e. two consecutive rounds of sorting were performed to purify the individual progenitor cells). Overall cloning efficiency was 25.6%. Medium and cytokines were refreshed every 7 days by replacing half of the media containing 2x cytokines. For stimulation assays, cells were stimulated with 81 nM phorbol 12-myristate 13-acetate and 1.34 μM ionomycin (eBioscience) plus 1 nM recombinant human IL-2 (Peprotech) for 6 hours. Alternatively and where indicated, cells were stimulated for 24 hours in ILC specific stimuli with IL-12 (10 ng/mL); IL-18 (100 ng/mL); IL-2 (1 nM); IL-25 (50 ng/mL); IL-33 (50 ng/mL); IL-1β (50 ng/mL); and/or IL-23 (50 ng/mL) (Bernink et al., 2015; Mjösberg et al., 2011; Trotta et al., 2005). Brefeldin A (BD Biosciences) was added four hours prior to collecting the cells. Intracellular staining was performed using Cytofix/Cytoperm (BD Biosciences) when analyzing cytokines or the transcription factor staining buffer set (eBiosciences) when analyzing transcription factors.

ILC transplantation into NSG mice—The *in vivo* experiments with NSG mice (The Jackson Laboratory) were performed in accordance with an approved OSU Institutional Animal Care and Use Committee protocol. Six to ten week old female NSG mice were treated with 25 mg/kg busulfan one day prior to injection of human tonsil-derived progenitor cells in order to promote engraftment. The next day 1,000–3,000 purified tonsil-derived Stage 2 $IL\text{-}1R1^+$ cells were injected intravenously into NSG mice immediately after sorting. At day 0 and twice weekly thereafter for five weeks the mice were given intraperitoneal injections of recombinant human IL-15 (0.5ug/mouse). The mice were then sacrificed, and their spleens, blood, and BM were harvested for flow cytometry immunophenotypic

analysis. During the latter, murine leukocytes were excluded using an anti-mouse CD45 antibody and human leukocytes were positively identified with an anti-human CD45 antibody.

Flow cytometry—Cells were analyzed using an LSRII cytometer (BD Biosciences) and FlowJo software (TreeStar) as previously described (Hughes et al., 2014). Nonspecific staining was determined through appropriate use of isotype controls, unstimulated controls, or, in the case of intracellular cytokine detection, a “cold competition” control whereby stimulated and subsequently fixed and permeabilized ILCs were first labeled with 20x amounts of unconjugated anti-cytokine antibodies for 1 hour at 4°C followed by addition of 1x amounts of fluorochrome-conjugated antibodies. No significant differences were observed comparing the unstimulated versus the stimulated “cold competition” controls (not shown). A list of antibodies utilized can be found in Supplemental Experimental Procedures.

RT-PCR—mRNA was isolated using the Total RNA Purification Kit Plus (Norgen Biotek). Reverse transcription utilized the Superscript VILO™ master mix (Life Technologies). qPCR was performed with a ViiA 7 real time PCR system (Life Technologies). Gene expression was normalized to 18S mRNA internal control (Ct=Ct gene of interest − Ct 18S). Relative mRNA expression for each gene tested was calculated as $2^{-(\Delta Ct)}$. A list of primers utilized is included in the Supplemental Experimental Procedures.

Cytotoxicity assays—$Lin^{-}CD161^{+}CD94^{-}$ and $Lin^{-}CD94^{+}$ ILCs were purified *ex vivo* from SLT or from *in vitro* cultures initiated with SLT $Lin^{-}CD34^{+}CD45RA^{+}$ progenitor cells. ^{51}Cr-release cytotoxicity assays against the K562 cell line were performed as previously described (Briercheck et al., 2015).

Statistical analysis—Statistical significance was determined with Student’s t-test or ANOVA analysis to correct for multiple comparisons when appropriate using Prism GraphPad software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- All human innate lymphoid cell subsets express *RORγt*
- RORγt is expressed in a subset of $CD34^{+}CD45RA^{+}$ progenitors found in human SLT
- The human $ROR\gamma t^{+}CD34^{+}CD45RA^{+}$ progenitor is restricted to ILC development

eTOC blurb

Human innate lymphoid cells (ILCs) mediate responses against pathogens and cancer; how they develop is unclear. Freud and colleagues identify a human RORγt^+ progenitor that selectively resides in secondary lymphoid tissues and exclusively generates all ILCs, including NK cells. These findings help define the pathways involved in human ILC development.

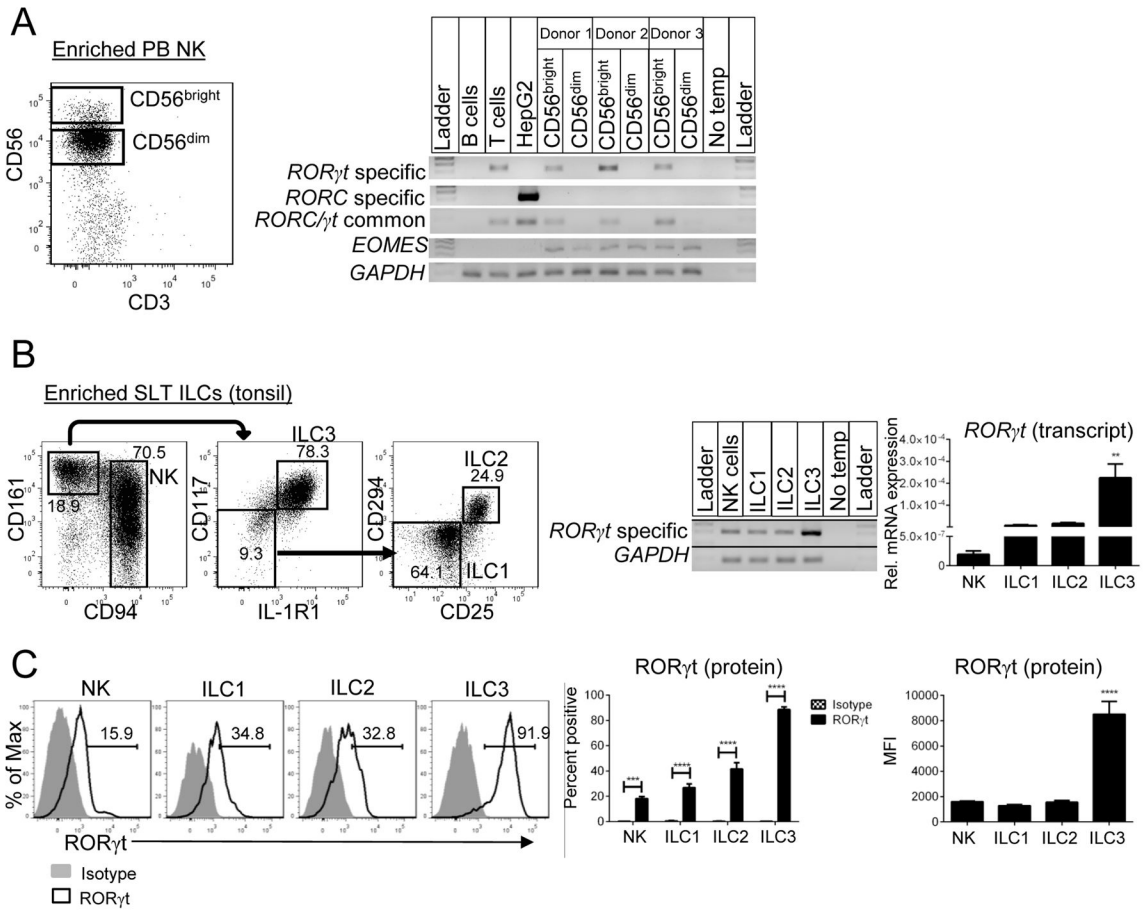

Figure 1. *RORγt* is expressed by PB CD56^{bright} NK cells and each SLT ILC subset

(A) cDNA preparations from 3×10^5 FACS purified B cells, T cells, PB CD56^{bright} NK cells, and CD56^{dim} NK cells were analyzed by RT-PCR using exon spanning primer sets for each gene tested (n=6 from 2 independent experiments with 3 donors shown). HepG2 cells were used as an *RORC* specific control (Ratajewski et al., 2012). PCR amplification products were analyzed on a 2% agarose gel. **(B)** Similar cDNA preparations were made from FACS purified ILCs isolated from human tonsils and RT-PCR or qPCR was performed for *RORγt* (n=4, from 2 independent experiments). *GAPDH* was included as a loading control. (**C**) RORγt protein analysis was performed by intracellular flow cytometry. Intracellular RORγt staining (black line histograms) of gated SLT NK cells (Lin$^-$CD94$^+$), ILC1s (Lin$^-$CD161$^+$CD94$^-$CD117$^-$IL-1R1$^-$CD294$^-$), ILC2s (Lin$^-$CD161$^+$CD94$^-$CD117$^-$IL-1R1$^-$CD294$^+$), and ILC3s (Lin$^-$CD161$^+$CD94$^-$CD117$^+$IL-1R1$^+$CD294$^-$) with respect to isotype control staining (shaded histograms) is presented. The two bar graphs quantitate the percentage of cells expressing RORγt compared to the isotype control (left graph) as well as a mean fluorescence intensity (MFI) (right graph) (n=4, from 2 independent experiments). Error bars indicated s.e.m. **p<0.01; ***p<0.001; ****p<0.0001. See also Figure S1.

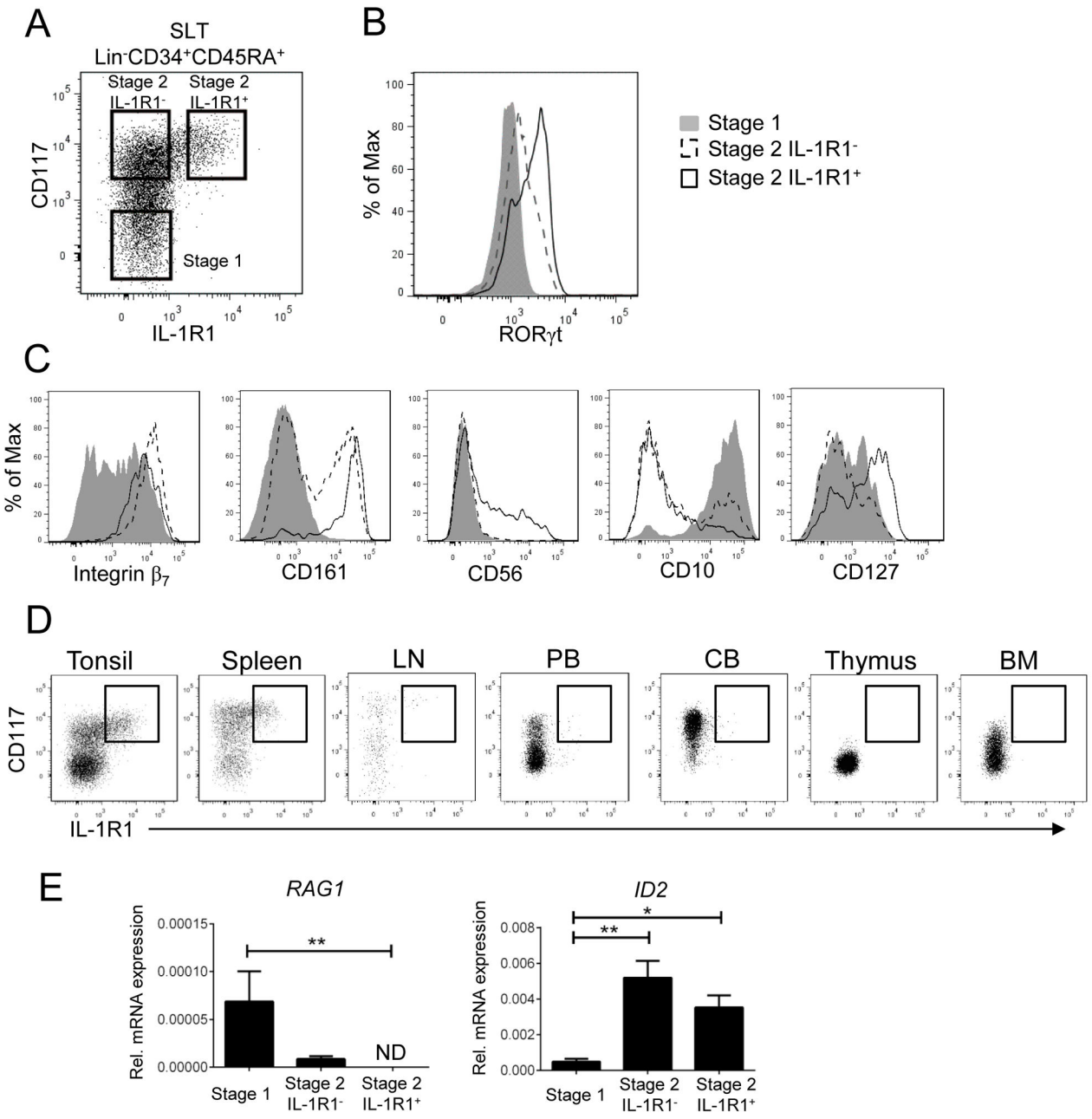

Figure 2. Identification of Lin$^-$CD34$^+$CD45RA$^+$CD117$^+$IL-1R1$^+$ cells in human SLTs

(A) Representative surface immunophenotypic analysis of total Lin$^-$CD34$^+$CD45RA$^+$ cells freshly enriched from human pediatric tonsils (n=86, from 33 independent experiments). (**B**–**C**) Representative donor depicting intracellular RORγt and extracellular markers within each progenitor population (n=6, from 3 independent experiments) **(D)** Representative surface immunophenotypic analyses of total Lin$^-$CD34$^+$CD45RA$^+$ cells isolated from pediatric tonsil, adult spleen (n=4), adult lymph nodes (n=3), adult PB (n=4), UCB (n=4), pediatric thymus (n=2), and adult BM (n=2) and analyzed for the presence of the Stage 2 IL-1R1$^+$ subset (black boxes) from 8 independent experiments. **(E)** qPCR analysis from FACS

purified Lin-CD34^{+}CD45RA^{+} SLT subsets (n=5, from 2 independent experiments). Not detected = ND. Error bars indicate s.e.m. *p<0.05, **p<0.01. See also Figure S2.

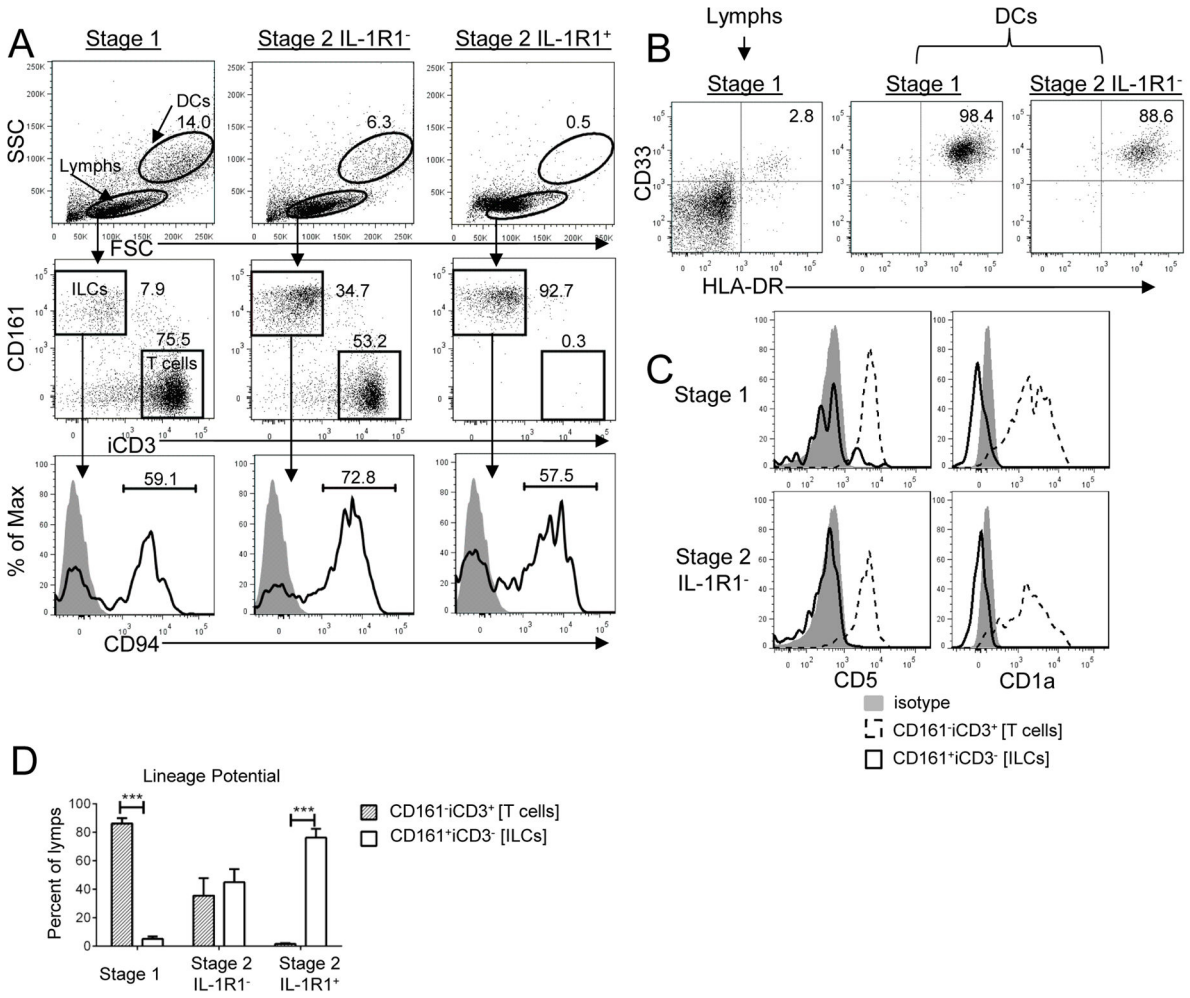

Figure 3. SLT Stage 2 IL-1R1$^+$ cells are ILC-restricted progenitors

(A–E) Representative flow cytometry immunophenotypic analyses of SLT Lin$^-$CD34$^+$CD45RA$^+$ subsets cultured for 28 days on non-irradiated OP9-DL1 stroma plus IL-7 and FL. **(A)** Top row: Analysis of hCD45$^+$ cells derived *in vitro* based on forward and side scatter. Middle row: Lin$^-$hCD45$^+$ lymphocytes were analyzed for iCD3 and CD161 expression, designating T cells and ILCs, respectively. Bottom row: Expression analysis of CD94 (black lines) by Lin$^-$hCD45$^+$CD161$^+$iCD3$^-$ ILCs (isotype control is grey shade). (**B**) Expression analysis of HLA-DR versus CD33 by gated lymphocytes (lymphs) or DCs (as indicated in part **A**) derived from Stage 1 and Stage 2 IL-1R1$^-$ cells. **(C)** *In vitro* derived hCD45$^+$ lymphs were comparatively analyzed for the T cell-associated markers CD5 and CD1a. **(D)** Percentages of T cells and ILCs among total Lin$^-$hCD45$^+$ lymphocytes (n=5, from 2 independent experiments). Error bars indicate s.e.m. ***p<0.001. See also Figure S3.

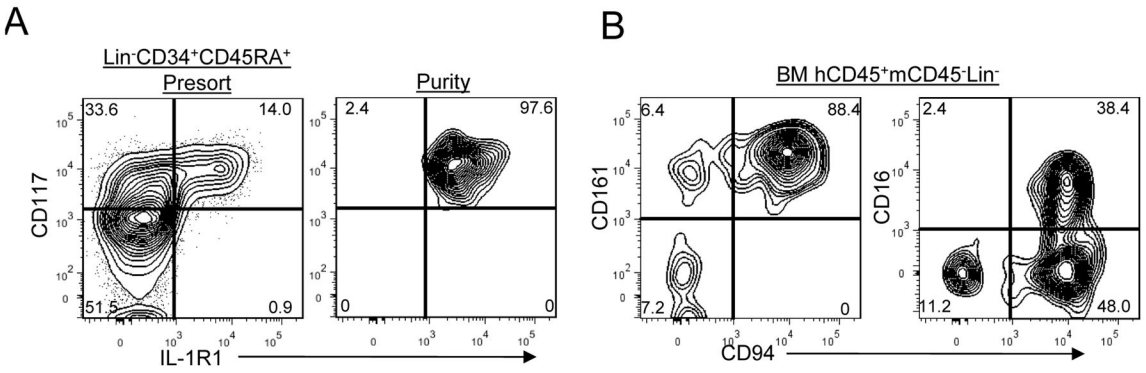

Figure 4. SLT Stage 2 IL-1R1^{+} progenitors give rise to Lin^{-}CD94^{+}CD16$^{+/-}$ NK cells *in vivo*

Freshly sorted Stage 2 IL-1R1^{+} cells (left) obtained from pediatric tonsils were injected into NSG mice that then received twice weekly hIL-15 injections. After 5 weeks the spleens, BM, and PB were harvested and analyzed by flow cytometry. Shown to the right is a representative flow cytometry analysis for expression of the indicated markers by Lin^{-}mCD45^{-}hCD45^{+} lymphocytes within the BM (n=3, from 2 independent experiments). Similar results were obtained from the spleen and PB specimens (data not shown).

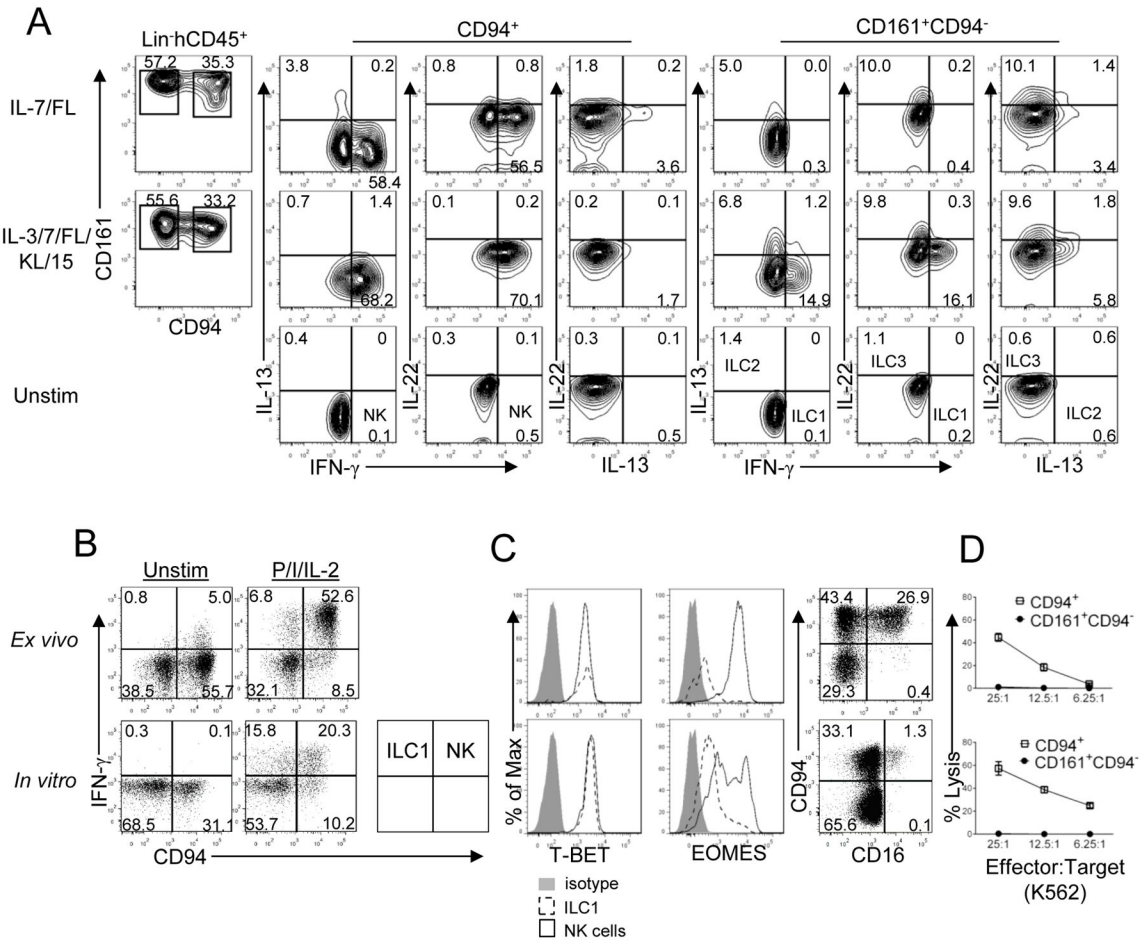

Figure 5. SLT Stage 2 IL-1R1$^+$ progenitors give rise to all four ILC subsets *in vitro*

(A) Representative functional analysis by intracellular flow cytometry following 6 hr P/I/2 stimulation of *in vitro*-derived progeny resulting from culture of tonsil Stage 2 IL-1R1$^+$ progenitor cells on OP9-DL1 stroma with either IL-7/FL or with IL-3 (first week only) plus IL-7/FL/KL/15 for 28 days. NK cells were defined as hCD45$^+$Lin$^-$CD94$^+$IFN-γ$^+$IL-13$^-$IL-22$^-$; ILC1s were defined as hCD45$^+$Lin$^-$CD94$^-$CD161$^+$IFN-γ$^+$IL-13$^-$IL-22$^-$; ILC2s were defined as hCD45$^+$Lin$^-$CD94$^-$CD161$^+$IFN-γ$^-$IL-13$^+$IL-22$^-$; and ILC3s were defined as hCD45$^+$Lin$^-$CD94$^-$CD161$^+$IFN-γ$^-$IL-13$^-$IL-22$^+$. Gates were determined using unstimulated controls (bottom panel; red labels indicate where the ILC subsets are represented in the above dot plots) (n=3 from 2 independent experiments). **(B–D)** NK cell versus ILC1 identification and validation. (**B**) Representative analyses of enriched SLT ILCs or ILCs derived from Lin$^-$CD34$^+$CD45$^+$ progenitor cells *in vitro* depicting ILC1 versus NK cell populations according to CD94 expression among Lin$^-$IFN-γ$^+$ events (following 6 hr P/I/2 stimulation) (n=5, from 3 independent experiments). (**C**) ILC1s and NK cells (as defined in **A**) were profiled according to their expression of T-BET, EOMES, and CD16 (n=3, from 2 independent experiments). (**D**) Comparison of cytotoxic potential of *ex vivo* and *in vitro*-derived CD94$^+$ NK cells versus CD161$^+$CD94$^-$ ILCs against ^{51}Cr labeled K562 target cells at the indicated effector to target cell ratios (n=4, from 2 independent experiments). See also Figures S4 and S5.

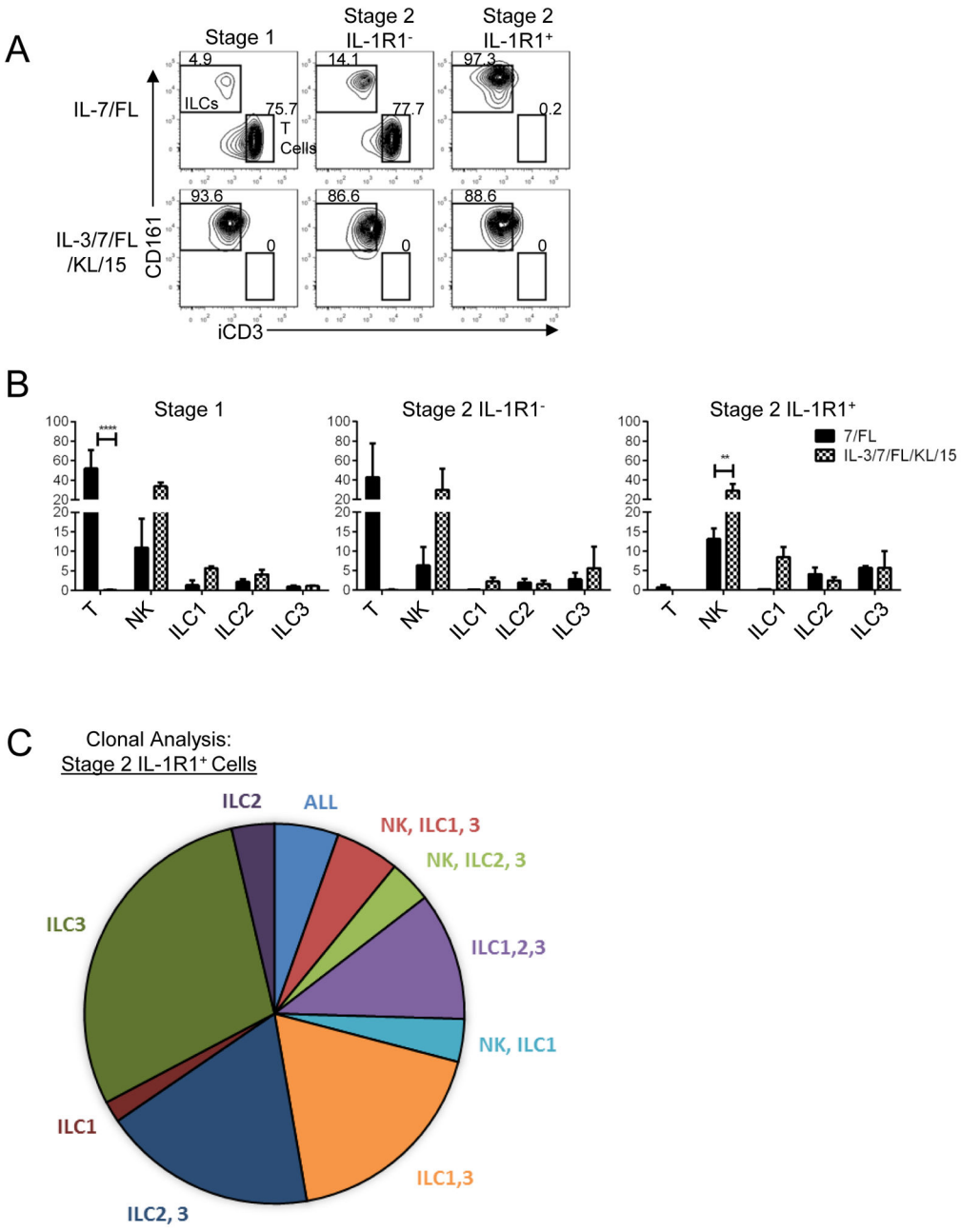

Figure 6. The cytokine milieu influences differentiation potential of Stage 2 IL-1R1$^+$ cells

(**A**) Representative flow cytometry immunophenotypic analysis of Lin$^-$hCD45$^+$ lymphocytes derived *in vitro* following 28 days of co-culturing SLT Stage 1, Stage 2 IL-1R1$^-$, or Stage 2 IL-1R1$^+$ progenitor subsets with non-irradiated OP9-DL1 feeder cells plus either IL-7/FL (top row) or IL-3(first week only)/IL-7/FL/KL/15 (bottom row) (n=3, except for Stage 2 IL-1R1$^-$ where n=2, from 2 independent experiments). (**B**) Cumulative data showing percentages of lymphocyte subsets derived *in vitro* from each indicated progenitor population under the two conditions described in **A**. T cells were identified as Lin$^-$hCD45$^+$iCD3$^+$CD161$^-$; ILCs were identified as Lin$^-$hCD45$^+$iCD3$^-$CD161$^+$ and further

subtyped functionally as in Figure 5 following a 6 hour P/I/IL-2 stimulation. (**C**) Clonal analysis of freshly purified SLT Stage 2 IL-1R1^{+} cells individually sorted into wells containing irradiated OP9-DL1 feeder cells plus IL-3/7/FL/KL/15 for 28 days. Clones were analyzed for functional ILCs as in Figure 5 (n=10, from 4 independent experiments; 55 individual clones were analyzed using this panel). Cloning efficiency was 25.6%. Error bars indicate s.e.m. **p<0.01; ****p<0.0001. See also Figure S6.