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Subsets of ILC3-ILC1-like cells generate a diversity spectrum of ILCs in human mucosal tissues.

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Author contributions.

C.S., P.L.C. and S.Z. contributed equally to this work. M. Cella designed, performed and interpreted experiments. R.G. and S.Z. analyzed scRNA-seq data and wrote methods for scRNA-seq analysis. C.S. generated Aiolos- and T-bet-transduced MNK3 cells. M.L.R. and V.P. analyzed the microarray data and RNA-seq data. K.Z. and M.N.A. provided bioinformatic support. J.K.B., K.Y. and V.C. helped in flow cytometry data presentation and analysis. C.F. and R.F. generated libraries for scRNA-seq. J.S. provided critical advice for Cytot analysis. W.G., L.-L.L. and M.B. provided critical insights to the study. S.G., R.A.F. and L.S. provided key reagents. P.L.C. performed cut and run experiment and interpreted data under supervision of E.M.O. S.A.J. and M. Colonna supervised the study. M. Cella, S.A.J. and M. Colonna wrote the manuscript and all the authors contributed editing and suggestions.

Competing Interests

R.G., S.Z., W.G., J.S., L.-L.L., M.B. and S.A.J. are Pfizer employees. M. Colonna received funding from Pfizer to study ILC biology in Inflammatory Bowel Disease. The other authors declare no conflicts of interests.

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Abstract

Innate lymphoid cells (ILCs) are tissue-resident lymphocytes categorized on the basis of their core regulatory programs and the expression of signature cytokines. Human ILC3s that produce the cytokine IL-22 convert into ILC1-like cells that produce interferon- γ in vitro, but whether this conversion occurs in vivo remains unclear. In the present study we found that ILC3s and ILC1s in human tonsils represented the ends of a spectrum that included additional discrete subsets. RNA velocity analysis identified an intermediate ILC3-ILC1 cluster, which had strong directionality toward ILC1s. In humanized mice, the acquisition of ILC1 features by ILC3s showed tissue dependency. Chromatin studies indicated that the transcription factors Aiolos and T-bet cooperated to repress regulatory elements active in ILC3s. A transitional ILC3-ILC1 population was also detected in the human intestine. We conclude that ILC3s undergo conversion into ILC1-like cells in human tissues in vivo, and that tissue factors and Aiolos were required for this process.

Innate lymphoid cells (ILCs) are tissue-resident lymphocytes that lack antigen-specific receptors and produce defined cytokines early during the immune response against pathogens^{1–3}. Their function is to immediately respond to pathogens and facilitate subsequent responses by antigen-specific T cells and B cells⁴. Three major groups of ILCs are distinguished by the signature cytokines they produce: ILC1s release interferon (IFN)- γ ; ILC2s secrete interleukin (IL)-5 and IL-13; and ILC3s produce IL-22 and IL-17. Each ILC group responds to distinct stimuli: IL-12, IL-18 and IL-15 trigger ILC1s; IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) trigger ILC2s; and IL-23 and IL-1 β trigger ILC3s. ILC subtypes are also defined by distinct transcriptional programs and the specific transcription factors that instruct these programs: T-bet and Hobit are critical for ILC1s, high expression of the transcription factor GATA-3 regulate ILC2s, and ROR γ t and Ahr control ILC3 identity and function⁵. The three ILC modules mirror the functional polarization of CD4⁺ T helper (T_H) cells into T_H1, T_H2 and T_H17 cells.

ILC diversity, however, extends beyond the strict definitions of ILC1s, ILC2s and ILC3s. Single cell RNA sequencing (scRNA-seq) has indicated substantial transcriptional heterogeneity in ILCs^{6,7}. Moreover, ILCs have been proposed to be plastic⁸. This attribute, which has been extensively studied in T cells^{9,10}, facilitates the adaptation of immune responses in disparate tissues to diverse pathogenic stimuli. ILC plasticity was first observed in ILC3s in vitro^{11,12}. Human ROR γ t⁺ ILC3s cultured in vitro with IL-2, IL-15 or IL-23 acquire ILC1-like features, such as the production of IFN- γ and the expression of the transcription factor T-bet^{11,13}. Fate mapping experiments in *Rorc* reporter mice have indicated that a subset of IFN- γ ⁺ ILC1s derive in part from Ror γ t⁺ ILCs. This subset, referred to as ex-ILC3s, requires a decrease in Ror γ t^{14–16}, along with a coordinate increase in T-bet^{14–17} and Notch signaling^{17–20}, for its generation. However, the extent and biological impact of human ILC3 plasticity in vivo, and the tissue factors that promote plasticity in humans, remain unresolved.

We hypothesized that, if conversion of ILC3s to ILC1s occurs in humans in vivo, transitional ILC populations with features of both ILC3s and ILC1s should be detectable. In human

mucosal-associated lymphoid tissues, ILC3s and intraepithelial ILC1s are CD56⁺NKp44⁺, but can be distinguished by the expression of CD196 (CCR6) and CD103 (αEβ7 integrin), respectively^{11,21}. In the present study, we show that flow cytometry, transcriptome profiling, mass spectrometry and scRNA-seq analyses identified additional ILC subsets, which lay between ILC3s and ILC1s. In vivo transfer experiments into a humanized mouse model demonstrated that ILC3s acquired transcription factors and cytokines characteristic of ILC1-like cells in a tissue-dependent fashion. The transcription factor Aiolos played an integral role in this process and cooperated with T-bet to suppress expression of IL-22 and RORγt. Importantly, the ILC3–ILC1 intermediate populations were not confined to the tonsils, but were also found in the lamina propria of the human ileum, suggesting that ILC3-to-ILC1 plasticity is common to mucosal tissues.

Results

Four subsets of ILCs are detected in human tonsils.

In the inflamed tonsils of children, CD3[−]CD19[−]CD56⁺NKp44⁺ cells include a subset of natural killer (NK) cells and two major ILC subsets: IL-22⁺ ILC3s¹¹ and IFN-γ⁺ intraepithelial ILC1s²¹. ILC3s were CD103[−]CD196⁺CD300LF⁺ (Fig. 1a)²², whereas most of the intraepithelial ILC1s were CD103⁺CD196[−]CD300LF[−] (Fig. 1a). We noticed that CD56⁺NKp44⁺CD103⁺ ILCs contained two additional populations that were CD196⁺CD300LF⁺ and CD300LF[−]CD196⁺ (Fig. 1a). Although their percentages varied, these subsets were present in all donors tested (n=25) and were less abundant than CD103[−]CD196⁺CD300LF⁺ ILC3s and CD103⁺CD196[−]CD300LF[−] ILC1s (Fig. 1b). Based on their relative similarities, we postulated that these populations represented intermediate subsets of the ILC3–ILC1 spectrum. Hereafter, we refer to CD103[−]CD196⁺CD300LF⁺ ILC3s as ILC3a and CD103⁺CD196⁺CD300LF⁺ as ILC3b, CD103⁺CD196[−]CD300LF[−] as ILC1b and CD103[−]CD196[−]CD300LF[−] ILC1s as ILC1a, unless otherwise specified. CD56⁺NKp44⁺ cells that were CD103[−]CD196[−]CD300LF[−] corresponded to conventional NK cells (Fig. 1a) and were not considered for further transcriptional analyses. Thus, the ILC3–ILC1 spectrum in human tonsil was more heterogeneous than previously anticipated.

ILC3b and ILC1b have intermediate gene expression signatures

Next, ILC3a, ILC3b, ILC1a and ILC1b were sorted from four human tonsils (see Supplementary Fig. 1) and their transcriptome profiles analyzed by microarray analysis. Hierarchical clustering of thousands of gene expression measurements distinguished two separate, but related, clusters of ILC3s (ILC3a and ILC3b) and ILC1s (ILC1a and ILC1b) (data not shown). In principal components analysis (PCA), the first principal component (PC1), which accounted for 73% of the variation, distinguished ILC3a, ILC3b, ILC1b and ILC1a, and highlighted differences between ILC3a and ILC1a (Fig. 2a). PC1 values for ILC3b and ILC1b were intermediate between those for ILC3a and ILC1a (Fig. 2a). The second principal component (PC2), which accounted for 10% of the variation, separated ILC1a from ILC1b and ILC3b from ILC3a; the third principal component (PC3) accounted for 5% of the variation and most likely reflected donor effects (Fig. 2a). We identified 599 transcripts differentially expressed between ILC3a and ILC1a: ILC3a abundantly expressed known ILC3 genes, such as *IL22*, *IL23R* and *IL1R1*, whereas known ILC1 genes, such as

IFNG, *GZMK* and *CCL4*, were more highly expressed by ILC1a (Fig. 2b). Examination of the most differentially expressed genes across the four subsets revealed a gradient of expression, which placed the ILC3b and ILC1b transcriptomes between those of ILC3a and ILC1a (Fig. 2c).

Within the ILC3 subsets, 93 transcripts were expressed at least twofold higher in ILC3a than in ILC3b (Fig. 2d); across all four subsets, expression of many of these transcripts was similar or lower in ILC1b and ILC1a than in ILC3b (Fig. 2e). Conversely, many of the 69 transcripts that were expressed more than twofold higher in ILC3b than in ILC3a were more abundantly expressed in ILC1b and ILC1a than in ILC3b (Fig. 2e). Within the ILC1 subsets, 105 transcripts were more highly expressed in ILC1a than ILC1b, and expression of many of these genes was even lower in ILC3b and ILC3a than in ILC1b (Fig. 2e). Moreover, most of the 76 transcripts that were more abundantly expressed in ILC1b than ILC1a were more highly expressed in ILC3b and ILC3a (Fig. 2e).

RNA-seq analysis of the same ILC3-ILC1 subsets indicated the increased expression of core ILC1a genes (*KLRD1*, *TIGIT*, *CD247*, *CD244*, *GZMK*, *GZMB*) in ILC3b as well as ILC3a genes (*RORC*, *KIT*, *ICOS*, *IL1R1*, *IL2*, *IL7R*) in ILC1b (see Supplementary Fig. 2), suggesting that ILC3b and ILC1b subsets expressed transcriptional signatures of both ILC1s and ILC3s. As RNA-seq detected more genes than microarray analysis, sets of genes were identified that were specifically enriched in either ILC1b (that is, *FCRL2*, *ABCG1*, *RABAC1*) or ILC3b (that is, *SCAP*, *ZBTB24*, *S100A8*), in addition to genes with graded expression within the ILC3-ILC1 spectrum (see Supplementary Fig. 2; data not shown).

Finally, intracellular staining was used to determine the expression of IL-22 and IFN- γ protein in sorted ILC3a, ILC3b, ILC1b and ILC1a after stimulation with phorbol 12-myristate 13-acetate (PMA) + ionomycin. Only ILC3a expressed IL-22 (Fig. 2f). The percentage of IFN- γ ⁺ cells gradually increased from ILC3b and ILC1b to ILC1a (Fig. 2f), consistent with the increased expression of an ILC1-like phenotype in these cells. Overall, these data suggested a spectrum of gene expression in which ILC3b and ILC1b had transcriptional programs that were intermediate between the program of ILC1a and that of ILC3a.

Expression of key proteins is graded across ILC3–ILC1 subsets

To understand how responsiveness to environmental stimuli and effector functions changed across the ILC3–ILC1 subsets, a search was made for cell surface receptors, soluble factors and lineage-defining transcription factors with expression modulated across these subsets. Transcripts that had decreasing expression from ILC3a to ILC1a encoded the cytokine receptors CD127 (*IL7R*), IL-1R (*IL1R1*) and CD117 (*KIT*) (Fig. 3a), the costimulatory molecule ICOS and the inhibitory receptors CD33, CD31 (*PECAM1*) and CD200R1 (Fig. 3a), the cytokines and chemokines IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF) (*CSF2*), B cell activating factor (*TNFSF13B*), leukemia inhibitory factor and chemokine ligand (CCL)20 (Fig. 3b), and the transcription factors ROR- γ t (*RORC*), Ahr and Tox2 (Fig. 3c). In contrast, transcripts that progressively increased expression from ILC3a to ILC1a encoded surface molecules indicative of TGF- β imprinting, such as TIGIT and CD9 (Fig. 3a); cytokines and chemokines, such as IFN- γ , CCL3, CCL4 and CCL5 (Fig. 3b);

granzymes and perforin (Fig. 3b); and the transcription factors T-bet (*TBX21*), Hobit (*ZNF683*) and Aiolos (*IKZF3*) (Fig. 3c).

Next, the transcriptional changes were validated by flow cytometry analysis of protein expression in tonsils from multiple donors (six to eight). This focused on cell surface and intracellular molecules for which validated antibodies were available. Among the proteins with progressively lower expression from ILC3a to ILC1a, the expression of CD127, CD117, ICOS, IL-1R, CD200R1, CD33 and CD31 (Fig. 3d,e) was validated. Among the proteins with incrementally increased expression from ILC3a to ILC1a, CD39, CD94, TIGIT, CD9, CD244 and SIRP- γ were consistent with the changes observed at the transcript level (Fig. 3f,g).

In addition, analysis of the data indicated that ILC3a selectively expressed the calcium binding protein S100A4 (Fig. 3a,d,e), which is secreted and can upregulate β 1-integrin expression on epithelial cells²³. ILC1b and ILC1a expressed the activating receptor NKG2C (encoded by *KLRC2*), as well as the activating adaptor CD247 (CD3 ζ) (Fig. 3a,f,g), the expression of which has been associated with memory or adaptive NK cells that are specific for human cytomegalovirus infection. It was concluded that several key markers of ILC3s were progressively downregulated from ILC3a to ILC3b to ILC1b to ILC1a; conversely, prototypical markers of ILC1s were gradually acquired in the same direction.

ILC3-ILC1 subsets are distinct from NK cells

To corroborate the variegated expression of ILC3 and ILC1 programs in the ILC subsets using an alternative approach, high-dimensional profiling of CD56⁺NKp44⁺ cells isolated from tonsils (six donors) was performed with an immunophenotyping panel of 36 markers, including CD56, NKp44, CD127, CD196 and CD161, which is expressed by all ILCs and NK cells¹². Cells were clustered by t-distributed stochastic neighbor embedding (t-SNE) using viSNE analysis²⁴. Six major clusters of cells were identified, with ILC3a and ILC1a distinguishable at the opposite sides of the CD127 t-SNE map (Fig. 4a). ILC3b and ILC1b subsets were identifiable within the CD103⁺ population based on expression gradients of CD127 and CD94 (Fig. 4a). Key cell surface markers (such as CD196 and CD117) decreased from ILC3a-ILC3b to ILC1b, whereas other markers (such as NKG2A) increased from ILC3a-ILC3b to ILC1b-ILC1a. (Fig. 4a,b). ILC3a contained both CD39⁺ and CD39⁻ cells (Fig. 4a). Although both subsets secreted IL-22 on IL-23 stimulation (data not shown), they may differ in their ability to degrade ATP, because CD39 is an ectonucleotidase. Two additional clusters of CD103⁻ cells were identified as 'others'. One was characterized by concomitant expression of CD127, CD117, CD94 and NKG2A and the second expressed CD94, NKG2A and CD16 (Fig. 4a). These features suggested that they, most probably, corresponded to blood CD56^{bright} and CD56^{dim} NK cells (Fig. 4a)^{25,26}. The ILC3b and ILC1b subsets were even more clearly demarcated when the analysis was restricted to CD103⁺ cells (Fig. 4c). Thus, mass spectrometry analysis confirmed the resolution of four ILC1-ILC3 subsets that were distinct from conventional NK cells.

ILC3 subsets convert into ILC1 subsets

To further resolve the heterogeneity of tonsil ILC3s and ILC1s, ILC3a, ILC3b, ILC1b and ILC1a cells were sorted from the same tonsil and processed them for scRNA-seq using the 10X Genomic platform. Two separate donors were processed in parallel and ~5,000–6,000 cells from each of the two donors were characterized. Unsupervised clustering by tSNE in donor 1 identified nine discrete clusters based on the most ten differentially expressed genes (Fig. 5a,b). Five ILC3 clusters expressed *RORC*, *IL23R*, *KIT*, *CD300LF* and *IL7R*. Among these, cluster 0 had a relatively large number of cells, whereas clusters 2, 3, 5 and 6 had fewer cells (Fig. 5a–c). Cells in cluster 5 had high expression of genes encoding cytokines, including *IL22*, *IL26* and *CSF2* (Fig. 5b,c), which suggest that it may comprise ILC3s that have been exposed to activating stimuli in vivo. The expression of actin cytoskeleton proteins in cluster 2 and L-selectin in cluster 6 (Fig. 5b) indicated that cells in these clusters may represent alternative activation states and/or reflect their location in different niches. A large ILC1a cluster (cluster 1) and a small ILC1a-related cluster (cluster 7) that expressed *IFNG*, *TBX21*, *KLRD1* and *CD160* (Fig. 5a–c) were identified. Cluster 7 was enriched in genes related to the cell cycle, including Ki67 (Fig. 5b,c), reflecting the presence of proliferating ILC1s. Importantly, cluster 4 expressed *CD300LF*, *IL7R* and *KLRD1* and represented a transitional population (Fig. 5b,c) that probably encompassed the ILC3b and ILC1b subsets. However, we did not distinguish two separate transitional populations (Fig. 5a–c), most likely because ILC3b and ILC1b share many overlapping transcripts and the sequencing depth of scRNA-seq (~1,500 transcripts/cell) was insufficient to separate cells with small, graded differences in transcript expression. Finally, cluster 8 was enriched in transcripts characteristic of B cells (immunoglobulins, major histocompatibility complex (MHC) class II and other transcripts related to antigen-presentation) (Fig. 5b) and, most likely, represented a minor contamination (~1.5%) with tonsillar B cells. Similar results were observed in cells isolated from donor 2 (see Supplementary Fig. 3).

Next, ILC trajectories and dynamics were analyzed using RNA velocity, a high-dimensional vector that predicts the future state of single cells based on intronic and exonic reads in the RNA-seq datasets²⁷. Projecting the RNA velocity field for cells from donor 1 on to its t-SNE plot evinced a clear directional flow, originating from cells in the transitional cluster 4 towards cluster 1 (ILC1s) (Fig. 5d,e). In addition, directional flow of some of the replicating *MKI67*⁺ cells in cluster 7 towards cluster 1 (Fig. 5d,e) indicated that these proliferating cells may be ILC1 precursors. Altogether, these data suggest that within the ILC3-ILC1 subsets, an intermediate population, cluster 4, was converting into ILC1-like cells, whereas additional cell clusters, such as cluster 5, 2, 6 and 7, expressed unique transcripts, perhaps reflecting proliferation, location into a specific tissue niche or exposure to activating stimuli.

ILC3-ILC1 subsets have a graded capacity to secrete IFN- γ

To explore the plasticity of ILC3-ILC1 subsets by functional analyses, ILC3a, ILC3b, ILC1b and ILC1a were cloned from two donors by limiting dilution in the presence of irradiated allogeneic feeder cells, IL-2 and IL-23. Growing clones were stimulated with PMA + ionomycin on day 15 of culture and the production of IL-22 and IFN- γ was assessed in cells derived from over 200 clones from the 2 individual donors. Cloning efficiencies were similar for ILC3a, ILC1b and ILC1a, whereas the efficiency for ILC3b was generally lower (data

not shown). Most ILC3a-derived clones contained IL-22⁺ cells, IFN- γ ⁺ cells and a few cells producing both cytokines (Fig. 6a and see Supplementary Fig. 4). Rarely (3 out of 64 clones, 4.6%), ILC3a-derived clones exclusively produced IFN- γ (Fig. 6a; data not shown). A similar trend was observed in cells derived from ILC3b, although clones that only generated IFN- γ ⁺ cells were slightly more frequent (5 of 36, 13.8%) (Fig. 6a; data not shown). Most ILC1b-derived clones produced IFN- γ and only a few contained IL-22⁺ cells (4 out of 57, 7%) (Fig. 6a; data not shown). ILC1a-derived clones exclusively produced IFN- γ (Fig. 6a). The percentage of IL-22⁺ cells progressively decreased from ILC3a-derived clones to ILC1a-derived clones, whereas the percentage of IFN- γ ⁺ cells followed the opposite pattern (Fig. 6b and see Supplementary Fig. 4). Overall, these data indicate that, although a certain percentage of cells in ILC3a-derived clones acquired the ability to produce IFN- γ , the number IFN- γ ⁺ cells progressively increased from ILC3a to ILC3b to ILC1b, and that ILC1a were fully committed to IFN- γ production.

The microenvironment impacts ILC3a plasticity

Next, the ability of ILC3a to generate IFN- γ ⁺ cells after transfer in vivo was tested. Human ILC3a were sorted at high purity from tonsils, cultured for 10 days in IL-7 and stem cell factor (SCF) to ensure selective expansion of IL-7R⁺CD117⁺ cells, further amplified in vitro for 10–15 days to attain adequate numbers of cells and transferred into MISTRG-6–15 mice^{28,29}, which express human M-CSF, IL-3/GM-CSF, signal regulatory protein (SIRP) α , thrombopoietin (TPO), IL-6 and IL-15 on a Rag2^{-/-} Il2rg^{-/-} background (see Supplementary Fig. 5). Before transfer, ILC3a were Ror γ ^{hi}Aiolos^{low/-}T-bet^{low/-} by intracellular staining and produced mainly IL-22 when stimulated with PMA+Ionomycin (see Supplementary Fig. 6a,b). At day 19 and day 39 post-transfer, ILCs, identified by the expression of human CD45 and MHCI, were detected in the spleen and liver, but not in the epithelium or lamina propria of the small intestine (see Supplementary Fig. 5 and 6c), indicating that MISTRG-6–15 lacked host factors to support the migration of human ILC3s into these mucosal sites. The percentage of human ILCs recovered from the liver was four- to fivefold higher than in the spleen at any time point analyzed (see Supplementary Fig. 5c and 6c). Based on intracellular staining for transcription factors, adoptively transferred ILCs had higher expression of Aiolos and T-bet in spleen than ILCs in the liver both at day 19 and day 39 (see Supplementary Fig. 6d–f). Spleen ILCs at day 39 expressed significantly more Aiolos and T-bet than ILCs at day 19 post-transfer (see Supplementary Fig. 6d–f). Transferred ILCs produced significantly more IFN- γ and less IL-22 in the spleen than in the liver, and this difference was more pronounced at day 39 than at day 19 (see Supplementary Fig. 6g–i). Altogether, these results indicate that human ILC3a transferred into humanized MISTRG-6–15 mice were more likely to acquire ILC1-like features in the spleen than in the liver, and that this process was time dependent, suggesting that plasticity reflected a sustained influence of tissue microenvironment.

Aiolos and T-bet extinguish the ILC3 program

Next, it was investigated whether plasticity within the ILC3-ILC1 subsets was governed by transcription factors upregulated early during the ILC3 to the ILC1-like cell transition. Transcripts for *IKZF3*, encoding Aiolos, a transcription factor of the Ikaros family³⁰, were absent in tonsil ILC3a and detected in ILC3b, and had the highest expression in ILC1b and

ILC1a (Fig. 3c and see Supplementary Fig. 2b). Known targets of Aiolos, such as *IRF8* and *MYC*^{31,32}, had the same expression pattern whereas *TBX21*, encoding T-bet, was expressed in ILC1b and increased in ILC1a (Fig. 3c). Intracellular staining for transcription factors confirmed that Aiolos was expressed in ILC3b, whereas T-bet was detectable in ILC1b (Fig. 7a,b). To test whether Aiolos and T-bet were involved in the ILC3 to ILC1-like transition, use was made of the ILC3-like mouse cell line MNK3 (ref.³³). Aiolos (MNK3-Aiolos), T-bet (MNK3-T-bet) or both (MNK3-Aiolos+T-bet) were lentivirally or retrovirally expressed in a selected MNK3 clone (MNK3-control) (see Supplementary Fig. 7a,b). MNK3-T-bet made slightly less IL-22 than MNK3-control in response to IL-23+IL-1 β -stimulation by intracellular staining and ELISA (Fig. 7c,d). However, production of IL-22 was even more reduced in MNK3-Aiolos+T-bet. MNK3-Aiolos cells did not produce more IFN- γ (data not shown) and did not secrete less IL-22 compared to MNK3-control (see Supplementary Fig. 7c,d). MNK3-T-bet produced more IFN- γ than MNK3-control (Fig. 7e). However, IFN- γ production occurred with a delayed kinetic (48 hrs post-stimulation) and required stimulation with IL-18 and IL-12 in addition to IL-23 (Fig. 7e; data not shown). Thus, Aiolos and T-bet cooperated to extinguish the ILC3 program, while T-bet alone induced production of IFN- γ .

To investigate whether expression of Aiolos directly repressed ILC3 genes, Cut and Run chromatin profiling³⁴ was performed for Aiolos and histone 3 lysine 27 acetylation (H3K27ac), a histone mark associated with transcriptional activity, in MNK3-Aiolos+T-bet, MNK3-T-bet and MNK3-control. Aiolos bound active regulatory elements (H3K27ac⁺ peaks) near the ILC3 genes *Il22* (Fig. 7f) and *Rorc* (see Supplementary Fig. 8a) in MNK3-Aiolos+T-bet. In addition, there was a limited but significant reduction of expression of *Roryt* by intracellular staining in MNK3-Aiolos+T-bet cells compared with MNK3-T-bet or MNK3-control (see Supplementary Fig. 8b,c). Aiolos binding in MNK3-Aiolos+T-bet was not detected at type 1 loci, such as *Ifng* and *Eomes*, in or near housekeeping genes, such as *b2m* and *Suv39h2*, or in MNK3-control (see Supplementary Fig. 8 d,e), suggesting that Aiolos selectively regulated ILC3-specific genes.

As IKZF (Ikaros family zinc finger) members suppress gene expression by changing the chromatin landscape via deacetylation of local histones³⁵, we leveraged our H3K27ac data to directly measure Aiolos-induced deacetylation in MNK3-Aiolos+T-bet, MNK3-T-bet and MNK3-control. Quantification of H3K27ac in MNK3-T-bet and MNK3-Aiolos+T-bet indicated that the relative H3K27ac was reduced exclusively at positions showing Aiolos peaks in MNK3-Aiolos+T-bet (Fig. 7f and see Supplementary Fig. 8f). To test whether Aiolos repressed human chromatin regions that transitioned from 'on' in ILC3s to 'off' in ILC1s, we identified direct Aiolos binding sites by running de novo motif prediction on Aiolos peaks detected in MNK3-Aiolos+T-bet. Similar to Ikaros (encoded by *Ikzf1*), Aiolos bound a TGTGGT motif (see Supplementary Fig. 8g). To analyze evolutionary conservation, we identified IKZF-binding motifs in the *IL22* and *RORC* loci. We compared ATAC-seq peaks (indicative of chromatin accessibility) from human tonsil ILC3s and intraepithelial ILC1s²² with mouse H3K27ac peaks from MNK3 cells. Consistent with direct binding, Aiolos peaks and repression were predominantly localized at the conserved motifs (Fig. 7f and see Supplementary Fig. 8a). Thus, Aiolos directly suppressed chromatin activity at

mouse-human-conserved distal regulatory elements of genes encoding ILC3 cytokines and transcription factors.

Next, it was assessed which tissue factors were most likely to control the expression of Aiolos and T-bet. The expression of Aiolos, T-bet and Ror γ t was quantified by intracellular staining in tonsil ILC3a expanded with IL-7 and SCF for 15–20 days, and subsequently stimulated with various cytokines for additional 5–10 days. ILC3a cultured with TGF- β and IL-23 expressed more Aiolos and T-bet and less Ror γ t compared to cells exposed to IL-7 and SCF only (Fig. 7g). TGF- β exposure alone was sufficient to partially induce Aiolos and downregulate Ror γ t (Fig. 7g). These data collectively suggest that TGF- β and IL-23 could represent the tissue factors that would coordinately regulate the expression of Aiolos, T-bet and Ror γ t driving the transition of ILC3s to ILC1-like cells.

ILC3–ILC1 subsets are present in the gut lamina propria

To test whether the ILC3–ILC1 intermediate subsets could be detected in other mucosal tissues, we sorted CD3⁺CD19⁺ cells from the lamina propria of ileal specimens derived from two patients with Crohn's disease (CD) and two control donors, and analyzed the cells by scRNA-seq using the 10X Genomic platform. We computationally merged the samples from all four donors and harmonized them to remove batch effects. We also computationally removed myeloid cells, mast cells and plasma cells, or other B cell-related clusters, based on transcripts known to be expressed by these cell types (that is, *LILRB4*, *TPSB2*, *CD19*, *SDC1* and *IGKC*). On reclustering, the tSNE plot showed ten discrete subsets of putative ILCs (Fig. 8a), which were detected in both control donors and CD patients (Fig. 8b). A heat map based on the most differentially expressed genes indicated a major ILC3 cluster (cluster 0), as well as smaller ILC3-related clusters (cluster 3, 5, 6 and 8), which selectively expressed MHC class II (*HLA-DPA1*), activation markers (*CD69*) or migration molecules (*SELL*) indicative of a specific state of activation or location (Fig. 8c). The analysis detected two major clusters of ILC1s (cluster 2 and 1) and a smaller cluster of ILC1s (cluster 9) that expressed very high amounts of *PRF1* and *FCGR3A* (Fig. 8c), and may therefore be closer to NK cells than to ILCs. Between the ILC3 and ILC1 clusters a group of cells, cluster 4, could be distinguished, which co-expressed markers of ILC3s and ILC1s, such as *IL7R*, *KIT*, *IL23R*, *KLRD1*, *NKG7*, *KLRC1*, *CCL5*, *XCL1* and *XCL2* (Fig. 8d). A small cluster of actively proliferating cells that mainly expressed ILC1 markers, such as *KLRD1* and *IFNG*, (cluster 7) was more abundant in CD patients than control samples (99 vs 26 cells) (Fig. 8e). These data indicate that an intermediate subset, co-expressing markers of ILC3s and ILC1s, could be detected in the lamina propria of human ileum.

Discussion

In the present study it was shown that ILC3s and intraepithelial ILC1s in the human tonsils represent the ends of a spectrum that included additional ILC3- and ILC1-related subsets. The presence of these discrete subsets reflected, at least in part, functional plasticity. Unbiased RNA velocity analysis of scRNA-seq data identified one ILC3–ILC1 intermediate cluster, which progressed towards an ILC1-like phenotype. Transfer of ILC3a into humanized mice indicated that ILC3 converted to ILC1-like cells more readily in the spleen

than in the liver, suggesting a role for the tissue microenvironment in this process. A discrete ILC3-ILC1 intermediate subset was also detected in the human intestinal mucosa. Although the detection of variegated ILC3-ILC1 subsets was indicative of plasticity, it cannot be excluded that certain ILC subsets may directly derive from the differentiation of rare progenitor cells undetected in this study. Whether conversion within the ILC3-ILC1 subsets is uni- or bi-directional also requires further investigation.

Previous studies have shown that the conversion of ILC3s into ILC1s requires a decrease in the expression of *Ror γ t*¹⁴, along with a coordinate increase in T-bet^{14–17} and Notch signaling^{18–20}. Hobit may also concur in the ILC3-to-ILC1 conversion, given its requirement for ILC1 development³⁶. The results pinpointed a role for Aiolos in this process. First, expression of Aiolos and known Aiolos targets was detected in ILC3b whereas the expression of T-bet became apparent in ILC1b. In gain-of-function experiments, concomitant expression of Aiolos and T-bet was required in the MNK3 cell line, a surrogate for ILC3s, to suppress the ILC3 signature genes *IL22* and *RORC*. Cut- and-run experiments in these cells showed that Aiolos bound active regulatory elements near the *IL22* and *RORC* genes, whereas H3K27ac levels, indicative of active chromatin, were reduced at the same positions. Finally, ATAC-seq experiments indicated that Aiolos-driven repression marked evolutionarily conserved regulatory elements that transitioned from accessible in human ILC3s to inaccessible in human ILC1s. Thus, Aiolos suppressed chromatin activity at regulatory elements of conserved signature ILC3 genes. It is unclear why the repressive function of Aiolos requires T-bet. We postulate that T-bet may be important for inducing chromatin modifications that facilitate Aiolos recruitment and/or its repressor activity. This interaction may occur at certain loci that are enriched in DNA-binding motifs for T-bet and Aiolos. Aiolos promotes NK cell maturation in mice, thus corroborating a role for this transcription factor in the acquisition of a type 1 functional program in a different context³⁷. In agreement with our data, a role for Aiolos in human ILC3 to ILC1-like plasticity was very recently reported³⁸.

The results of the present study provide insights into the impact of tissue microenvironment in controlling ILC heterogeneity and diversity. Adoptive transfer of ILC3a in humanized mice showed that the ILC3-to-ILC1 transition was tissue-dependent, because it occurred more effectively in the spleen than in the liver. TGF- β could be one of the tissue factors that could control transition. ILC1s express TGF- β -imprinted molecules, such as Aiolos, Hobit, CD103, CD9, CD39 and TIGIT. Thus, ILC3 transition to ILC1-like cells may reflect the adaptation of ILC3s to tissue niches that are enriched in TGF- β , such as the tonsil epithelial cell layer. The inflammatory cytokine IL-23 also contributes to the transition of human ILC3s to ILC1s in vitro¹¹ as well as mouse ILC3s to ILC1s in vivo¹⁴, although the mechanism remains unclear. It was found that IL-23 enhanced the capacity of TGF- β to induce both Aiolos and T-bet, which may depend on the IL-23-mediated activation of STAT3 and STAT4 (refs.^{38,39}); both have been shown to bind the promoters of Aiolos and T-bet, respectively.

The integration of flow cytometry and mass cytometry provided useful criteria to distinguish ILC1s, ILC3s and intermediate ILC subsets from conventional NK cells, which has been problematic^{41–44}. Although ILC3s share several markers with CD56^{bright} NK cells,

including CD127 and CD117^{26,43}, we found that ILC3s lacked CD94 and NKG2A, while uniquely expressing CD196, CD300LF and IL-22. Intraepithelial ILC1s and NK cells also have many markers in common and both produce IFN- γ ⁴⁴. However, ILC1s and ex-ILC3s uniquely expressed TGF- β -induced markers of tissue residency, like CD103. Omics analyses of sorted ILC populations have also revealed novel facets of ILC biology. In addition to ILC3 subsets producing IL-22, IL-26 and GM-CSF, ILC3s produced IL-2, which may impact other immune cells, such as NK cells and T_{reg} cells⁴⁵. ILC3s also produced S100A4, which may modify the barrier function of mucosal surfaces by increasing the expression of b1-integrin²³. Similar to ILC3s, ILC1s were also functionally heterogeneous and encompassed subsets that produced CCL3, CCL4, XCL1 and XCL2 chemokines. Thus, in addition to IFN- γ production, ILC1s may be important for attracting other immune cells into the tissue during a pathogenic insult, as it has been recently proposed for tissue resident T cells⁴⁶. Beyond the tonsil, scRNA-seq of human intestinal samples from CD patients and controls revealed the presence of an intermediate ILC3-ILC1 cell population in the lamina propria of the ileum. It will be important to further characterize whether microbiome, inflammatory conditions that occur during CD or other conditions impact ILC3-ILC1 diversity and plasticity. In conclusion, the present study shows that ILC3 conversion to an ILC1-like phenotype occurs in vivo in humans and reveals a role for Aiolos in this process; it also emphasizes the relevance of the tissue niche in creating a microenvironment that promotes ILC diversity and functional adaptation to local stimuli.

Methods

Preparation of CD56⁺ cells from tonsils.

Children's tonsils were processed as previously reported¹¹. Briefly, tissue was mechanically disrupted, passed through 0.0059 inches opening sieve, and filtered extensively through 70 μ m cell strainers. CD56⁺ cells were enriched via positive selection with CD56 microbeads (Myltenyi), according to manufacturer's recommendations. Cells were then stained for sorting or for flow cytometry analysis. Sorting was performed on BD FACS Aria II and flow cytometry analysis was performed on a BD LSR Fortessa. Data were analyzed by FlowJo Software (TreeStar).

Preparation of single cell suspension from lamina propria (LP) of terminal ileum surgical explants.—Mucosal tissue was separated from the muscular layer and serosa and cut in small pieces. Intraepithelial lymphocyte cells were extracted by rotating the tissue at room temperature for 40 min in Hank's balanced salt solution, 10% FCS and 5mM ethylenediaminetetraacetic acid. Cells were filtered through 100- μ m cell strainers and dithiothreitol was added at a final concentration of 5 mM. After intraepithelial lymphocyte removal, LP cells were extracted by digesting tissue in complete RPMI medium containing 1 mg ml⁻¹ Collagenase IV (Sigma, C-5138) at 37 C for 1 h under agitation. Cells were filtered and subjected to density gradient centrifugation using 40% and 70% Percoll solutions. CD3⁺CD19⁻ cells were sorted and processed for scRNA-seq. Control patients for the present study were patients undergoing abdominal surgery for colon cancer or polyposis, which had non-involved terminal ileum removed, as part of the surgical procedure.

All human studies were conducted under the approval of Institutional Review Boards of Washington University. Ileum samples were provided as surgical waste with no identifiers attached on informed consent to the Digestive Disease Research Cores Center at Washington University.

Antibodies.—The following anti-human/mouse antibodies were used for staining:

BD Pharmingen: anti-NKp44-PE (558563), NKp44-AlexaFluor647 (558564), CD56-PE/Cy7 (557747), CD103-FITC (550259), CD103-PE (550260), CD33-PE (347787), CD16-FITC (555406), CD117-PE (555714), MIP1-b-PE (561120), GM-CSF-PE (18595A), IFN- γ -FITC (554700); T-bet-BV421 (563318); **Biolegend:** anti-CD196-BV421 (353408), CD103-BV605 (350218), CD45-AlexaFluor700 (368514), CD127-BV421 (351309), CD172g-PE (336606); CD117-BV510 (313219), Aiolos-PE (371104), CD161-BV510 (339922), CD161-BV421 (339916), CD62L-BV605 (304833), TIGIT-BV605 (372711), CD127-BV605 (351334), NKp44-PE/Cy7 (325116), CD19-PerCP-Cy5.5 (302230), ILT3-PerCP-Cy5.5 (333014), CD94-FITC (305504), CD56-APC/Cy7 (318331), CD117-FITC (313231), CD39-FITC (328205), TIGIT-PE (372703), S100a4-PE (370003) IL-2-PE (500307), GM-SCF-PacificBlue (502313), anti-mouse-IFN- γ -APC (505810); **eBioscience:** anti-CD300LF-eFluor660 (50-3008-42), ICOS-PE (12-9949-81), CD247-PE (12-2479-82), CD3-PerCP-Cy5.5 (8045-0036-120), CD19-PerCP-Cy5.5 (45-0198-42), CD161-PE (12-1619-42), CD161-AlexaFluor488 (53-1619-42), CD127-FITC (11-1278-42), CD200R-PE (12-9201-41), CD56-PE (12-0567-42), IFN- γ -APC (17-7319-82), T-bet-PE (12-5825-82); anti-mouse IL-17A eFluor450 (48-7179-42), mouse IL-22-PE (516404); **Immunotech:** CD9-FITC (1755), CD94-PE (2276), CD31-FITC (1431), CD244-PE (1608); **R&D:** anti NKG2C-PE (FAB138P), IL-1R1-PE (FAB269P), IL-22-PE (IC7821P). Staining for transcription factors was performed using the Foxp3 transcription factor staining kit, as recommended (eBioscience, 00-5523-00).

Statistical analysis.—Statistical analysis was performed with the Prism v.7 software, as indicated in the figure legends.

RNA extraction, microarray and bulk RNA-seq analysis.—RNAs from the sorted ILC subsets were prepared by using RNeasy Plus Micro kit (Qiagen), following the manufacturer's instructions. RNAs were then amplified and hybridized to the Affymetrix Human Gene (v.1.0) ST arrays. Data were analyzed with GenePattern software (Broad Institute) as previously described⁴⁷. Due to the small size of the ILC3b and ILC1b subsets and their variability among donors, ILC subsets were pooled from two donors to obtain enough RNA for microarray analysis. Alternatively, for bulk RNA-seq analysis, RNA was prepared using SMARTer Universal Low Input RNA Kit (Clontech). Sequences were aligned and gene count tables were produced using STAR aligner. Differential expression was analyzed using DESeq2 R Package. Genes with a sum of fewer than ten counts among all samples were excluded from analysis. Gene expression was modeled as a sum of the effects of the cell type and batch, that is data from the experiment. Differentially expressed genes were estimated using the Wald's test and *P* values were automatically adjusted by DESeq2 using independent filtering for a false discovery rate of 0.05.

Culture of cells *in vitro*.—ILC3a were expanded in human IL-7 (10ng ml⁻¹, Peprotech 200–07) and SCF (10ng ml⁻¹, Peprotech 300–07). They were expanded for 15–20 days prior to exposure to IL-23 (10ng ml⁻¹, R&D 1290-IL) and/or TGF-β (1ng ml⁻¹, Peprotech 100–21) for additional 5–10 d. For short term expansion ILC subsets were cultured in IL-7, SCF and IL-2 (10ng ml⁻¹, Peprotech 200–02) to promote maximal proliferation in a 3-d culture timeframe; they were then stimulated with PMA + ionomycin (Cell activation cocktail, Biolegend, 423302) for 2 h and further incubated for 4 h with GolgiPlug (BD, Cytofix/Cytoperm Plus, 555028), before intracellular staining for cytokines.

Cloning of ILC populations.—ILC populations were cloned by limiting dilutions in the presence of irradiated feeder cells (3,000R), IL-2 (1000U ml⁻¹) and IL-23 (1ng ml⁻¹). Growing colonies were stimulated with PMA + ionomycin in the presence of GolgiPlug for 6 h before intracellular staining for cytokines.

In vivo transfer of ILC3a into humanized mice.—MISTRG-6 and IL-15 humanized mice were generated by Regeneron Pharmaceutical as described^{28,29} and intercrossed at Washington University to obtain MISTRG-6–15. FACS-sorted ILC3a were amplified in IL-7 and SCF as above, and then further expanded in the presence of IL-2 to increase their numbers. Cells (3–6X10⁶) were injected into MISTRG-6–15 mice retro-orbitally. MISTRG-6–15 mice were stimulated 24 h before adoptive transfer with 50μg of poly(I:C) (polyinosinic:polycytidylic acid; Amersham, 27–4732-01). Cells were retrieved from the spleens and livers on collagenase D digestion. Lymphocytes from the liver were enriched by density gradient centrifugation using 40% and 70% Percoll solutions. Intraepithelial lymphocytes and LPL were prepared as previously described⁴⁸. All animal experiments were approved by the Animal Studies Committee of Washington University in St. Louis.

Cytof analysis.—In some experiments cells were enriched with CD56 microbeads and directly stained for Cytof. In other experiments cells were FACS sorted before staining for Cytof, to remove contaminating B and T cells. The Cytof panel included: NKp44–167Er, which was conjugated in-house (eBioscience, 16–3369-85). All the following antibodies were purchased from Fluidigm and included in the panel CD45–089Y (3089003B); CD196–141Pr (3141003A); CD19–142Nd (3142001B); CD117–143Nd (3143001B); CD4–145Nd (3145001B); CD8a–146Nd (3146001B); CD25–149Sm (3149010B); FcεR–150Nd (3150027B); CD138–150Nd (3150012B); CD103–151Eu (3151011B); TCRgd–152Sm (3152008B); TIGIT–153Eu (3153019B); CD3–154Sm (3154003B); CD85j–156Gd (3156020B); CD194–158Gd (3158032A); CD161–159Tb (3159004B); CD39–160Gd (3160004B); CD27–162Dy (3162009B); CD45RO–165Ho (3165011B); CD34–166Er (3166012B); CD127–168Er (3168017B); CD159–169Tm (3169013B); CD45RA–170Er (3170010B); CD226–171Yb (3171013B); CD354(TREM1)–172Yb (3172022B); CD94–174Yb (3174015B); CD14–175Lu (3175015B); CD56–176Yb (3176009B) and CD16–299Bi (3209002B). The following antibodies were originally included in the panel: CD303–147Sm (3147009B); CD1b–155Gd (3155007B); DR3–161Dy (3161003B); CD294–163Dy (3163003B); CD141–173Yb (3173002B), but they were not found to produce reliable signal and therefore substituted later with: CD7–147Sm (3147006B); PD1–155Gd (3155009B); CD26–161Dy (3161015B); CD57–163Dy

(3163022B); and CD158b-173Yb (3173010B). In addition, ICOS-148Nd (3148019B) was added to the panel. Cells were washed with Cy-FACS buffer (CyPBS, Rockland, MB-008; 0.1% BSA, Sigma, A3059; 0.02% Sodium Azide, Sigma, 71289, 2mM ethylenediaminetetraacetic acid, Hoefer, GR123–100) stained on ice for 1 h. After two washes cells were stained with cisplatin (Enzo Life Sciences, NC0503617) for 1 min, washed again twice, fixed in 4% PFA (Electron Microscopy Sciences, 15710) for 15 min, spun down and re-suspended in Intercalator-Ir125 (Fluidigm, 201192A) overnight. Cells were washed and counted and analyzed on a Cytos 2 mass cytometer (Fluidigm). Data were processed using Cytobank and Flowjo v.10.0.7 (ref.²⁴).

MNK3 and viral transduction.—The MNK3 cell line has previously been described³³. Cells were cloned by limiting dilution twice and a clone, which reproducibly responded to IL-23 and IL-1b stimulation, was selected. The lentiviral and retroviral vectors PLVX-IKZF3-IRES-mCherry and PMX-Tbx21-IRES-GFP were used for the ectopic expression of the human Aiolos and the murine T-bet. These vectors were constructed by sub-cloning the IKZF3 and Tbx21 open reading frames in the multiple cloning site of the commercially available PLVX-EF1alpha-IRES-mCherry (Clontech no. 631987) and PMX-IRES-GFP (Cell Biolabs no. RTV-013) plasmids, respectively. Both vectors have an internal ribosome entry site (IRES) sequence that allows the expression of the gene of interest and the reporter gene in a bicistronic messenger RNA (mRNA). For the lentiviral particle production the accessory psPAX2 (Addgene no. 11260) and pCMV-VSV-G (Addgene no. 8454) plasmids were cotransfected into the packaging cell line along with the PLVX vectors. For lentiviral production, the 293T packaging cell line (ATCC, CRL-3216) was transiently transfected with the lenti-vectors and accessory plasmids using Lipofectamine 2000 (Invitrogen, 11668–019) according to manufacturer's protocol. For retroviral production, the Phoenix amphotropic packaging cell line (kindly provided by the Nolan's laboratory, Stanford) was used. The virus-containing cell supernatants were collected 48 h after transfection and used for spin infection of MNK-3 cells with 8 $\mu\text{g ml}^{-1}$ of Polybrene (Sigma H-9268) at 900 rcf for 1 h 40 min at room temperature; 72 h after the spin infection the transduced cells were sorted, based on the expression of green fluorescent protein (GFP) and/or mCherry reporters. MNK3 cells were stimulated with murine IL-23 (10ng ml^{-1} , R&D 1887-ML), IL-1b (10ng ml^{-1} , Peprotech 211–11b) IL-12 (10ng ml^{-1} , R&D 419ML) and IL-18 (10ng ml^{-1} , MBL B-002–5). Cytokines in supernatants were measured by BD Cytometric Bead Array (Mouse Th1/Th2/Th17 kit, 560485) or IL-22 Elisa (Biolegend mouse IL-22 Deluxe set, 436304). Transduction was repeated twice to ensure reproducibility.

The scRNA-seq and data analysis.—ILC3a, ILC3b, ILC1b and ILC1a cells were sorted from enriched tonsil CD56⁺ based on NKp44, CCR6, CD300LF and CD103 expression. Sorted cells were sequenced using 10X Genomics platform. Cell Ranger pipeline (<https://support.10xgenomics.com/single-cell-gene-expression/software/overview/welcome>) was used to process chromium single-cell RNA-seq output to align reads and generate gene-cell expression matrices. Briefly, short sequencing reads were aligned to the GRCh38 reference genome and Ensembl⁴⁹ transcriptome by STAR⁵⁰. The uniquely aligned reads were used to quantify gene expression levels for all Ensembl genes. We filtered out

low-quality cells from the dataset if the number of genes detected was less than 500 or more than 3,000. We also excluded those cells with high percentage of mitochondrion reads (>15%). Mitochondrion and ribosomal genes usually consumed a large fraction of reads in our dataset, and their relative abundance showed large variations from sample to sample. Such genes were not interesting in the present research, and thus were excluded for downstream data analysis. In addition, all genes that were not detected in at least 1% of all our single cells were discarded.

The scRNA-seq downstream analysis: downstream analyses were performed using the R software package Seurat (<http://satijalab.org/seurat/>). Raw reads in each cell were first scaled by library size and then log-transformed. To improve downstream dimensionality reduction and clustering, first any unwanted source of variation arising from the number of detected molecules was regressed out. Then highly variable genes were identified and selected for principal component analysis reduction of high-dimensional data. Based on elbow plot, the top 25 principal components were selected for unsupervised clustering of cells. The resolution in the FindClusters function in Seurat was set to 0.6 and the clustering results were shown in a t-SNE plot. Accordingly, the marker genes in each cluster were identified using Student's t-test implemented in the Seurat v.2.3 package. RNA velocity analysis was performed as described²⁹ using the following pipelines: RNA velocity (<https://github.com/velocyto-team/velocyto.R>); and RNA velocity of single cells (<https://www.nature.com/articles/s41586-018-0414-6>); quantification of spliced and un-spliced reads (<https://github.com/hms-dbmi/dropEst/>).

dropEst: pipeline for accurate estimation of molecular counts in droplet-based single-cell RNA-seq experiments (<https://genomebiology.biomedcentral.com/articles/10.1186/s13059-018-1449-6>). Harmonization of gut samples from merged donors was performed as described (<https://doi.org/10.1101/461954>).

Cut and run.—Cut and run was performed as described³⁴ using 2 µg of antibody, 2X10⁶ cells per cut and run. Briefly, MNK3 cells were permeabilized with 0.02% of DMSO-dissolved digitonin, bound to concanavalin A-beads, incubated with antibody, protein-A Mnase, and chromatin was digested with ice-cold ethylenediaminetetraacetic acid for 25 min. Antibodies were as follows: H3K27ac (Abcam ab4729), IKZF3 (Biolegend 371002) and rabbit-anti-mouse immunoglobulin G (Invitrogen 31188). Antibody or protein-A Mnase incubation, as well as washes, were done at room temperature for 5 min without rotation. Libraries, generated from soluble DNA, were sequenced on a Hi-seq 3000 using 1×50 reads and aligned using novaAlign. Peak calling, de-novo motif calling and creation of IKZF3 motif tracks were performed using HOMER. Visualization and relative H3K27ac subtraction were done using the UCSC genome browser. Genome-wide pileup and reads per kilobase million (RPKM) normalization were done using deepTools.

Data Availability.

Microarray data, bulk RNA sequencing and chromatin profiling data have been deposited in the GEO repository under accession code GSE130775. All the other data will be available upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Identification of ILC3a, ILC3b, ILC1b and ILC1a by flow cytometry.

a (left), Gating strategy to identify ILC3s, intraepithelial ILC1s and putative transitional population based on NKp44 and CD103 expression. A gate was applied on CD56⁺CD3⁻CD19⁻ cells. Top right, expression of CD300LF and CD196 on NKp44⁺CD103⁻ cells; P1=ILC3a. Bottom right, expression of CD300LF and CD196 on NKp44⁺CD103⁺ cells. P2=ILC3b, P3=ILC1b, P4=ILC1a. One donor representative of 25 tested is presented. **b**, Percentages of ILC3a, ILC3b, ILC1b and ILC1a populations in children's inflamed tonsils of different donors ($n=25$). Data are mean $\pm$ s.d.

Fig. 2. Transcriptome analysis places ILC3b and ILC1b between ILC3a and ILC1a.

a, PCA of the ILC subsets using the top 10% most variable genes. Numbers along the axes indicate relative scaling of the principle variables. **b**, Volcano plot identifying genes significantly ($P < 0.05$, Student's t -test) expressed more than twofold in ILC3a versus ILC1a (red) or ILC1a versus ILC3a (blue) (322 and 277 genes, respectively). **c**, Heat maps representing the relative abundance of genes identified in **b** across the different ILC subsets. **d**, Transcripts upregulated in single comparisons between ILC1a/ILC1b and ILC3a/ILC3b or in two comparisons combined together (colors in plot match key). Caption indicates the numbers of transcripts upregulated at least twofold in each comparison. **e**, Heat maps representing the relative abundance across the ILC subsets of transcripts differentially expressed in ILC3a>ILC3b (first panel on the left), ILC3b<ILC3a (second panel on the left), ILC1a>ILC1b (third panel on the left) or ILC1b<ILC1a (right panel). **f**, Intracellular content

of IL-22 and IFN- γ in ILC3a, ILC3b, ILC1b and ILC1a after short-term in vitro expansion. APC, allophycocyanin; PE, phycoerythrin. One experiment representing two is shown. Data in **a**, **b**, **c**, **d**, and **e** are derived from independent biological replicates (ILC3a, $n=4$; ILC3b, $n=3$; ILC1b, $n=4$; ILC1a, $n=4$).

Fig. 3. Molecules with progressively decreased or increased expression in the ILC3-ILC1 spectrum.
a-c, Heat maps representing the mRNA expression of selected genes across the ILC subsets. Genes were grouped in three categories: cell surface receptors (**a**); soluble factors and molecules relevant to cytotoxicity (**b**); transcription factors (**c**). Each column represents an individual biological replicate. **d,e**, Flow cytometry validation of cell surface receptors and intracellular molecules (S100a4) gradually decreasing from ILC3a to ILC3b to ILC1b to ILC1a. **d**, Histograms for one representative donor; **e**, Mean fluorescent intensity (MFI) in different donors ($n=8$). **f,g**, Flow cytometry validation of cell surface receptors and intracellular molecules (CD247) gradually increasing from ILC3a to ILC3b to ILC1b to ILC1a. **f**, Histograms for one representative donor. **g**, MFI or percentages of positive cells in different donors ($n=6-8$). Significance was calculated using an ordinary, one-way analysis of variance (ANOVA), multiple comparison test with Prism v.7 software. * P 0.05, ** P 0.001, *** P 0.005, **** P 0.0001. **e, g**, Data are mean $\pm$ s.d.

Fig. 4. Mass spectrometry analysis separates the ILC3-ILC1 spectrum and NK cells.

a, The viSNE analysis of tonsil NKp44⁺ cells based on 36 selected markers and colored by intensity of expression. Each plot shows expression of the indicated cell surface marker. Populations corresponding to ILC3a, ILC3b, ILC1b and ILC1a are delineated in the CD127 plot. Cell subsets that lack CD103, but express CD94, NKG2A and CD127 are indicated as “Others”. **b**, Heat maps show graded expression of the indicated markers in the ILC subsets. Two donors analyzed in parallel on the same day are shown for consistency. **c**, The viSNE map of NKp44⁺CD103⁺ cells shows 3 clusters in which the degree of expression of CD127 and CCR6 inversely correlates with that of CD94. The viSNE analysis also identifies a small ILC1 cluster expressing CD16. Data are representative of six donors. Cells were gated based on NKp44 and CD161 (**a,b**) or NKp44, CD161 and CD103 (**c**) before running viSNE. Contaminating CD3⁺CD19⁺FcεR⁺TREM1⁺CD14⁺ cells were excluded by manual gating.

Fig. 5. The scRNA-seq of the ILC3-ILC1 spectrum identifies an intermediate cluster transitioning to ILC1s.

a, Unsupervised t-SNE analysis of ILC3-ILC1 subsets to define clusters. **b**, Heat map showing the top ten differentially expressed genes in each cluster. **c**, The t-SNE of representative ILC3-related or ILC1-related genes. **d, e**, RNA velocity analysis based on spliced and unspliced mRNAs to predict cell dynamics. One donor representing two is shown.

Fig. 6. Cytokine production in clones derived from ILC3a, ILC3b, ILC1b and ILC1a.

a, IFN- γ and IL-22 production by two of the most represented clones (panels on the left and in the middle) and by one of the least represented clones (panels on the right) derived from ILC3a, ILC3b, ILC1b and ILC1a. **b**, Percentages of cells producing IL-22 only, IFN- γ + IL-22, or IFN- γ only in each clone tested. Data are mean $\pm$ s.d. A total of 109 clones was tested (ILC3a=31; ILC3b=25; ILC1b=28; ILC1a=25). One donor representing two is shown. The significance was calculated using ordinary, one-way ANOVA, multiple comparison test using the Prism v.7 software. * P 0.05, ** P 0.001, *** P 0.005, **** P 0.0001.

Fig. 7. Aiolos represses ILC3 lineage genes in cooperation with T-bet.

a, Histograms showing expression of Aiolos and T-bet in ILCa/b subsets ex vivo from one donor that represent six. **b**, Cumulative data for percentages and MFIs of Aiolos and T-bet expression in different donors. Circles with the same color indicate the same individual ($n=6$). Data are mean $\pm$ s.d. Significance was calculated using an ordinary, one-way ANOVA, multiple comparison test with Prism v.7 software. * P 0.05, ** P 0.001, *** P 0.005, **** P 0.0001. **c**, Intracellular content of IL-22 and IL-17a in control MNK3 cells or MNK3 transduced with T-bet or both Aiolos and T-bet, after stimulation with IL-23 and IL-1b. **d**, **e**, Quantification of IL-22 (**d**) and IFN- γ (**e**) in culture supernatants of control MNK3 and MNK3 transduced with T-bet, or both Aiolos and T-bet; cells were stimulated with IL-23+IL-1b or a combination of IL-23, IL-12 and IL-18 ($n=3$). Significance was calculated using an ordinary, one-way ANOVA, multiple comparison test with Prism v.7 software.

* P 0.05, ** P 0.001, **** P 0.0001. One experiment representing four (**c**) or two (**d**, **e**) is shown. **f**, UCSC snapshots of the *IL22* locus. Tracks represent cut and run for H3K27ac (green, 0–300 RPKM), Aiolos (blue, 0 to 100 RPKM) or ATAC-seq (red, 0 to 100 RPKM). Fold change plots below H3K27ac tracks represent differences between H3K27ac in MNK3 expressing T-bet and both Aiolos and T-bet. Conserved IKZF3 motifs between mouse loci (top snapshots) and human loci (bottom snapshots) are indicated as dashed lines. Relative kilobase scale and gene locations are indicated on top. Two independent cut and run experiments were performed. rep=replicate; ieILC, intraepithelial ILCs. **g**, TGF- β and IL-23 stimulation induces expression of Aiolos and T-bet while downregulating Ror γ t in ILC3a. Numbers indicate MFI. One experiment representing five is shown.

Fig. 8. Identification of ILC clusters in small intestinal lamina propria of controls and CD patients.

a, Unsupervised t-SNE analysis of ILC subsets. ILCs were pooled from two controls and two CD patients with similar number of total cells (2068 control, 1836 CD). **b**, Color representation of single cells in each cluster based on disease status. **c**, Heat map showing the top ten differentially expressed genes in each cluster. **d**, The t-SNE of representative ILC3-related or ILC1-related genes. **e**, Number of cells detected in each cluster and separated by disease status. Ctr, control.