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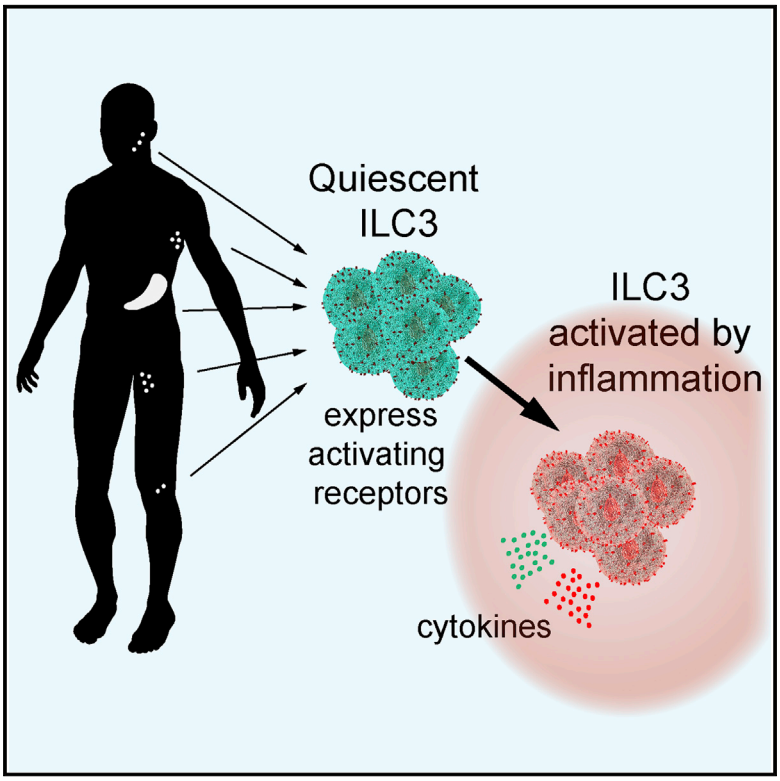
Cross-Tissue Transcriptomic Analysis of Human Secondary Lymphoid Organ-Residing ILC3s Reveals a Quiescent State in the Absence of Inflammation

Graphical Abstract



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In Brief

Bar-Ephraim et al. describe a cross-tissue transcriptional comparison of human ILC3s and show that ILC3s in lymph nodes and spleen share a transcriptional profile that is distinct from that of tonsil ILC3s. Lymphoid organ ILC3s are a substantial pool of resting cells that can be recruited into immune responses upon local activation.

Highlights

- Human ILC3s in lymph nodes and spleen lack the transcription of classical ILC3 cytokines
- Human ILC3s in lymph nodes and spleen can respond to inflammatory cytokines
- NKp44 expression and cytokine production are separately regulated

Data and Software Availability

E-MTAB-5909

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Cross-Tissue Transcriptomic Analysis of Human Secondary Lymphoid Organ-Residing ILC3s Reveals a Quiescent State in the Absence of Inflammation

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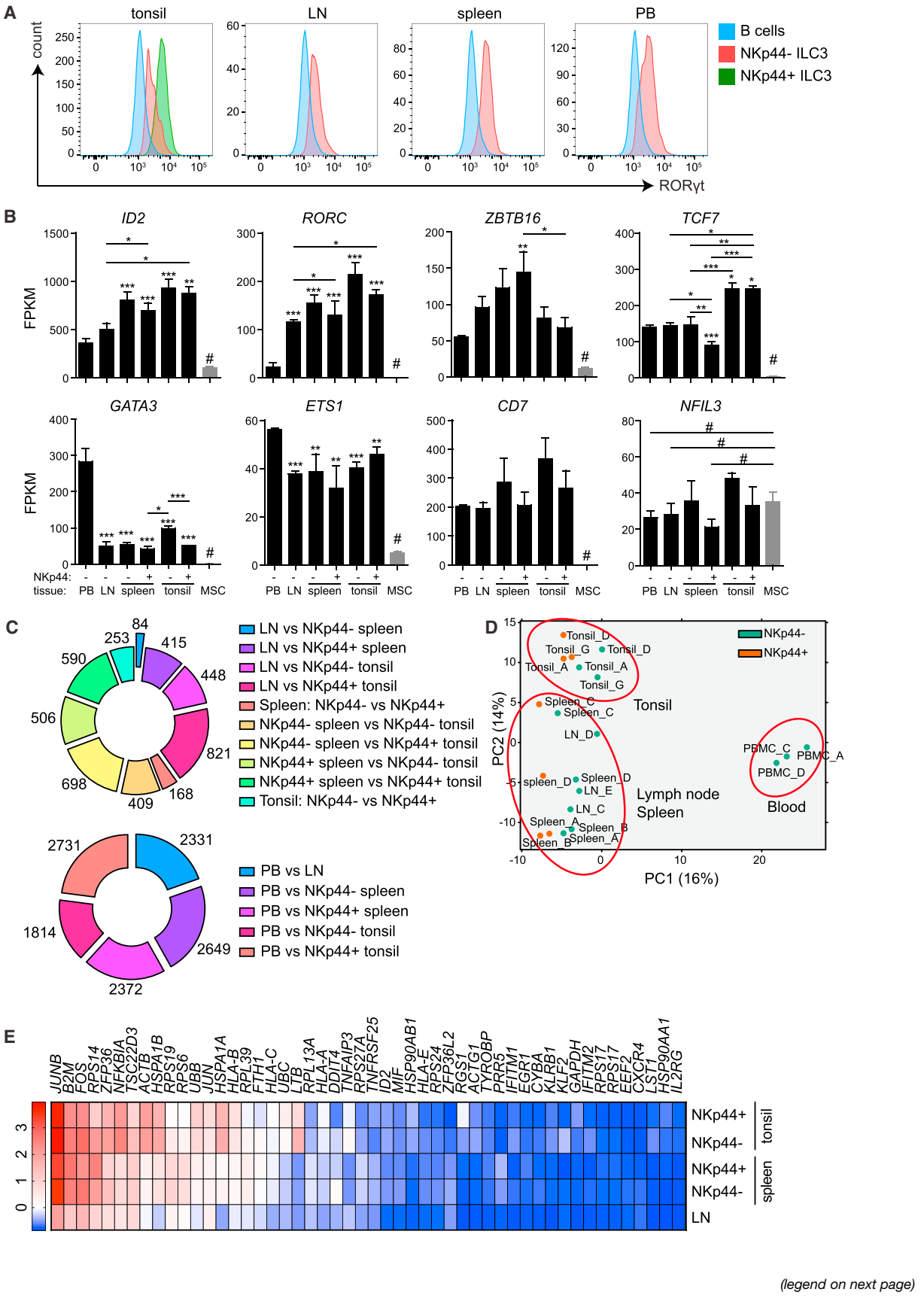
SUMMARY

A substantial number of human and mouse group 3 innate lymphoid cells (ILC3s) reside in secondary lymphoid organs, yet the phenotype and function of these ILC3s is incompletely understood. Here, we employed an unbiased cross-tissue transcriptomic approach to compare human ILC3s from non-inflamed lymph nodes and spleen to their phenotypic counterparts in inflamed tonsils and from circulation. These analyses revealed that, in the absence of inflammation, lymphoid organ-residing ILC3s lack transcription of cytokines associated with classical ILC3 functions. This was independent of expression of the natural cytotoxicity receptor NKp44. However, and in contrast to ILC3s from peripheral blood, lymphoid organ-residing ILC3s express activating cytokine receptors and have acquired the ability to be recruited into immune responses by inflammatory cytokines. This comprehensive cross-tissue dataset will allow for identification of functional changes in human lymphoid organ ILC3s associated with human disease.

INTRODUCTION

In recent years, group 3 innate lymphoid cells (ILC3s) emerged as cytokine-secreting effector cells at mucosal barriers that are involved in maintenance of barrier integrity and initiation of immune responses upon infection (Artis and Spits, 2015). ILC3s are cells of lymphoid origin (Montaldo et al., 2014; Scoville et al., 2016) that are defined by lack of known lineage markers but marked by expression of the alpha chain of the interleukin-7 (IL-7) receptor (IL-7Rα, CD127) and the nuclear hormone receptor retinoic acid orphan receptor (ROR) γt (Artis and Spits, 2015; Spits et al., 2013). ILC3s have been extensively described as resident cells at epithelial and mucosal surfaces, where they safeguard barrier integrity by secretion of IL-22 in both mice and humans (Artis and Spits, 2015; Crellin et al., 2010a, 2010b; Hazenberg and Spits, 2014). In addition to mucosal tissues, ILC3s are found in secondary lymphoid organs (SLOs). During fetal development in mouse and man, ILC3s interact with endothelial and mesenchymal tissue cells and are responsible for the initiation of lymph node and Peyer's patch organogenesis (Cupedo et al., 2009; van de Pavert and Mebius, 2010; Onder et al., 2017). After birth, ILC3s reside in SLOs throughout life, embedded within a specialized mesenchymal stromal niche (Hoorweg et al., 2015). Elegant studies on mouse ILC3s have clarified some functions of these SLO-resident ILC3s. Located at the inter-follicular areas, ILC3s interact with memory T cells, enhancing their survival (Kim et al., 2003; Withers et al., 2011). Moreover, splenic ILC3s are involved in the activation of marginal zone B cells and influence the ensuing production of antibodies (Magri et al., 2014). Finally, lymph node ILC3s can process and present antigens in major histocompatibility complex class II (MHC class II) and induce either T cell deletion or T cell activation (Hepworth et al., 2015, 2013; von Burg et al., 2014). Combined, a picture emerges in which mouse SLO-residing ILC3s have distinct functional features that can potentially affect multiple aspects of innate and adaptive immunity. In contrast to these data on mouse ILC3s, knowledge on SLO-residing human ILC3s is scarce.

Data on human SLO-residing ILC3 functionality is almost exclusively derived from cells isolated from tonsils. Human lymph node or splenic ILC3s have been described (Hoorweg et al., 2012; Magri et al., 2014), but the full function and phenotype of these cells remains to be established. The ILC3s that reside in palatine tonsils resemble mucosal tissue ILC3s in phenotype and function. They express the natural cytotoxicity receptor (NCR) NKp44 and, in line with the association of this receptor with active cytokine secretion, are avid producers of ILC3 signature cytokines, including IL-22 and



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granulocyte-macrophage colony stimulating factor (GM-CSF) (Glatzer et al., 2013; Hoorweg et al., 2012).

Lymph nodes and spleen have long been recognized as essential sites for activation and regulation of adaptive immune cells. On average, the human body contains more than 500 lymph nodes (Trepel, 1974) yet only 5 tonsils. This implies that at any moment, the combined number of ILC3s residing in lymph nodes will outnumber the sum of tonsil-resident ILC3s.

Insight in the immune phenotype of ILC3s residing in non-reactive human SLOs is pertinent for a full understanding of ILC3 biology and function. In this study, we set out to analyze the immune phenotype and functionality of human ILC3s from resting SLOs in the absence of inflammation or immune activation compared to those of ILC3s from inflamed tonsils and peripheral blood (PB). By massive parallel sequencing of RNA transcripts, we show that ILC3s in resting SLOs lack transcription of the major ILC3 signature cytokine genes, irrespective of NKp44 expression or activated phenotype. Nonetheless, this substantial pool of resting ILC3s has the ability to produce ILC3 signature cytokines in response to inflammatory stimuli. Activation capability is controlled by the SLO microenvironment, because PB ILC3s lack the appropriate cytokine receptors and are cytokine unresponsive. Altogether, these data provide a comprehensive multi-tissue transcriptomic dataset of human ILC3s residing in SLOs and revise current views on human ILC3s by identifying SLO-residing ILC3s as a substantial pool of resting, non-cytokine-producing immune cells with the ability to rapidly respond to activating signals from cells present in the local microenvironment.

RESULTS

Signature Cytokines Are Absent from the Shared SLO ILC3 Immune Transcriptome

To identify immunological functionalities of SLO-residing ILC3s, we characterized the transcriptome of ILC3s ($Lin^-CD45^+CD127^+cKit^+CRTH2^-$ $NKp44^-$ or $NKp44^+$) by RNA sequencing of purified populations of cells from non-reactive hepatic lymph nodes and spleens, from recurrently inflamed pediatric tonsils, and from healthy adult PB buffy coats. In lymph nodes and blood, most ILC3s lacked expression of the NCR NKp44, while spleens and tonsils contained both $NKp44^-$ and $NKp44^+$ ILC3 populations (for gating strategy and distributions, see Figure S1). ILC3 identity was confirmed by expression of RORγt protein, with the highest expression in tonsil $NKp44^+$ ILC3s (Figure 1A), and transcription of key transcription factors associated with the ILC3 lineage, including *ID2*, *RORC*, *NFIL3*, and *GATA3* (Figure 1B). Human bone marrow-derived stromal cells (Chen et al., 2016) were used as a control population in these analyses. ILC3 lineage genes were either absent or expressed at background levels in these cells, with the exception of *NFIL3* (Figure 1B). The latter is in line with public transcript data on stromal cell populations in the mouse (http://www.immgen.org).

To identify transcripts associated with ILC3 tissue residency, we analyzed differentially expressed genes between ILC3s isolated from SLOs (lymph nodes, spleens, and tonsils) and from PB. $NKp44^-$ ILC3s from resting lymph nodes and spleen were transcriptionally comparable, with only 84 genes expressed at significantly different levels (Figure 1C). In contrast, $NKp44^-$ ILC3s from inflamed tonsils differed in more than 400 genes from the phenotypically similar ILC3s from either lymph nodes or spleen (Figure 1C). The highest numbers of significantly different genes were observed between $NKp44^+$ cells from inflamed tonsils and $NKp44^-$ cells from either resting lymph nodes (821 genes) or spleens (698 genes) (Figure 1C). In part, these differences are likely explained by the inflamed environment of the tonsils compared to the homeostatic environment of the lymph nodes and spleens. The transcriptome of PB ILC3s was significantly different compared to all SLO ILC3 subsets, with at least 1,800 genes expressed differentially (Figure 1C).

We decided to further focus on the transcripts related to immunological functions of ILC3s from the different SLOs and performed principle component analysis (PCA) based on genes annotated within the gene ontology (GO) term "immune system process" (for the list of genes used, see Table S1). ILC3s from resting SLOs (lymph nodes and spleens), tonsils, and PB formed distinct clusters (Figure 1D; Figure S2), suggesting that ILC3 immune transcriptomes are shaped by local tissue microenvironments (for genes contributing to PC1 and PC2, see Table S2).

To identify immunologically relevant genes expressed in ILC3s and associated with SLO residency, we compiled an immune gene signature common to all SLO-residing ILC3s, irrespective of NKp44 expression or inflammatory status of the donor tissue. To do so, the 50 genes with the highest expression across all SLO-residing ILC3s and contained within the GO term "immune system process" were selected (Table S3). These included genes previously associated with ILC3 biology, e.g., *LTB*, *ID2*, *TNFRSF25* (encoding DR3), *KLRB1* (encoding CD161), *IL2RG* (CD132, common γ chain of the IL-2 receptor), and *TYROBP* (encoding DAP12) (Figure 1E) (Ahn et al., 2015; Björklund et al.,

Figure 1. SLO-Residing ILC3s Share a Transcriptional Signature

(A) Tonsil, lymph node, spleen, and blood samples were stained for RORγt expression. $CD19^+$ B cells were used as a negative staining control. Data representative of at least n = 3 are shown (full gating in Figure S1A).

(B) Transcription of key ILC3 transcription factors analyzed by RNA sequencing and plotted as FPKM (fragments per kilobase of exon per 10^6 reads mapped) in sorted ILC3 populations. As a negative control, bone marrow mesenchymal stromal cells (MSCs) were taken along (n = 10). For PB, lymph node (LN), and tonsil, n = 3; for spleen, n = 4. *p < 0.05, **p < 0.01, ***p < 0.001 compared to PB or between indicated groups; #p < 0.05 between MSC and any ILC3 populations. Error bars represent mean ± SEM.

(C) Number of genes with significantly different expression (p < 0.05) between the indicated ILC3 samples. The upper circle shows SLO ILC3s, while the lower circle shows PB ILC3s versus SLO ILC3s.

(D) Principal component analysis (PCA) of ILC3s using genes within the gene ontology (GO) term "immune system process" (excluding *NCR2*). Each dot represents an individual sample, with green dots marking $NKp44^-$ ILC3s and red dots marking $NKp44^+$ ILC3s. See also Figure S2 and Tables S1 and S2.

(E) 50 highest transcribed genes common to all SLO ILC3s within the GO term "immune system process." *Z* scores were calculated based on the average FPKM value of all depicted genes across the samples (n = 3–4, as in C). See also Tables S1 and S3.

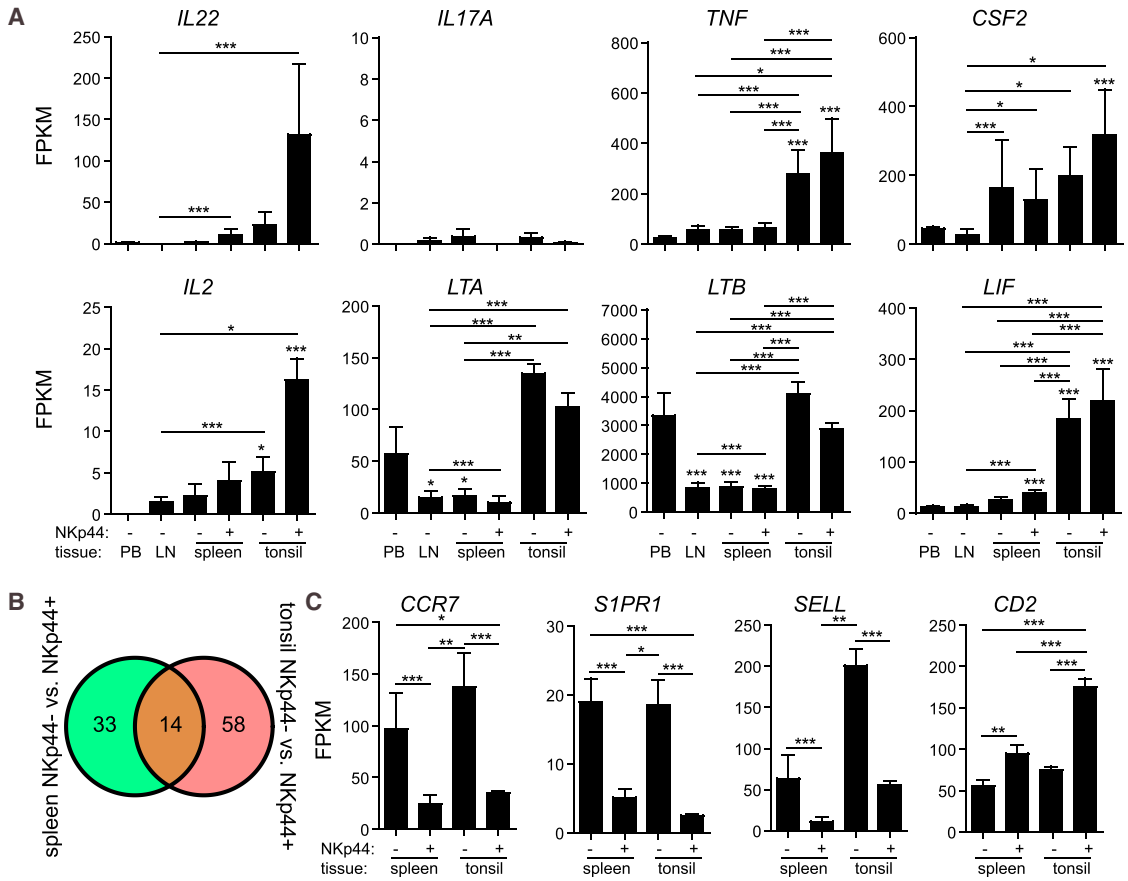


Figure 2. Cytokine Transcription Is Independent of NKp44 Expression or Activation State

(A) Expression levels (FPKM) of selected cytokine genes previously associated with human ILC3s.

(B) Venn diagram showing the overlap of the number of genes that are significantly different ($p < 0.05$) between NKp44$^+$ and NKp44$^-$ ILC3s in spleens (33 genes; green circle) and tonsil (58 genes; red circle), as well as the genes differentially expressed in both organs (14 genes). See also Table S4.

(C) Expression levels (FPKM) of selected genes from B associated with T cell activation.

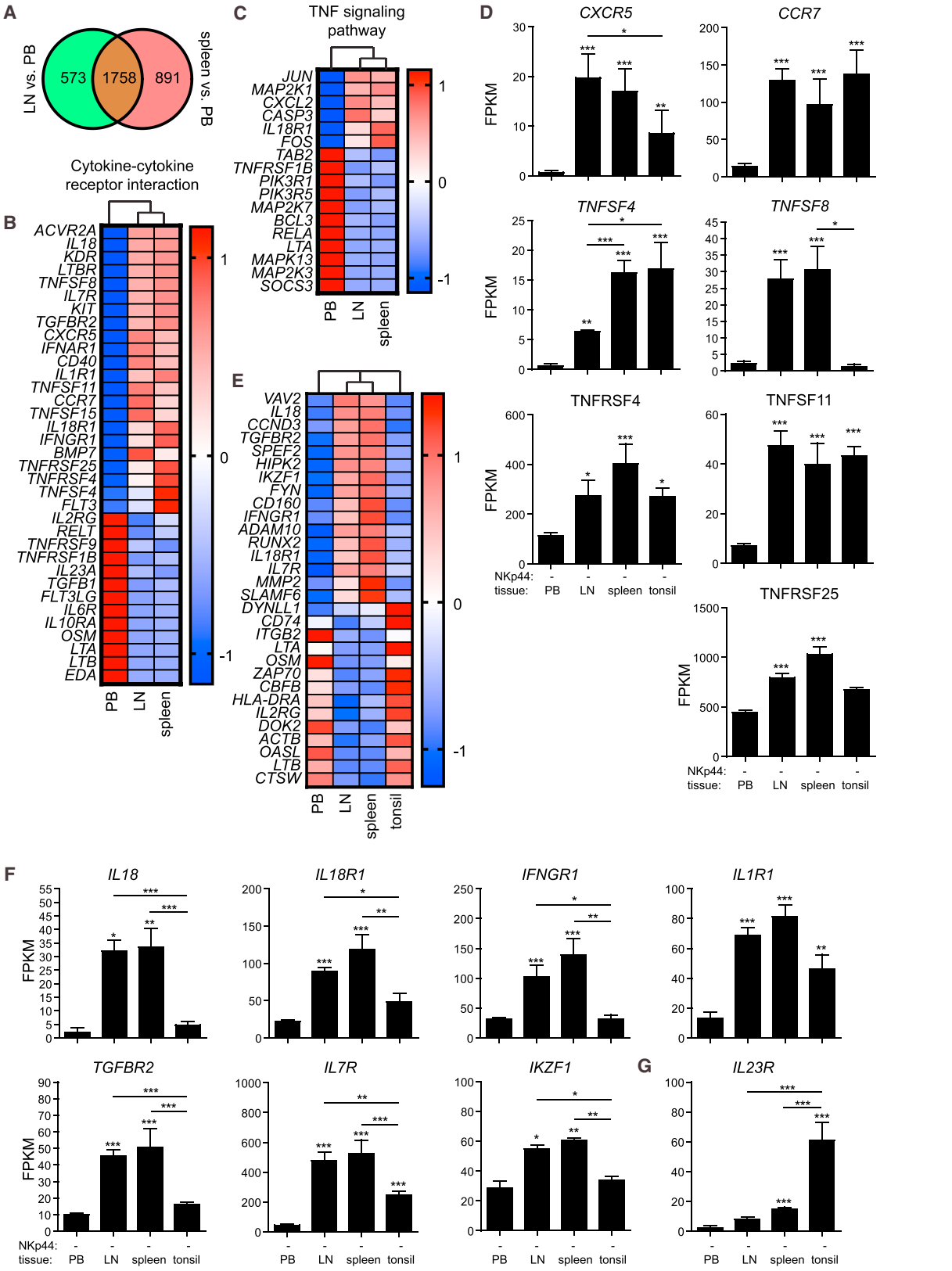
*$p < 0.05$, **$p < 0.01$,***$p < 0.001$ compared to PB or between indicated groups. (A and C) Error bars represent mean ± SEM.

2016). We also identified genes not previously associated with ILC3 function, such as *TNFRSF18* (GITR) and *CXCR4* (Figure 1E). However, most striking was the absence of all ILC3 signature cytokines, including *IL22*, *CSF2* (encoding GM-CSF), and *TNF*, which are considered to be constitutively expressed in inflamed tonsillar ILC3s and intestinal ILC3s (Artis and Spits, 2015; Hazenberg and Spits, 2014). Altogether, these data show that ILC3s from human SLOs share a functional immune signature that is distinct from PB ILC3s and does not include cytokine genes commonly associated with ILC3 function.

Cytokine Transcription Is Independent of NKp44 Expression

The SLO ILC3 immune transcriptome did not contain any ILC3 signature cytokines that are constitutively expressed by ILC3s in inflamed tonsil and intestines. This could be explained by only a low number of ILC3s within the tonsil transcribing these cytokines, as is the case for non-inflamed tonsils (Björklund et al., 2016), or by differences in cytokine production between the non-reactive SLOs and the inflamed tonsils. To elucidate the cytokine profile of ILC3s residing in non-reactive SLOs, we subsequently focused on transcription of cytokine genes in ILC3s from the different SLOs. Signature genes such as *IL22*, *LTA*, *LTB*, and *TNF* were transcribed by tonsil ILC3s yet either absent or expressed at very low levels in lymph node and spleen ILC3s (Figure 2A). Additional cytokines previously associated with ILC3 function, such as *LIF* and *IL2* (Cella et al., 2009; Crellin et al., 2010b), followed a similar pattern of transcription (Figure 2A). The cytokine transcriptome of PB ILC3s was comparable to that of ILC3s from lymph nodes and spleen, with the exception of *LTA* and *LTB*, which were transcribed at higher levels in PB ILC3s, similar to tonsil ILC3s (Figure 2A). These findings indicate that ILC3s within human non-reactive SLOs are not constitutively transcribing high levels of cytokine transcripts but rather appear to be in a more resting immunological state.

Secretion of cytokines has been associated with expression of NKp44 in ILC3s isolated from intestines or tonsil (Glatzer et al., 2013; Hoorweg et al., 2012). Here we show that in a non-inflamed environment such as the spleen, IL-22 transcription is low in both NKp44$^+$ and NKp44$^-$ ILC3s (Figure 2A), indicating that active



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cytokine transcription is associated with inflammation, rather than NKp44 expression. We next asked whether NKp44$^+$ splenic ILC3s show alternative signs of cellular activation in the absence of cytokine transcription. We identified 14 immune genes that were differentially expressed between NKp44$^+$ and NKp44$^-$ ILC3s from both resting spleens and inflamed tonsils (Figure 2B; Table S4). Many of these commonly different genes are involved in cell migration, with NKp44$^+$ ILC3s expressing lower levels of *SELL* (encoding CD62L), *CCR7*, and *S1PR1*. This suggests that NKp44$^+$ and NKp44$^-$ ILC3s differ in their migration capacity, possibly influencing their localization. High expression of *SELL* was previously associated with a subset of more naive ILC3s in resting tonsils (Björklund et al., 2016), reinforcing the notion that *SELL*^{lo} NKp44$^+$ tonsil and spleen ILC3s in our analysis represent a more activated population. However, additional activation-associated transcripts involved in cytoskeletal function or intracellular protein processing, previously linked to an activated ILC3 subset in resting tonsils (Björklund et al., 2016), were similar between NKp44$^-$ and NKp44$^+$ ILC3s from either spleens or inflamed tonsils (Figure S3). The only transcript associated with cellular activation that we observed to be higher in NKp44$^+$ ILC3s was the T cell activation marker *CD2* (Figure 2C). Altogether, these data show that NKp44 expression does not associate with cytokine transcription or overt signs of cellular activation and that splenic and tonsil NKp44$^+$ ILC3s differ from their NKp44$^-$ counterparts in genes involved in cell migration.

ILC3s in Human SLOs Transcribe Genes Involved in Cellular Interactions

ILC3s from non-inflamed SLOs seem to function independently of classical ILC3 cytokines, suggesting that these ILC3s could have alternative functional roles. To address this possibility, we analyzed expression of genes involved in functional properties suggested for mouse lymphoid organ-residing ILC3s, including cytokine production and support of adaptive immune cells (Cella et al., 2009; Hepworth et al., 2015, 2013; Kim et al., 2003; Magri et al., 2014; von Burg et al., 2014; Withers et al., 2011). We determined genes specifically expressed in both lymph node and splenic NKp44$^-$ ILC3s compared to the phenotypically similar NKp44$^-$ cells present in PB. This yielded 1,758 differentially expressed genes (Figure 3A). Subsequently, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified cytokine-cytokine receptor interactions and tumor necrosis factor (TNF) signaling pathways as significantly enriched in this gene set (Figures 3B and 3C). In mouse and human SLOs, ILC3s localize to the T-B interface (Hoorweg et al., 2015; Kim et al., 2011). In line with this spatial positioning, lymph node and spleen ILC3s expressed both *CXCR5* and *CCR7* (Figure 3D). In addition, ILC3s from resting SLOs expressed high levels of *TNFSF11* and of *TNFRSF25* (encoding RANKL and death receptor [DR] 3, respectively). Lymph node ILC3s in mouse can influence memory T cell survival through expression of TNF superfamily members, independent of inflammatory cytokines (Kim et al., 2011; Withers et al., 2012). Similar to those of mouse, human SLO ILC3s expressed *TNFSF8* and *TNFSF4* (encoding the T cell costimulating molecules CD30L and OX40L, respectively) (Figure 3D) (Kim et al., 2003, 2005; Withers et al., 2011, 2012). ILC3s also transcribed the T cell-associated molecule *TNFRSF4* (encoding OX40) (Figure 3D). All these TNF family members, except for *TNFSF8*, were also transcribed by tonsil ILC3s (Figure 3D) and should thus be considered part of a more general homeostatic SLO ILC3 transcriptional signature, expressed independently of an inflammatory environment.

In the search for ILC3 immune genes associated specifically with presence in non-inflamed SLOs, we focused on transcripts expressed to significantly higher levels in NKp44$^-$ ILC3s from lymph node and spleen in comparison to phenotypically similar cells from tonsil and PB (Figures 3E and 3F). This revealed that ILC3s in resting SLOs differentially transcribed several cytokine receptor genes, including *IL1R1*, *IL18R1*, *IL7R*, *IFNGR1*, and *TGFBR2*. In addition, the cytokine *IL18* and the transcription factor *IKZF1*, involved in inhibiting IL-2 production in T cells (Bandyopadhyay et al., 2007; O'Brien et al., 2014; Thomas et al., 2007), were transcribed to significantly higher levels in NKp44$^-$ ILC3s from lymph node and spleen compared to ILC3s from tonsil or blood (Figure 3F). The finding that *IL1R1*, a major activating receptor for ILC3s, was more highly expressed in lymph node and spleen ILC3s compared to tonsil ILC3s led us to analyze transcription of another important ILC3-activating receptor: *IL23R*. In contrast to *IL1R1*, *IL23R* transcription followed a pattern associated with the degree of inflammation. Highest expression of *IL23R* transcription was observed in tonsil ILC3s, lower levels were seen in lymph node and spleen ILC3s, and transcription was very low in PB ILC3s (Figure 3G).

Altogether, these transcriptomic profiles predict functional roles for human SLO-residing ILC3s that include intercellular communication with memory T cells via TNF family members and receptiveness to environmental signals through expression of cytokine receptors.

Figure 3. SLO ILC3s Transcribe Genes Involved in Immune Interactions and Cytokine Responsiveness

(A) Venn diagram showing the number of genes that are significantly different ($p < 0.05$) between PB ILC3s and LN ILC3s (2,331 genes; green circle) and between PB ILC3s and NKp44$^-$ splenic ILC3s (2,649 genes; red circle) and the genes differentially expressed in both datasets (1,758 genes) that were used for KEGG pathway analysis.

(B and C) Heatmaps of genes obtained from KEGG pathway analysis of cytokine-cytokine receptor interaction (B) and TNF signaling pathway (C) that were significantly different ($p < 0.05$) in both LN and splenic ILC3s when compared to PB ILC3s. *Z* scores were calculated per gene based on average FPKM per ILC3 population.

(D) Expression levels (FPKM) of selected genes from (B).

(E) Heatmap of genes that were significantly different ($p < 0.05$) between both LN and splenic ILC3s versus PB and NKp44$^-$ tonsillar ILC3s. *Z* scores were calculated per gene based on average FPKM per ILC3 population.

(F) Expression levels (FPKM) of selected genes from (D).

(G) Expression levels (FPKM) of IL-23R in ILC3s from the analyzed tissues.

$^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to PB or between indicated groups. (D, F, and G) Error bars represent mean ± SEM.

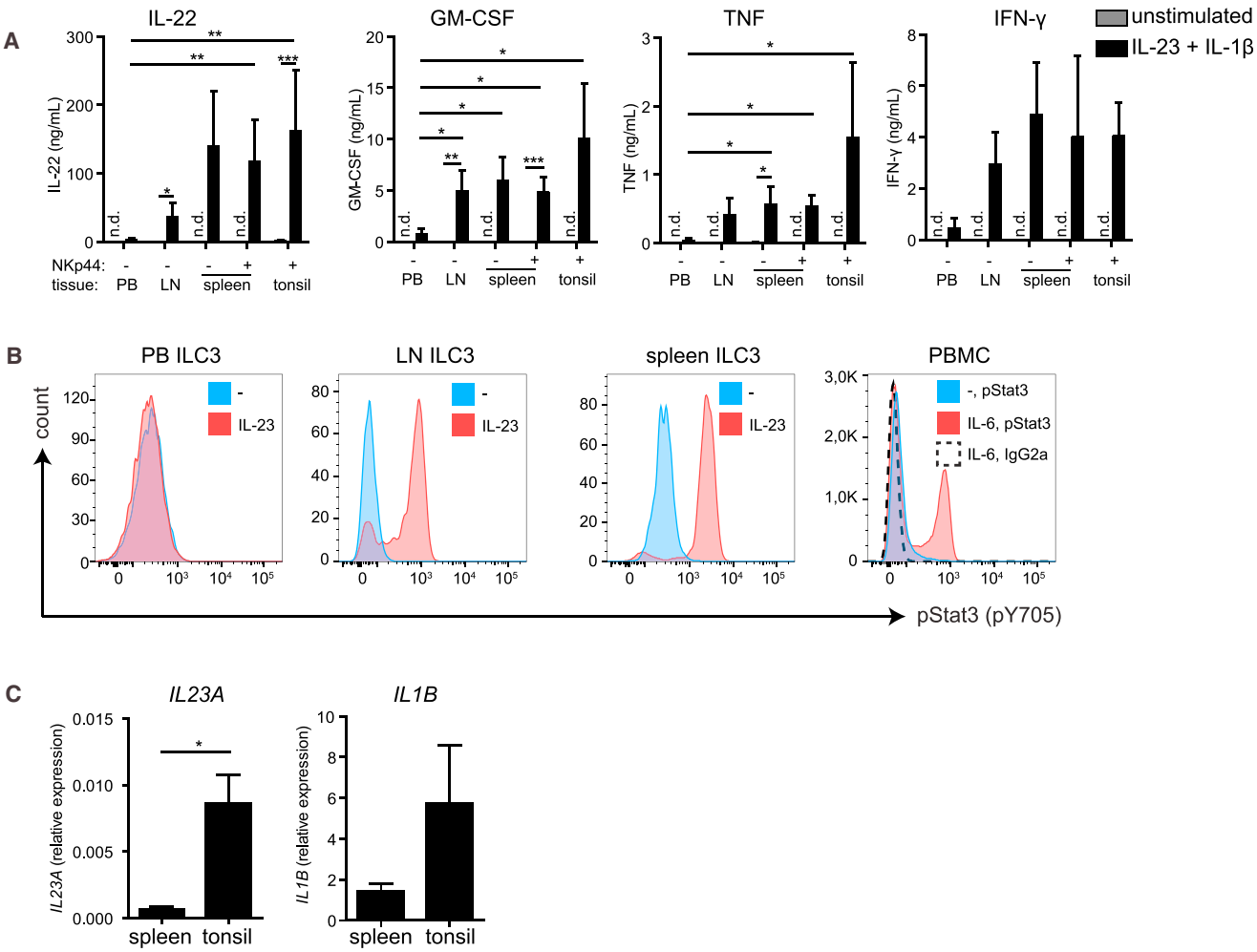

Figure 4. Non-reactive SLO ILC3s Can Be Activated

(A) Purified human ILC3s isolated from indicated tissues were cultured for 3 days with IL-23 and IL-1β (black bars) or left unstimulated (gray bars), and indicated cytokines were measured in the culture supernatant using ELISA. Differences between unstimulated and stimulated conditions were calculated with the Wilcoxon signed-rank test; differences between ILC3 stimulated conditions were calculated by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to PB or between indicated groups. For IL-22, n = 5–13; for GM-CSF, n = 5–12; for TNF, n = 3–8; for IFN-γ, n = 4–7; ND, not detected. See also Figure S4A.

(B) Purified ILC3s from PB, lymph nodes, and spleen were stimulated with IL-23 for 15 min (or left unstimulated), and phosphorylation of STAT3 (pStat3) was measured by flow cytometry. Unfractionated blood mononuclear cells (MNCs) (PBMCs) stimulated with IL-6 were used as a positive assay control. Data shown are representative of n = 3.

(C) qPCR analysis of *IL23A* and *IL1B* expression in mononuclear phagocytes ($CD3^-CD19^-CD11b^+HLA\text{-}DR^+$) (for full gating, see Figure S4B) sorted from tonsils (n = 4) and spleens (n = 6). *$p < 0.05$, Mann-Whitney U test.

(A and C) Error bars represent mean ± SEM.

SLO-Residing ILC3s Can Be Activated by Inflammatory Signals

Our transcriptional definition of the immune status of ILC3s in resting and inflamed human SLOs suggests that active cytokine production is the result of local inflammation-related signals. Furthermore, ILC3s in non-inflamed SLOs lack signature cytokines yet transcribe genes associated with cellular interactions and cytokine responses. Because SLOs are the primary sites of immune cell activation, we hypothesized that ILC3s within resting SLOs should be able to initiate cytokine production in response to signals associated with infection or inflammation. To test this hypothesis, we purified ILC3s from lymph nodes, spleen, tonsils, and PB and stimulated these cells with IL-1β and IL-23. As expected, tonsil ILC3s responded to cytokine stimulation by secretion of IL-22, GM-CSF, TNF-α, and interferon γ (IFN-γ) (Figure 4A). ILC3s from resting lymph nodes and spleens, which do not transcribe cytokines directly ex vivo, had the ability to respond to stimulation with IL-1β and IL-23 and secreted high levels of all 4 cytokines (Figure 4A). Stimulation with IL-23 alone was insufficient to evoke cytokine production in either splenic or tonsil $NKp44^+$ ILC3s, while IL-1β alone led to production of GM-CSF, but not IL-22, in both cell types (Figure S4A). Altogether, these

results show that even though ILC3s in resting SLOs are not actively transcribing cytokines, they are receptive to inflammatory signals and can convert to reactive cytokine-secreting cells.

In contrast to SLO ILC3s, the ILC3s isolated from PB showed virtually no production of IL-22, GM-CSF, TNF-α, or IFN-γ in response to IL-23 and IL-1β (Figure 4A). This was in line with the finding that transcripts for the receptors for IL-23 and IL-1 were virtually absent from this population (Figures 3F and 3G). To determine whether transcriptional levels of *IL23R* translated into IL-23 responsiveness, STAT3 phosphorylation was measured shortly after cytokine exposure of ILC3s. Upon stimulation with IL-23, STAT3 was phosphorylated in ILC3s from lymph nodes and spleens, similar to activation of total PB mononuclear cells (PBMCs) with IL-6 (Figure 4B). In contrast, exposure of PB ILC3s to IL-23 did not induce STAT3 phosphorylation (Figure 4B). Because all SLO-residing ILC3 populations can respond to cytokine stimulation, steady-state differences in cytokine transcription between inflamed and non-inflamed lymphoid tissues are likely a reflection of the local cytokine milieu. To corroborate this notion, we purified mononuclear phagocytes from non-inflamed spleens and inflamed tonsils and assessed transcription of the ILC3-activating cytokines IL-23 and IL-1β (for gating, see Figure S4B). Tonsil phagocytes transcribed more *IL23*, and there was a trend toward more *IL1B* (Figure 4C), indicating that the inflamed environment of the tonsil is likely enriched for ILC3-activating signals. These findings fit the proposed scenario in which the circulating ILC3 pool is enriched for precursors to the ILC lineage (Lim et al., 2017) and highlight the importance of signals emanating from the SLO microenvironment to induce full ILC3 receptiveness to cytokine production, regulated at least partly through acquisition of IL-23R expression and responsiveness to local cytokines.

DISCUSSION

Here we present a cross-tissue transcriptomic comparison of human ILC3s. We provide compelling evidence indicating that most SLO-residing ILC3s are characterized by a resting phenotype and, in contrast to mucosal tissue ILC3s, are not constitutively transcribing the cytokines that are often considered synonymous with ILC3 function.

Although mainly described as mediators of inflammation and guardians of homeostasis in epithelial and mucosal tissues, a substantial population of ILC3s is present in SLOs in both mice and humans (Bar-Ephraïm and Mebius, 2016). In humans, functional studies on SLO-resident ILC3s mainly relied on cells isolated from inflamed tonsils. Although these ILC3s are likely a relevant population in the context of mucosal immunity and barrier integrity, functional similarity to their more numerous counterparts in non-inflamed SLOs might be limited. This is not unlike adaptive immune cells that exist mostly as naive cells in resting SLOs, while in tonsils, both germinal center B cells and distinct populations of activated and memory T cells are present (Nave et al., 2001; van Kempen et al., 2000). By using resting, non-reactive hepatic lymph nodes and spleens, we could identify functional traits of SLO-residing ILC3s in a direct comparison with ILC3s from circulation or from inflamed palatine tonsils.

ILC3s from non-inflamed SLOs, inflamed tonsils, and PB all formed distinct clusters in PCA, and this was irrespective of NKp44 expression status. The differential clustering of lymph node and spleen ILC3s versus tonsil ILC3s was also reflected in transcriptional changes of immune-related genes. ILC3s from inflamed tonsils are the most immunologically active ILC3 population in our analyses, with the highest levels of transcription of cytokines and activation markers. In contrast, ILC3s in non-inflamed SLOs lack transcription of most activation markers and of signature cytokines such as IL-22 and GM-CSF. A similar absence of cytokines and activation markers was obvious in PB ILC3s, even though the highly divergent clustering of these innate lymphoid cells (ILCs) is likely influenced by this population containing ILC precursors with the ability to differentiate into all ILC subsets (Lim et al., 2017). This was confirmed by the low levels of *RORC* transcription in the PB ILC subset (Figure 1B). Curiously, this did not translate into lower levels of RORγt protein (Figure 1A). Whether this is due to technical issues or reflects a true biological phenomenon remains to be asserted. We did not detect transcripts associated with T or B cell lineage development (*RAG1*, *RAG2*, *EBF*, or *LMO2*) in PB ILC, suggesting that these cells are committed to the ILC lineage (data not shown) (Lim et al., 2017).

Through the analyses of ILC3s in non-reactive spleens, we had the opportunity to investigate the functional association of NKp44 expression with ILC3 cytokine transcription. In line with previous findings (Glatzer et al., 2013; Hoorweg et al., 2012), our dataset links NKp44 expression to cytokine secretion in ILC3s from inflamed tonsils. However, in contrast to tonsil-residing ILC3s, NKp44 expression on ILC3s from non-reactive spleens is uncoupled from cytokine production, because IL-22, GM-CSF, and other signature cytokines are absent from both NKp44$^+$ and NKp44$^-$ splenic ILC3s. Moreover, in both spleen and inflamed tonsil, NKp44 expression did not enrich for activation-associated genes, as previously defined on resting tonsil NKp44$^+$ ILC3s (Björklund et al., 2016). This notwithstanding, NKp44$^+$ ILC3s were reported to appear in lymph nodes of patients with rheumatoid arthritis (Rodríguez-Carrio et al., 2017).

The ability to respond to activating cytokines, in particular IL-23 and IL-1β, is a feature that is shared by all SLO-residing ILC3s, regardless of whether they are in an inflamed or a non-inflamed environment. Only ILC3s from inflamed tonsils actively secrete the cytokines downstream of IL-23 and IL-1β activation, which revealed that the lymph nodes and spleen collectively contain a substantial reservoir of ILC3s with the capacity to be activated by local cytokine signals. This was corroborated by our in vitro activation studies, which showed that ILC3s from lymph nodes and spleen can respond to cytokine stimulation and secrete high amounts of IL-22, GM-CSF, TNF-α, and IFN-γ. Furthermore, mononuclear phagocytes from tonsils produced more IL-23 compared to phagocytes from spleen, again emphasizing that differences in the ILC3 microenvironment determines their overall activation profile.

The ability to respond to cytokines was absent from PB ILC3s. It is likely that this is largely because of their precursor status (Lim et al., 2017), yet STAT3 phosphorylation and IL-23R responsiveness are absent, which also suggests that any mature ILC3s in blood might need to initiate cytokine receptor transcription to

become responsive to inflammatory cytokines. It is tempting to speculate that this will be regulated by local signals in the SLO microenvironment, but this will need to be experimentally addressed.

Besides avid cytokine producers, mouse ILC3s have been implicated in other immune-related functions within SLOs. In human non-inflamed SLOs, ILC3s express transcripts for many TNF family members known to affect T cell function (i.e., *TNFSF4* and *TNFSF8*, encoding OX40L and CD30L, respectively). Although expression of OX40L by ILC3s and its functional consequences have been described in mice (Kim et al., 2003, 2005; Withers et al., 2011), human SLO ILC3s also transcribed *TNFRSF4* (OX40). The functionality and consequences of OX40 expression on ILC3s remains unexplored, but it could allow for co-activation of ILC3s by antigen-presenting cells or by neighboring ILC3s and warrants future investigation. Human SLO-residing ILC3s also transcribed *IKZF1*, which restricts IL-2 production in activated $CD8^+$ and anergic $CD4^+$ T cells (Bandyopadhyay et al., 2007; O'Brien et al., 2014; Thomas et al., 2007). This observation might represent a previously unrecognized regulator of ILC3 proliferation and activation.

Altogether, our findings redefine current views on human in situ ILC3 immunology and provide a detailed dataset of human SLO-residing ILC3s. These cells are in a resting state yet form a significant pool of cells capable of responding to inflammatory signals. Whether they remain in the lymphoid environment upon activation or migrate to affected tissues, similar to adaptive lymphocytes, has yet to be investigated, although data from mouse studies provide evidence for the latter (Kim et al., 2015; Yang et al., 2016). Altogether, the findings presented in the current study allow for investigation of the functional consequences of ILC3 activation and for exploring of whether alterations in ILC3 phenotype can be exploited as sensitive indicators of local immune activation in response to infection or malignancy.

EXPERIMENTAL PROCEDURES

Human Tissues

PBMCs were isolated from buffy coats of anonymous adult blood donors (Sanquin Blood Supply, the Netherlands) by gradient centrifugation on Lymphoprep (d = 1.077, Fresenius Kabi Norge, Berg i Østfold, Norway). PBMCs were subsequently washed twice in cold PBS (B. Braun Melsungen, Melsungen, Germany) supplemented with 10% v/v donor plasma.

Palatine tonsils were obtained during routine pediatric tonsillectomies at the Department of Otolaryngology, Slotervaart Hospital, Amsterdam, the Netherlands, and Sophia Children's Hospital, Rotterdam, the Netherlands. Hepatic lymph node and spleen tissues were collected post-mortem during multi-organ transplantation procedures at the Erasmus University Medical Center, Rotterdam, the Netherlands. Tissue was cut into small pieces, and cell suspensions were prepared by disrupting the tissue with a GentleMacs dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence of 0.5 mg/mL Collagenase D (Sigma Aldrich, St. Louis, MO, USA). Mononuclear cells were further isolated by gradient centrifugation on Lymphoprep (d = 1.077, Fresenius Kabi Norge).

All human tissues were collected after approval by the Medical Ethical Committee of the VU University Medical Center (Amsterdam, the Netherlands) or the Erasmus University Medical Center (Rotterdam, the Netherlands), in accordance with the Declaration of Helsinki and according to Dutch law. Tissues are used anonymously, and no information is available on age or gender of the donors.

Flow Cytometry

For cell sorting of ILC3s, mononuclear cell fractions of all tissues were enriched for ILC3s using the CD117 MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol, followed by positive selection using LS columns (Miltenyi Biotec). Cell suspensions were subsequently labeled with the following antibodies: CD45-phycoerythrin (PE)-Cy7 (clone HI30), CD127-APCe Fluor 780 (eBioRDR5), and CD94-FITC (DX22) (all eBioscience, San Diego, CA, USA); NKp44-Alexa Fluor 647 (P44-8, BioLegend, San Diego, CA, USA); a lineage cocktail containing CD3 (MEM57), CD19 (LT19), CD14 (MEM15), and CD34 (4H11) (all PE-Texas red, all Exbio, Vestec, Czech Republic); and CRTH2-PerCP-Cy5.5 (BM16, BD Biosciences, Mountain View, CA, USA). From the CD117-negative fraction, mononuclear phagocytes were sorted after labeling with CD11c-PE (Bu15, BioLegend), HLA-DR/DP/DQ-FITC (Tu39, BD Biosciences), CD14-Alexa Fluor 700 (HCD14, BioLegend), and CD19-PE-Texas red (LT19, Exbio). For both ILC3 and dendritic cell (DC) sorting, dead cells were excluded using DAPI. Sorting was performed using a FACSAria III sorter (BD Biosciences, Mountain View, CA, USA).

For flow cytometric determination of RORγt, anti-RORγt-PE (AFKJS-9) was added to the antibody cocktail. Fixation and permeabilization were performed using the Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBioscience, San Diego, CA, USA).

Cell Culture and Stimulation

Fluorescence-activated cell sorting (FACS)-purified ILC3s (25,000 cells) were cultured in 200 μL DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal calf serum (HyClone, GE Healthcare Life Sciences, Loga, UT, USA), penicillin, streptomycin, and L-glutamine in the presence of IL-7 (PeproTech, Rocky Hill, NJ, USA) and stem cell factor (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) and IL-1β (10 ng/mL) in the presence of IL-7 and SCF (10 ng/mL), unless indicated otherwise, for 3 days before measurement of cytokine release by ELISA. Quantification of IL-22, TNF-α, and GM-CSF was performed using the DuoSet ELISA Kits (R&D Systems, Minneapolis, MN, USA) according the manufacturer's protocol.

For detection of phosphorylated STAT3 (pSTAT3), FACS-purified ILC3s were stimulated for 15 min with IL-23 (100 ng/mL) (R&D Systems, Minneapolis, MN, USA) or left untreated. Next, cells were fixed and permeabilized (BD Cytofix/Cytoperm, BD Biosciences, Mountain View, CA, USA) for 15 min at 37°C, followed by additional fixation with 90% (v/v) methanol (−20°C) on ice for 30 min. Cells were then washed with permeabilization and wash solution (BD Cytofix/Cytoperm), and pSTAT3 was stained for 1 hr at room temperature (RT) using mouse-anti-STAT3-Alexa Fluor 647 (pY705, clone 4/P-STAT3, BD Phosflow) IgG2a antibody, or, isotype control antibodies.

Analysis was performed on a LSR II (BD Biosciences, Mountain View, CA, USA). Further analysis was done using FlowJo v.10 for Microsoft (Tree Star, San Carlos, CA). Gates were set based on isotype controls and/or unstimulated cells.

Sample Preparation and RNA Sequencing

For RNA sequencing, ILC3s were sorted from PB (n = 3), hepatic lymph nodes (n = 3), spleens (n = 4), and tonsils (n = 3). Cells were FACS sorted directly into lysis buffer containing tris(2-carboxyethyl)phosphine (TCEP; Macherey-Nagel, Düren, Germany) and stored at −80°C until further processing. RNA was isolated according the manufacturer's protocol (NucleoSpin XS Kit, Macherey-Nagel), omitting the addition of carrier RNA. RNA quality was measured on a RNA 6000 Pico Kit (Agilent Technologies, Amstelveen, the Netherlands) using a 2100 Bioanalyzer (Agilent Technologies). 100 pg of high-quality RNA (RNA integrity number [RIN] = 8.37 ± 0.55 [average ± SD]) was converted into cDNA using the SMART-Seq (v.3) Ultra Low Input RNA Kit (Clontech Laboratories, 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) and further processed according the TruSeq Nano DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Samples were sequenced on the HiSeq 2500 platform (Illumina, San Diego, CA, USA). Data were processed as described previously (Chen et al., 2016). In brief,

SMARTer adapters were trimmed with Cutadapt, and the resulting sequences were aligned to the human RefSeq transcriptome and reference genome (build HG19) using TopHat2 (Kim et al., 2013). Normalization and quantification were performed using Cufflinks, resulting in a per gene abundance estimation measured in fragments per kilobase of exon per 10^6 reads mapped (FPKM) (Trapnell et al., 2012). Fragment counts were determined per gene with HTSeq-count and subsequently used for differential expression analysis using the DESeq2 package (Anders and Huber, 2010). Multiple testing correction was performed with the Benjamini-Hochberg procedure to control the false discovery rate (FDR) as part of the DESeq2 procedure. PCA was performed on the fragment counts using the R environment.

RNA sequencing data from human bone marrow stromal cells (n = 10) were generated in a similar manner and published previously (Chen et al., 2016).

RNA Isolation, cDNA Synthesis, and qPCR

Mononuclear phagocytes 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 manufacturer's protocol (NucleoSpin XS Kit, Macherey-Nagel). RNA was converted into cDNA using the SensiFAST cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer's instructions. For qPCR, the SensiFAST SYBR Lo-ROX Kit (Bioline) was used according the manufacturer's instructions with the addition of $MgCl_2$ to a final concentration of 4 mM on a 7500 Fast Real-Time PCR System (Applied Biosystems). All reactions were performed in duplicate and were normalized to the expression of *GAPDH*. Relative expression was calculated by the cycling threshold (CT) method as 2^{-dCt}. Primer sequences are as follows: *GAPDH* (forward, 5′-GTCGGAGTCAACGGATT-3′; reverse, 5′-AAG CTTCCCGTTCTCAG-3′), *IL23A* (forward, 5′-TGGGACACATGGATCTAAG-3′; reverse, 5′-GCTCCCCTGTGAAAATATC-3′), *IL1B* (forward, 5′-CCTGCCCA CAGACCT-3′; reverse, 5′-GGACCAGACATCACCAAG-3′).

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA sequencing data reported in this paper is ArrayExpress: E-MTAB-5909.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.09.070.

AUTHOR CONTRIBUTIONS

Conceptualization, R.E.M. and T.C.; Investigation, Y.E.B.-E., F.C., N.P., and T.K.; Formal Analysis, Y.E.B.-E., F.C., R.M.H., M.A.S., and B.A.W.; Resources, J.M.M.D.H., M.G., and J.K.; Writing – Original Draft, Y.E.B.-E. and F.C.; Writing – Review & Editing, R.M.R., R.E.M., and T.C.; Funding Acquisition, F.C., R.E.M., and T.C.; Supervision, R.E.M. and T.C.

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