Chapter 3:

Transcriptome analysis of human peripheral blood type III ILCs reveals a naïve-like phenotype

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Type III innate lymphoid cells (ILC3s) are key regulators of mucosal and epithelial homeostasis, as part of a larger family of immune effector cells. ILC3s are also crucial during development of secondary lymphoid organs (SLOs) and present in adult SLOs in humans and mice, while they are additionally found in the circulation. Interestingly, in contrast to ILC3s found in SLOs, ILC3s present in human peripheral blood (PB) did not respond to classical activation stimuli by production of effector cytokines. Drawing the parallel to circulating naïve and tissue resident memory T cells, this led us to question whether PB ILC3s are a less mature, circulating counterpart of SLO ILC3s. Using RNAseq analysis we found that PB ILC3s are clearly distinct from SLO (i.e. tonsil, spleen and lymph node) ILC3s, while ILC3s that reside in the different lymphoid tissues are separate populations with tissue specific transcription signatures. In line with our expectations, PB ILC3s showed a more immature-like transcription signature supporting a model that these cells need additional stimuli to differentiate to fully matured effector cells.
INTRODUCTION

In recent years the family of innate lymphoid cells (ILCs) were demonstrated to be first-line cytokine secreting effector cells at mucosal barriers, where they take active part in the maintenance of tissue homeostasis and the initiation of immune responses upon pathogenic infection. ILCs are cells of lymphoid origin, defined by lack of known lineage markers, but positively marked by the expression of the alpha chain of the IL-7 receptor (IL-7α, CD127). In contrast to B and T cells, ILCs lack a rearranged antigen receptor. In accordane, ILCs were shown to be driven to cytokine production by either cytokine signals or by ligation of activating receptors and not upon encounter of specific antigenic epitopes. In parallel to T cells, the family of ILCs is composed of a cytotoxic member (classical natural killer (cNK) cells) and a variety of helper-like subsets, defined by expression of transcription factors and secretion of effector molecules. Group 1 ILCs (ILC1) are defined by expression of the transcription factor T-bet and production of interferon (IFN) γ. Like Th2 cells, group 2 ILCs (ILC2) take part in the immune response against worms and parasites by producing large amounts of the type 2 cytokines IL-5 and IL-13, and are distinguished by high level expression of GATA3. Finally, group 3 ILCs (ILC3) are dependent on retinoic acid orphan receptor (ROR)yt and produce large amounts of IL-22 and / or IL-17 when triggered. This latter group also encompasses the first helper-like ILC to be described, the lymphoid tissue inducer (LTI) cell, which is crucial for the formation of most secondary lymphoid organs (SLOs) during development. Based on recent findings, the division of the ILC family into different groups is less strict than previously thought. The initially defined subsets of ILCs were found to be heterogeneous in both mice and humans. Similar as seen in T cells, functional plasticity between the different subsets, which is controlled by environmental cues, was also observed. In addition, phenotypically similar ILC subset members were found to have distinct transcriptional profiles and function differently in different tissues.

Although ILCs are known to reside near mucosal and epithelial barriers, all ILC subsets are present in both SLOs and within peripheral blood (PB) in both mice and humans. In human lymphoid tissue, functionally and transcriptionally distinct populations of ILC3, based on expression of the activation marker NKp44 (NCR2), are present. Remarkably though, while all ILC subsets can be found in human PB, NKp44+ ILC3 are undetectable at steady-state. NKp44+ ILC3s are only detectable in PB of patients suffering from inflammatory conditions. The latter seems to be in line with reports of ILCs receiving tissue homing instructions in SLOs in case of immune activation, much like naïve T cells, and raises the possibility that SLOs can serve as sites for ILC education. As such, PB ILCs would then, like...
naive T cells, be activated in SLOs prior to their migration as effector cells to peripheral tissues. In line with this, ILC2 were found to enter lungs from the PB upon parasitic infection24.

Here, using RNA sequencing, we show that ILC3 in human PB were transcriptionally distinct from ILC3 found in human SLOs (i.e. tonsils, lymph nodes and spleens). The cells in PB had a less mature phenotype and were incapable to produce the prototypic ILC3 cytokines, IL-22 and IL-17A, upon stimuli with classical cytokine signals, i.e. IL-1β and IL-2325-27. These results support a model in which PB ILC3s are naïve-like and require SLOs for subsequent maturation into cytokine-producing effector ILC3s.

RESULTS

Human PB ILC3s do not produce IL-22 in response to conventional stimuli

Although ILC3s are mostly known for their effector functions in epithelium containing barrier tissues, such as lung, skin and intestine, these cells are also present in (human) PB and various SLOs (for gating see Fig. 1A)16. It has been previously shown in mice that ILCs from different tissues, phenotypically belonging to the same subset, behave differently13,14. To further assess the characteristics of ILC3s in the different lymphoid tissues and PB we analyzed this population for the expression of NKp44 (NCR2), which has been demonstrated to mark active, cytokine producing cells18. While NKp44+ cells were abundantly present in tonsils and spleens, these cells were underrepresented in PB and LNs (Fig. 1B), as has been shown previously18. In contrast to sorted ILC3s from SLOs, irrespective of NKp44 expression, PB ILC3s did not produce IL-22 upon stimulation with IL-1β and IL-23, or PMA and ionomycin (Fig. 1C and D), nor did these cells produce GM-CSF (CSF2) or IL-17A upon stimulation. Notably, the type I cytokine TNFα was secreted by PB ILC3s at low levels upon stimulation with IL-23 and IL-1β (Fig. 1C), but was uniformly produced upon additional stimulation with PMA and ionomycin (Fig. 1D). In contrast, no IFNγ production was detected upon stimulation with both IL-23 and IL-1β and PMA and ionomycin (Fig. 1D). In line with the lack of cytokine production following stimulation with IL-23 and IL-1β, the majority of PB ILC3s showed impaired phosphorylation of STAT3, a signaling event downstream of the IL-23 receptor (IL23R). While STAT3 phosphorylation occurred in the entire population of ILC3 derived from the spleen(Fig. 1E). Importantly, when we analyzed expression of the lineage defining transcription factor RORγt by flow cytometry, and used B cells from the same donors as negative controls, we confirmed the ILC3 identity of these cells, despite lower RORγt expression levels as compared to NKp44+ cells from tonsils (Fig. 1F).
In summary, RORγt⁺ ILC3s isolated from different lymphoid tissues and PB show heterogeneity in cytokine producing profiles. While PB NKp44⁺ ILC3s were unable to produce IL-22, NKp44⁻ ILC3s from LNs, tonsils and spleens did, implying that tissue-origin further sub-defines functionally different populations of ILC3.

**PB ILC3s show a naïve-like transcriptional profile as compared to SLO ILC3s**

To further assess tissue-specific differences amongst ILC3s, we performed RNA-sequencing (RNA-seq) on purity sorted NKp44⁺ and NKp44⁻ ILC3s from tonsils and spleens, and NKp44⁺ ILC3s from PB and hepatic LNs (for gating see Supplementary Fig. S1A). Remarkably, principle component analysis (PCA) revealed that the cells clustered foremost on the tissue they originated from, and not per se on the expression of NKp44, with PB ILC3 forming a distinct cluster, clearly separated from SLO ILC3s (Fig. 2A). Recently, it has been shown that both at the functional and transcriptional level, allergen non-experienced and allergen-experienced ILC2s mirror differences between naïve and memory T cells²⁸. As PB ILC3s expressed lower levels of RORγt, produced less cytokines and showed a distinct transcriptional profile, we hypothesized that these could represent a naïve subset of ILC3s, in parallel with the classification made for T cells. To address this, we investigated the expression of ILC3-specific genes, as previously determined in ILC3s from human tonsils⁶ (Fig. 2B). As expected, based on the PCA, PB ILC3s clustered together, apart from the rest of the samples. Interestingly, PB ILC3s were relatively devoid of expression of ILC3-related genes. Most notably, PB ILC3s lacked expression of IL23R (Fig. 2B), supporting our observations that stimulation with this cytokine mostly did not induce STAT3 phosphorylation nor IL-22 production. The relatively lower expression of ILC3-associated genes in PB ILC3s is in line with these cells representing a naïve-like ILC3 population, in parallel with allergen-inexperienced ILC2s in mice²⁸. To assess whether PB ILC3s resemble a naïve-like ILC3 subset in human tonsil, we analyzed the expression of the ‘naïve’ ILC3 signature and an ‘effector’ ILC3 signature, as recently described by Bjorklund et al. (2016)⁶ in our samples (Fig. 2C). PB ILC3s expressed most of the ‘naïve’ genes, while not expressing ‘effector-like’ genes, with a few notable exceptions (e.g. NCR3, IL-32 and IFI6). Together, these data imply that ILC3s from PB form a naïve population of ILC3s, which may need to receive additional signals before they gain full effector functions.

**PB ILC3s are less mature than SLO ILC3s**

The analysis pointed out that PB ILC3s expressed most genes associated with an immature, resting phenotype, in contrast to ILC3s isolated from the different SLOs. The majority of PB ILC3s also express CD45RA and CD62L
Figure 1. PB ILC3s do not produce IL-22 in response to conventional stimuli. (A) Gating strategy for ILC3s in PB of healthy donors. A gate for PBMCs was set based on FSC and SSC profile. Subsequently, doublets and dead cells were gated out, and a gate was set on lineage (Lin)- cells expressing CD127. Shown are plots of a representative sample. (B) Expression of NKp44 on ILC3s from human tonsil, PB, LN and spleen. Shown are plots of representative samples (left) and mean percentage of NKp44+ cells as part of the ILC3 population. Numbers within plots represent percentages of the gated ILC3 population. (C) Production of ILC3-associated cytokines IL-22, GM-CSF and TNFα by purity sorted ILC3 from PB, LN, spleen...
(Fig. 3A), both markers of naïve T cells. Importantly, the latter is a homing molecule to SLOs. Drawing the parallel to naïve T cells further, we speculated that PB ILC3s circulate through SLOs as NKp44+ ILC3s in search of an activation signal in much the same. If this would be the case, the population of SLO NKp44+ ILC3s would at least partially express the machinery to enter and leave SLOs. Indeed, like PB ILC3s, SLO NKp44+ ILC3s expressed SELL (coding for CD62L) and S1PR1, which is necessary for lymph node egress29, to higher levels as compared to NKp44+ ILC3s isolated from the same donors (Fig. 3A). At the same time, the tissue retention and activation molecule CD69 was expressed at similar levels by NKp44+ and NKp44+ SLO ILC3s from spleen and tonsils, while expressed at lower levels on LN and PB ILC3s (Fig. 3A). The latter observations show that at least part of the NKp44+ SLO ILC3s have the machinery to enter and leave the SLOs, further supporting a model in which PB NKp44+ ILC3s re-circulate between SLOs and PB. We therefore set out to identify from our RNA-seq analysis candidate signals, derived from the lymphoid tissue environment, which could induce maturation of PB ILC3s. Based on previous results, SLO resident NKp44+ ILC3s are closest in maturation status to PB ILC3s1,18 and our PCA confirms this notion (Fig. 2A). We therefore focused on the differences between PB ILC3 and NKp44+ ILC3s in SLOs. To exclude tissue-specific genes from our analysis we analyzed gene expression differences between PB ILC3s and NKp44+ ILC3s from LNs, tonsils and spleens separately. This analysis revealed a shared differential expression of 1050 genes between PB ILC3s and SLO NKp44+ ILC3s (Fig. 3B). Using gene ontology, we identified 160 genes which were annotated as immune-related (Suppl. Table 1). Supporting our hypothesis, 14 out of 15 genes that are involved in ILC3 biology6, including RORC, were expressed lower in PB ILC3s, with the exception of ITGB7, a homing receptor for mucosal tissues30,31 (Fig. 3C). The latter is therefore probably a reflection of the selective absence of mucosal homing cells in SLOs rather than differential expression of the gene. Of the 160 immune-related genes, 62 were expressed higher in all SLOs as compared to PB. We could further categorize these into different classes among which signaling molecules and transcription factors known to be and tonsil. Cells were cultured for 3 days in IL-7 and SCF with or without IL-1β and IL-23, after which cytokine production was measured by ELISA. (B, C): *: p < 0.05, **: p < 0.001, ****: p < 0.0001, Kruskall Wallis test followed by Dunn’s test for multiple comparisons. (D) Production of IL-22, TNFα, IL-17A and GM-CSF by PB ILC3s, as determined by intracellular cytokine staining. Cells were purity sorted and cultured for 3 days in IL-7 and SCF with or without IL-1β and IL-23. Upon culture, cells were re-stimulated with PMA and ionomycin for 6 hrs. Plots are representative for at least three individual experiments. (E) Sorted PB and spleen NKp44+ ILCs were FACS sorted and stimulated with rhIL-23 for 15 minutes after which phosphorylation of STAT3 was assessed by intracellular flow cytometry. (F) PB ILC3 and tonsil NKp44+ ILC3 were FACS sorted after which expression of RORγt was assessed by intracellular flow cytometry and compared to CD19+B cells from the same donor.
Figure 2. PB ILC3s show a distinct transcription profile as compared to SLO ILC3s. (A) Hierarchical clustering and principal component analysis of gene expression by NKp44+ and NKp44- ILC3 from different tissues. Annotation along the axes points to the tissue origin of the cells. (B) Heatmap of expression of ILC3 specific genes, as published previously by Bjorklund et al. (2016) in the different populations using unsupervised clustering. (C) Heatmap of expression of gene signatures associated with naïve-like and effector ILC3 in the different ILC3 populations using unsupervised clustering. Red marking in the right margin annotates genes associated with either the naïve-like or effector cluster. (A-C) Neg and Pos annotate NKp44- and NKp44+ samples, respectively.
involved in receptor tyrosine kinase (RTK) signaling (Fig. 3D and E). Five cytokine/chemokine transcripts were differentially expressed between PB and SLO NKp44+ ILC3s, of which only CXCL2 was higher in SLO ILC3s, while PB ILC3s expressed higher levels of TGFβ1, IL-32, OSM and FLT3LG (Fig. 3F). Six transcripts coding for cytokine receptors were expressed differentially, of which IL-6R and TGFBR3 were expressed higher in PB ILC3s (Fig. 3G). As could be expected by the lack of response to IL-1β in PB ILC3s, IL1R1, coding for the IL-1β receptor, was transcribed to a much lower level in PB ILC3s. In addition, IL7R and KIT (coding for IL-7Rα and cKit, respectively) were also expressed higher in SLO ILCs, even though both markers were used for sorting of ILC3s from all tissues analyzed (Fig. 3G and Supplementary Fig. S1). Furthermore, seven TNF superfamily members were differentially expressed (Fig. 3H), as well as nine homing-related transcripts and fourteen transcripts coding for membrane-associated molecules (Fig. 3I and J respectively). Further analysis of the 62 genes expressed higher in SLO ILC3s and 98 expressed higher in PB ILC3s using DAVID pathway analysis of GO terms, revealed enrichment for transcripts involved in immune development and differentiation in PB ILC3s compared to SLO ILC3s, while SLO ILC3s were enriched for genes involved in immune activation (Fig. 3K). Together, these data show that SLO ILC3s mostly differ from PB ILC3s in maturation status, gaining full effector functions.

**NKp30 triggering and cytokine signals are not sufficient for upregulation of IL23R and IL1R1 on PB ILC3s**

Cytokine secretion by SLO- and tissue-resident ILC3s is triggered by cytokine signals (typically IL-1β in combination with IL-23) whereas triggering of activating receptors (e.g. NKp44 or TLR2) can enhance it1,2,25,26,32. Considering that transcripts of IL23R are virtually absent from PB ILC3s and the receptor for IL-1β (IL1R1) is expressed at low levels (Fig. 4A), we set out to determine what factors could enhance expression of these receptors on PB ILC3s that enter SLOs. In CD4+ T cells, expression of IL23R is induced by IL-23 itself, as well as by TGF-β-dependent down regulation of the transcription factor Gfi-133,34, or in the absence of TGF-β, by IL-1β and IL-635. In human T cells, triggering via the T cell receptor or the cytokines IL-7, IL-15 and TGF-β can up-regulate IL-1R136. While ILCs do not express rearranged antigen receptors, the signaling cascade downstream of NCRs (i.e. NKp30, NKp44 and NKp46) is reminiscent of TCR signaling37. Although PB ILC3s do not express NKp44, NKp30 is transcribed by these cells at higher levels compared to SLO ILC3s (Fig. 4B), as well as the relevant cytokine receptors IL6R, IL6ST, TGFBR1, II and III, IL15RA and IL7R (Fig. 4B). Considering this, we stimulated purity sorted PB ILC3s with
Figure 3. PB ILC3s show a less mature transcriptional profile as compared to SLO ILC3s. (A) Expression of CD62L and CD45RA in PB ILC3s and heatmap of expression of SELL, S1PR1 and CD69. Plot is representative for multiple individual stainings. (B) Venn diagram demonstrating genes differentially expressed (adj. p < 0.05) between SLO NKp44- ILC3s and PB ILC3s. Numbers represent the amount of genes expressed differentially. (C-J) Expression of genes which were differentially expressed by PB ILC3s and SLO ILC3s and annotated as immune-related by Gene ontology (GO:0002376). Heatmaps show expression of genes known to be involved in ILC3 biology (C), encoding for transcription factors (D), signaling related molecules (E), cytokines (F), cytokine receptors (G), belonging to the TNF superfamily (H), related to homing or adhesion (I) or membrane-associated molecules (J). (K) DAVID pathway analysis of GO terms enriched in the immune-related transcriptional profiles of SLO ILC3s (SLO) or PB ILC3s (PB).
a cytokine cocktail containing IL-2, IL-6, IL-15, IL-23, IL-1β, IL-7, SCF (cKit ligand) and TGF-β, in the presence or absence of a stimulating anti-NKp30 monoclonal antibody (mAb). However, no up-regulation of IL23R or IL1R1 was seen (Fig. 4C) and no IL-22 transcripts were detected (data not shown). Additionally, stimulation with TNFα, which activates NFκB and induces pro-inflammatory gene transcription or IL-2 alone, which is known to activate lymphocytes, did not result in up-regulation of IL23R or IL1R1 (Fig. 4D).

In conclusion, the cytokine signals known to drive IL23R and IL1R1 transcription in naïve CD4+ T cells were not able to induce expression in PB ILC3s, suggesting that a different mechanism regulates expression of these genes in ILCs.

**Cellular interactions are not sufficient for PB ILC3s activation**

The SLO micro-environment is formed by hematopoietic cells and non-hematopoietic stromal cells, which take an active part in regulating immune responses. To investigate whether the stromal compartment of the SLOs can induce the expression of IL23R and/or IL1R1, we co-cultured purity sorted PB ILC3s with in vitro cultured tonsil-derived stromal cells (TSC). However, no up-regulation of IL23R or IL1R1 was observed (Fig. 5A). Finally, as signals derived from dendritic cells (DCs) determine T cell skewing upon activation and DCs have been shown to interact and influence ILC phenotype, we investigated whether activated DCs could induce IL23R and/or IL1R1 expression on PB ILC3s. However, upon co-culture with LPS stimulated monocyte-derived DCs, no up-regulation of IL23R and IL1R1 was seen, either in the presence or absence of NKp30 triggering (Fig. 5B).

While we propose that PB ILC3s are naïve ILC that need additional stimuli within the SLO to be able to produce IL-22, it could be argued that PB ILC3s are no genuine ILCs. We therefore analyzed the expression profile of the various transcription factors associated with ILC maturation in mouse and man, i.e. ID2, ZBTB16 (coding for PLZF), RUNX3, TOX, GATA3, RORA, TCF7 (coding for TCF1) and NFIL3. From this analysis one can appreciate that all transcription factors are expressed in all populations analyzed, although some differences in levels of transcription are apparent (Fig. 6). However, together with the previously shown expression of ROBy (Fig. 1F), these data do confirm that PB ILC3s can genuinely be classified as ILCs.

Collectively, while our data suggest that ILC3s undergo further maturation within SLOs, the in vitro systems used here to test for essential signals in ILC3 interaction with either stromal cells or DCs were not sufficient to induce up-regulation of IL23R or IL1R1 in PB ILC3s.
Figure 4. Cytokine signals do not induce maturation of Nkp44- PB ILC3s. (A) Relative expression of IL23R and IL1R1 in Nkp44- ILC3s from PB, LN, spleen and tonsil, as determined by fragments per kilobase of exon per million fragments mapped (FPKM) values based on RNA-seq analysis. (B) Relative expression of IL6R, IL6ST, IL15RA, IL7R, TGFBRI, TGFBRII, TGFBRIII and NCR3 (NKp30) in Nkp44- ILC3s from PB, LN, spleen and tonsil as determined by FPKM values, based on RNA-seq analysis. (C) Relative expression as determined by rtPCR of IL23R and IL1R1 in Nkp44- ILC3 from PB ex-vivo or after stimulation with αNKP30 mAb or isotype control, with or without a cytokine cocktail containing recombinant human (rh) IL-2, IL-6, IL-15, IL-23, IL-1β, IL-7, SCF (Kit ligand) and TGF-β for 24 hrs. (n = 3). As a positive control ex-vivo NKP44- LN ILC3 and ex-vivo NKP44- and NKP44+ tonsil ILC3 were included. Values represent expression relative to GAPDH. (D) Relative expression of IL23R and IL1R1 in Nkp44- ILC3 from PB with(out) stimulation with rhTNFα (n = 3) or rhIL-2 (n=2) for 24 hrs. Values represent expression relative to GAPDH. For all plots: *: p < 0.05, **: p < 0.01, Kruskall Wallis test, followed by Dunn’s test for multiple comparisons.
ILCs are mainly viewed as tissue resident cells which maintain tissue homeostasis and form a first line of defense in case of a pathogenic infection. However, recent reports show that ILCs are also present in PB, and are especially prominent in case of an ongoing immune reaction. Furthermore, SLOs play an important role in ILC homeostasis and migration, parallel to what is known for adaptive lymphocytes. Here, we show that PB ILC3s have a transcription profile distinct from ILC3s present in human SLOs. The main difference between SLO and PB ILC3s pointed towards their maturation status, with PB ILC3s showing a more immature phenotype. This is in agreement with our observation that PB ILC3s could not be induced to produce cytokines, with the notable exception of IL23 and IL-1β are the main factors driving IL-22 production in ILC3s in humans and mice and transcripts for both receptors were nearly absent in PB ILC3s, we set out to determine the signals responsible for up-regulation of both molecules on PB ILC3s. However, signals described for naïve CD4+ T cells did not result in up-regulation of IL23R or IL1R1 in PB ILC3s. Although we used signals known to skew naïve CD4+ T cells to a RORyt+ Th17 phenotype upon activation (i.e. signaling reminiscent of TCR stimulus in combination with cytokine signals), we may have missed the proper combination or timing of signals needed for transcription of the...
same genes in ILCs. Noteworthy in that respect, human ILCs utilize different master transcription factors than effector T cells⁴² and may thus require different signals. Nevertheless, maturation status was the main difference between PB and SLO ILC3s implying that further maturation takes place within SLOs for the PB ILC3s to get ready to produce cytokines. As mentioned, we show here that PB ILC3s express homing receptors for SLOs and have a transcription signature more reminiscent of CD62L⁺ naïve-like ILC3 found in human tonsils⁶, as well as lower expression of transcripts generally associated with mature ILC3s (e.g. IL-23R, IL1R1, IL22, RORC, see Fig. 2). As such, these data provide room for a model in which PB ILCs receive additional signals before they can become activated, similar as for naïve CD4⁺ T cells. Interestingly, analysis of regulatory elements in mouse ILCs showed a broad chromatin accessibility of loci coding for lineage defining transcription factors (i.e. Gata3, T-bet and Rorγt) across ILC lineages. At the same time, all ILC lineages in humans depend on GATA3, and express RORγt to some extent⁴³-⁴⁵, thus further indicating that the division between the

Figure 6. PB ILC3s express transcription factors implicated in ILC development and function. Relative expression of ID2, ZBTB16, RUNX3, TOX, GATA3, RORA, TCF7 and NFIL3 in the different populations of ILC3s analyzed, as determined by FPKM values based on RNA-seq analysis. For all plots: *: p < 0.05, **: p < 0.01, Kruskall Wallis test, followed by Dunn’s test for multiple comparisons.
different ILC lineages is not as strict. ILCs have also been shown to acquire effector functions depending on differential cytokine microenvironments controlling the final phenotype of the cells\textsuperscript{8,11,12,46,47}. In parallel to T cells, these observations suggest that rather than a strict developmental program, ILC effector functions are governed by environmental stimuli. Keeping the T cell parallel in mind, further research will have to elucidate whether PB ILC3s are indeed pre-destined to mature into ILC3s alone, or that they can mature to different ILC subsets as well depending on environmental cues.

SLOs play a crucial role in the development of an immune response. Spread throughout the body, these organs provide a structural scaffold for close interactions between antigen presenting cells and adaptive lymphocytes searching for their cognate antigen\textsuperscript{38,48}. Primarily, all ILC subsets are a resident population in adult SLOs\textsuperscript{16}. Apart from their leading role in lymphorganogenesis\textsuperscript{49}, ILCs have been shown to regulate T cell responses in SLOs\textsuperscript{13,17,50-52} and to initiate lymphoid tissue restoration upon LCMV infection\textsuperscript{52}. In recent years a number of reports have emerged which show that ILCs are recruited to (affected) tissues upon priming in SLOs\textsuperscript{22,23}. Also, following lung infection, ILCs derived from the bloodstream infiltrate the lungs\textsuperscript{24}, while ILC2 activation was observed in lung draining LNs of challenged mice\textsuperscript{28}. Although circumstantial, these data support a model in which SLO activation leads to ILC activation, causing a population of ILCs to rapidly proliferate\textsuperscript{7,28}, upon which they go back to the blood stream to infiltrate the diseased tissue and contribute to the immune response. Interestingly, the tissue retention and activation molecule CD69 was absent on PB ILC3s while expressed on NKp44 - SLO ILC3s (Fig. 3A). Similar as PB ILC3s, NKp44 - ILC3s express transcripts for molecules involved in leukocyte recirculation (CD62L for SLO entry via high endothelial venules and S1PR1 for SLO egress via afferent lymphatics, Fig. 3A). It therefore seems that this population of NKp44 - SLO ILCs is most probably a mixed population of tissue resident CD69\textsuperscript{+} and circulating CD62L\textsuperscript{+}S1PR1\textsuperscript{+} ILC3s. Two scenarios can be the cause of this observation. In one model, the PB ILC3 population recirculates between PB and SLOs and can mature into SLO resident NKp44 - ILC3s once an immune stimulation occurs. In that case, SLO resident NKp44 - ILC3s and PB ILC3s represent two separate populations which can be present in the SLOs at the same time. A different option is that PB ILC3s undergo a transient up-regulation of tissue related markers once they migrate into the SLOs turning into \textit{bona-fide} SLO resident NKp44 - ILC3s, after which they lose expression of these markers as they return to the circulation. Supporting this latter hypothesis, our STAT3 phosphorylation experiments revealed heterogeneity within the PB ILC3 population, as a small portion of this population seemed to react to the presence of IL-23 by phosphorylating STAT3 (Fig. 1E). This was however not reflected by IL-22 production, as we
have not observed cytokine production by intracellular flow cytometry nor by ELISA (Fig. 1C-D). Therefore, this might reflect a population that is susceptible to a subsequent crucial trigger for activation or differentiation. Importantly, as we analyzed bulk ILC3 populations, we cannot rule out either option. Single cell RNA-seq analysis of ILC3s from the different tissues will have to shed light on the heterogeneity of these populations.

In conclusion, we present here evidence for a model in which PB ILC3s are naïve-like ILCs and in need of further maturation to gain full ILC3 effector functions. These observations further support the notion that PB ILC3s recirculate through SLOs where, upon activation, they either mature to fully functional SLO resident ILC3s or exit the SLO and get recruited to affected tissues. Here, we were not able to determine the (combination of) stimuli needed for maturation of PB ILC3s to fully active SLO ILC3s. A number of potential mechanisms can be postulated, based on literature. One of these candidates is NOTCH signaling, which was shown to be needed for human ILC2 differentiation in the thymus as well as for differentiation of mouse ILC3s. In both cases, the final step of the differentiation involving NOTCH signaling was found to happen outside the bone marrow. Another candidate could be signaling via the aryl hydrocarbon receptor (AHR), which has been shown to be essential for ILC3 function in mouse. Intriguingly, in the colon of Ahr−/− mice, both IL-7R and cKit were expressed at lower levels on ILC3s, as is the case in PB ILC3s (Fig. 3C). Interestingly, in recent years RORytc+CD34+ cells found in human tonsils were reported to differentiate into all ILC subsets, depending on culture conditions. Importantly, these investigations involved weeks of culturing and thus culture introduced artefacts cannot be ruled out. Importantly, duration, order and combination of different stimuli can also be determining factors, as well as a 3D in vivo environment which is nearly impossible to mimic *ex vivo*. Further research will have to unveil the exact conditions leading to maturation of PB ILC3s as well as the determination whether PB ILC3s are indeed an ILC3-specific precursor or that these cells have the potential to differentiate into other ILC subsets.

**Materials and methods**

**Human tissues**

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin Blood Supply, Amsterdam / Rotterdam, the Netherlands) by gradient centrifugation on lymphoprep (d=1.077, Fresenius Kabi Norge AS, Berg i Østfold, Norway). The PBMCs were subsequently centrifuged and washed twice in cold PBS (B. Braun Melsungen AG, Meslungen, Germany) supplemented with 10% (v/v) donor plasma.
Palatine tonsils were obtained from the department of Otolaryngology, Slotervaart Hospital, Amsterdam, the Netherlands and the Sophia Children’s Hospital, Rotterdam, the Netherlands. Hepatic lymph node (LN) and spleen tissue were collected post-mortem during multi-organ transplantation procedures at the department of gastroenterology, Erasmus Medical Center, Rotterdam, the Netherlands. Tissue was cut into small pieces, digested in 0.5mg/mL Collagenase D (Sigma Aldrich, St. Louis, MO, USA) at 37°C and cell suspensions were prepared by disrupting the tissue with a GentleMacs dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Mononuclear cells were further isolated by filtering over a 70µm nylon cell strainer (Falcon, Wiesbaden, Germany) and gradient centrifugation on lymphoprep (d=1.077, Fresenius Kabi Norge AS). All human tissues were collected according to the guidelines of the Medical Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands) or the Medical Ethical Committee of the Erasmus University Medical Center (Rotterdam, the Netherlands), in accordance with the Declaration of Helsinki and according to Dutch law.

**FACS sorting**
Mononuclear cell fractions of all tissues were enriched for ILC3 using the CD117 MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer’s protocol, followed by positive selection using LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). ILC3 were subsequently stained with the following antibodies: CD45-PE-Cy7 (clone HI30), CD127-APCeFluor®780 (eBioRDR5), CD94-FITC (DX22, all eBioscience, San Diego, CA, USA), NKp44-AlexaFluor®647 (P44-8, Biolegend, San Diego, CA, USA), a lineage cocktail containing CD3 (MEM57), CD19 (LT19), CD14 (MEM15), CD34 (4H11, all PE-TexasRed, all Exbio, Vestec, Czech Republic), CRTH2-PerCP-Cy5.5 (BM16, BD Bioscience, Mountain View, CA, USA). Dead cells were excluded using DAPI. Sorting was performed using a FACSAria III sorter (BD Bioscience, Mountain View, CA, USA).

**Cell culture**
FACS-sorted ILC3s were cultured in DMEM (Gibco, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal calf serum (FCS, Hyclone, GE Healthcare Life Sciences, Logan, UT, USA), penicillin, streptomycin and L-glutamine, in the presence of IL-7 (Peprotech, Rocky Hill, NJ, USA) and SCF (R&D Systems, Minneapolis, MN, USA; both at 10 ng/ml) for 3-5 days before stimulation.
Cells were stimulated with IL-23 (50 ng/mL), IL-1b (10 ng/mL), IL-6 (10 ng/mL; all R&D Systems, Minneapolis, MN, USA), IL-15 and/or TGF-b1 (3 ng/mL; both Peprotech Rocky Hill, NJ, USA) in presence of IL-7 and SCF (10 ng/mL), unless indicated otherwise, for 24 h when analyzed by qPCR and for 3 days
when cytokine-release was measured by ELISA. In some experiments, ILC3 were stimulated through NKp30. Hereto, mouse-anti-human NKp30 (R&D Systems, Minneapolis, MN, USA) or mouse IgG2a isotype control antibodies (5 mg/mL in 200 mL PBS) were plate-bound for 3hrs. at 37°C.

**Sample preparation and RNA sequencing**

For RNA-sequencing analysis, NKp44− ILC3 were sorted from PB (n = 3) and LN (n = 3), and both NKp44− and NKp44+ cells were sorted from spleens (n = 4) and tonsils (n = 3). Cells were FACS-sorted directly into lysis buffer containing TCEP (Macherey-Nagel, Düren, Germany) and stored at -80°C until further processing. RNA was isolated according the manufacture’s protocol (NucleoSpin XS Kit, Macherey-Nagel, Düren, Germany), omitting the addition of carrier RNA. RNA quality was measured on a RNA 6000 Pico Kit (Agilent Technologies, Amstelveen, the Netherlands) using a 2100 Bioanalyser (Agilent Technologies, Amstelveen, the Netherlands). 100 pg high-quality RNA (RIN = 8.37 ± 0.55 [average±stddev]) was converted into cDNA using the SMART-Seq (v3) Ultra Low Input RNA Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) with 15 cycles of amplification. Amplified and Covaris-fragmented cDNA was quality-checked on a High Sensitivity DNA chip (Agilent Technologies, Amstelveen, the Netherlands) and further processed according the TruSeq Nano DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Samples were sequenced on a HiSeq 2500 platform (Illumina, San Diego, CA, USA). Data was processed as described previously. In brief, SMARTer adapters were trimmed with cutadapt (Marcel M. et al. EMBnet journal. 2011) and the resulting sequences were aligned to the human RefSeq transcriptome using TopHat2. Normalization and quantification was performed using Cufflinks. FPKM counts were determined per gene with HTSeq-count, and subsequently used for differential expression analysis using the DESeq2 package. Multiple testing correction was performed with the Benjamini-Hochberg procedure to control the False Discovery Rate (FDR). Principle component analysis was performed on the fragment counts using the R environment.

**Enzyme linked immunosorbent assay**

Quantification of IL-22, TNFα and GM-CSF was determined using the DuoSet ELISA Kits (R&D Systems, Minneapolis, MN, USA) according the manufacturer’s protocol.

**Generation of monocyte derived DCs (moDCs)**

PBMCs from buffy coats were stained with CD14-biotin (61D3, eBioscience, San Diego, CA, USA) and monocytes were isolated (>98%) using streptavidin-beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were cultured for 6 d in IMDM (containing 10% FCS, L-glutamin, 1% penicillin/streptomycin and 50
μM β-mercapto-ethanol) supplied with GM-CSF (800 U/mL, Invitrogen) and IL-4 (40 U/mL, R&D Systems, Minneapolis, MN, USA), which was replenished at day 3. At day 6, moDC were matured with 100 ng/mL LPS. 3,000 moDC were co-cultured with 17,000 PB ILC3 for 2 d in DMEM (containing 10% FCS and 1% penicillin/streptomycin) in presence of IL-7 and SCF (10 ng/mL). Where indicated, co-cultures were performed in wells pre-coated with anti-human NKp30 (5 μg/mL) antibodies or mIgG1 isotype control antibodies.

Real time polymerase chain reaction (rTPCR)
RNA was isolated from cell homogenates using the Ambion miRvana™ miRNA isolation kit (Life Technologies, Carlsbad, C, USA) according to manufacturer’s protocol. cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to manufacturer’s protocol. Real-time (rt)PCR was performed using SYBR Green mastermix (Foster City, CA, USA) on StepOne real-time PCR systems from Applied Biosystems (Bleiswijk, The Netherlands). Used primer sequences are listed in table 1.

Generation of tonsil stromal cell lines (TSC)
Stromal cells were isolated from tonsils as published previously for mouse and human LN62. In short, tonsils were cut into pieces of approximately 5×5 mm and incubated in warm RPMI-1640 (Gibco, Thermo Fischer Scientific, Weltham, MA, USA) supplemented with 0.6mg/mL collagenase P, 2.4mg/mL dispase and 0.3mg/mL DNAse I (all Roche, Basel, Switzerland). Every 5 min. tissue was re-suspended, supernatant was collected in PBS supplemented with 2% h.i. FCS and 5mM EDTA in a separate tube and fresh digestion medium was added to the tissue. This process was repeated 4-5 times, after which the collected supernatant was centrifuged and the cells were suspended in RPMI-1640 supplemented with 10% (v/v) h.i. FCS, penicillin, streptomycin and L-glutamine and cultured o/n in T75 culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) At day 1 and 2 post isolation the cells were extensively washed with PBS to wash away CD45+ cells after which the adherent cells were allowed to grow to confluence and after which they were transferred to new flasks. For transfer, cells were detached from the flask by using 0.2% (v/v) trypsin (Invitrogen, Thermo Fischer Scientific, Weltham, MA, USA) supplemented with 5mM EDTA in PBS.

Table 1: primer sequences used for rTPCR

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<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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Transcriptional profiling of human SLO and PB ILC3s
**Flow Cytometry**

FACS analysis was done using the following antibodies: a lineage cocktail containing CD3 (UCHT1), CD11c (3.9), CD19 (HIB19), CD14 (61D3), CD34 (4H11), CD94 (DX22, all FITC labeled, and obtained from eBioscience, San Diego, CA, USA), IL-17A-biotin (eBio64DEC17), CD127-PE or APC (eBioRDR5), RORyt-PE (AFKJS-9), CD117-PeCy7 (104D2), GM-CSF-eFluor®660 (DAVKAT), TNFα-eFluor® 450 (MAb11, all eBioscience, San Diego, CA, USA), CRTH2-PE-CF594 (BM16), CD62L-BV421 (DREG56, both BD Bioscience, Mountain View, CA, USA), IFNy-PE (4S.B3) and CD45RA-APC (H1100, Biolegend, San Diego, CA, USA).

Exclusion of dead cells was done using staining with Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA, USA). Fixation and permeabilization of all cells for intracellular cytokine and transcription factor stainings were performed using the Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience, San Diego, CA, USA).

For detection of phosphorylated STAT3 (pSTAT3), FACS-sorted ILC3 from PB and spleen were cultured overnight to reduce potential background staining induced by the sorting procedure. The following day, cells were stimulated for 15 minutes with IL-23 (50 ng/mL; R&D Systems, Minneapolis, MN, USA) or left untreated. Next, cells were fixed and permeabilized (BD Cytofix/Cytoperm, BD Bioscience, Mountainview, CA, USA) for 15 minutes at 37°C followed by additional fixation with 90% (v/v) methanol (-20°C) on ice for 30 minutes. Cells were washed with perm/wash solution (BD Cytofix/Cytoperm) and pSTAT3 was stained for 1 hour at RT using mouse-anti-STAT3-Alexa Fluor 647 (pY705, BD Phosflow, BD Bioscience, Mountainview, CA, USA) IgG2a antibody, or isotype control antibodies. Analysis was performed using either a LSR-Fortessa X20 (BD Bioscience, Mountain View, CA, USA) or a Cyan ADP (Beckman Coulter, Woerden, the Netherlands) flow cytometer. Further analysis was done using Flowjo Software version 10 for Microsoft (Tree Star, San Carlos, CA). Gates were set based on Fluorescence Minus One (FMO) or relevant isotype controls.

**Statistics**

Results are given as the mean ± SEM or SD. Statistical analysis was done using GraphPad Prism 4 Software (La Jolla, CA, USA). Due to small sample size we could not assume normal distribution and/or equal variance and thus used Kruskall-Wallis tests for comparisons between multiple groups, as described in the figure legends. Significance is indicated by * p ≤ 0.05, ** p < 0.01 or *** p<0.001. We did not use statistical methods to predetermine sample size of human samples, nor were the investigators blinded to sample identity or results. Samples were not randomized, except for the in vivo experiments in mice, as described above.
References


Supplemental figure S1. Sort strategy for ILC3s from PB and SLOs. (A) ILC3 sorts were performed from samples enriched for cKit expressing cells. A gate for PBMCs was set based on FSC and SSC profile. Subsequently, doublets were gated out, and a gate was set on live CD45+ cells, followed by gating on Lineage-cKit+ cells, CD94+ cells CD127+ cells and CRTH2+ cells. Finally, a gate was set on either NKp44- or NKp44+ population. Shown are plots of a representative sample. The lineage cocktail includes monoclonal antibodies for: CD3, CD11c, CD14, CD19 and CD34. (B) Post sort analysis of a representative sorted NKp44+ population. (C) Post sort analysis of a representative sorted NKp44- population.
Supplemental Table 1: Immune-related genes expressed differentially between SLO and PB NKp44+ ILC3

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