Crohn's Disease Is Associated With Activation of Circulating Innate Lymphoid Cells

Luca Mazzurana, MSc,* Ferdinando Bonfiglio, PhD,[†,‡] Marianne Forkel, PhD,[*,§] Mauro D'Amato,[†,‡]
Jonas Halfvarson,[¶,a] and Jenny Mjösberg, PhD[*,a]

Background: Inflammatory bowel disease (IBD) is associated with disturbed mucosal innate lymphoid cell (ILC) composition, which is correlated to the degree of intestinal inflammation. However, it remains unclear whether circulating ILCs are dysregulated in patients with IBD.

Methods: Blood samples from 53 patients with Crohn's disease (CD), 43 patients with ulcerative colitis (UC), and 45 healthy control subjects (HC) were analyzed by flow cytometry for markers of ILC subsets (ILC1, ILC2, and ILC precursors [ILCp]) and selected IBD-relevant proteins, as predicted by previous genome-wide association studies. A dimensionality reduction approach to analyzing the data was used to characterize circulating ILCs.

Results: The frequency of ILCp expressing the ILC3 activation markers NKp44 and CD56 was increased in CD versus HC and UC (NKp44) or in CD versus HC (CD56), whereas the CD45RA⁺ ILCp were reduced in CD versus UC. Furthermore, the activation marker HLA-DR was increased on ILC1 and ILC2 in CD versus HC. Interestingly, the IBD-related protein SLAMF1 was upregulated on ILC2 from both CD and UC samples as compared with HC samples. In active CD, SLAMF1⁺ ILC2 frequency was negatively correlated with disease severity (Harvey-Bradshaw index). The characterization of SLAMF1⁺ ILC2 revealed a higher expression of the ILC2 markers CRTH2, CD161, and GATA3 as compared with SLAMF1⁻ ILC2.

Conclusions: In line with the systemic nature of CD inflammation, our findings point toward the activation of ILCs in the blood of patients with CD. Furthermore, in active CD, circulating SLAMF1⁺ ILC2 are increased in patients with less active disease, introducing SLAMF1⁺ ILC2 as interesting therapeutic targets deserving further exploration.

Key Words: Crohn's disease, ulcerative colitis, innate lymphoid cells, ILC, SLAMF1, NKp44

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic relapsing–remitting disease characterized by an activation of the intestinal immune system, which subsequently causes inflammation. It encompasses the predominant forms of Crohn's disease (CD) and ulcerative colitis (UC). Although CD often causes widespread intestinal inflammation, UC is characterized by inflammation restricted to the colon.[1]

Innate lymphoid cells (ILCs) are particularly prominent in the mucosal linings, where they contribute to both homeostasis and inflammation.[2,3] They are defined by a lack of rearranged antigen-specific receptors and an absence of lineage markers.[4] The most recent nomenclature separates ILCs into five distinct subsets that mirror the T-cell family in terms of transcription factor dependence and cytokine profiles. Natural killer (NK) cells make up the innate equivalent of CD8⁺ T cells.[4] Like NK cells, ILC1 contribute to antimicrobial responses[5] and inflammation[6] by producing interferon-gamma (IFN-γ) and are regulated by the transcription factor T-bet.[5,6] However, in contrast to NK cells, ILC1 show low cytotoxicity.[5,6] ILC2 are regulated by the transcription factor GATA3 and play a role against parasitic infections and in allergy by releasing the Th2-associated cytokines interleukin (IL)-4, IL-5, IL-9, and IL-13.[7-9] They are stimulated by Th2 polarizing cytokines, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). ILC3, which constitute the innate equivalents of Th17/22 cells, are dependent on the transcription factor RORγt and are stimulated by IL-23 and IL-1β.[10,11] They produce IL-22,

granulocyte colony stimulating factor, IL-17A, and IL-17F, cytokines important for tissue repair and the promotion of barrier immunity but also inflammation.[4] Recent studies suggest that fully differentiated mucosal ILC subsets derive from naïve ILCs that are recruited from the circulation to tissues where they undergo full maturation,[12] in a process reminiscent of T-cell differentiation.

All known ILC groups have been shown to be present in the healthy human intestine, with ILC3 constituting the dominating ILC population.[6,13,14] In mouse models mimicking an inflammatory environment in the gut, it was shown how IL-22, produced by ILC3, decreased colon inflammation (induced by chemical or bacterial agents),[15] and a lack of IL-22 led to increased susceptibility to intestinal bacterial infections.[16] In patients with CD, IL-22-producing NKp44+ ILC3 have been found to decrease in frequency in the intestinal mucosa. Research has suggested that this decrease results from transdifferentiation into IFN-γ producing ILC1, which accumulate at sites of intestinal inflammation in both humans[6,13,17] and mice[5] and have been associated with disease activity among patients with CD.[13,18] These studies imply an important role of ILC1 in driving and of NKp44+ ILC3 in preventing intestinal inflammation in CD. Because mucosal ILCs can be derived from

circulating precursors, a thorough investigation of circulating ILCs is warranted because they may serve as potential biomarkers and/or targets of therapy.

In this study, using multidimensional flow cytometry, we investigated ILC phenotypes, frequencies, and counts in the peripheral blood of patients with CD and UC and compared them with that of age-matched healthy patients.

MATERIALS AND METHODS

Study Participants

We prospectively recruited adult patients with CD (n = 53) and UC (n = 43) from the population-based IBD cohort of Örebro University Hospital, Sweden.[19,20]

Clinical demographics of patients with IBD are reported in Table 1. Diagnosis was based on internationally accepted clinical, endoscopic, radiologic, and histologic criteria.[21,22] Medical notes were scrutinized to classify disease characteristics according to the Montreal classification.[23] To evaluate the degree of clinical disease activity of the patients with CD, we used the Harvey-Bradshaw Index[24] (HBI), which accounts for several parameters including patient well-being, presence of abdominal pain, number of liquid or soft stools, abdominal mass,

TABLE 1. Clinical Characteristics of All Patients With CD and UC, Stratified by Disease Activity, and of HC

	Active CD, n = 17	CD in Remission, n = 36	Active UC, n = 4	UC in Remission, n = 39	HC, n = 45
Sex (n, %)					
Males	11 (64)	19 (53)	2 (50)	22 (56)	26 (58)
Age, y ± SD	64 ± 14	62 ± 13	58 ± 19	59 ± 12	56 ± 10
Location (number of patients)					
Ileal (L1)	5	13	—	—	—
Colonic (L2)	1	7	—	—	—
Ileocolonic (L3)	11	16	—	—	—
Behavior (number of patients)					
Nonstricturing, nonpenetrating (B1)	3	13	—	—	—
Stricturing (B2)	13	19	—	—	—
Penetrating (B3)	1	4	—	—	—
Extent (number of patients)					
Proctitis (E1)	—	—	—	4	—
Left-sided colitis (E2)	—	—	1	19	—
Extensive colitis (E3)	—	—	3	16	—
Average score ± SD					
HBI	8.2 ± 3.1	2.2 ± 1.4	—	—	—
Partial Mayo score	—	—	4 ± 0.8	0.6 ± 0.7	—
Medications (number of patients)					
5-ASAs/SASP	3	3	1	32	—
Corticosteroids	4	3	—	3	—
Immunomodulators	6	18	—	12	—
Anti-TNF agents	1	1	—	—	—

5-ASA indicates 5-aminosalicylates; SASP, sulfasalazine; TNF, tumor necrosis factor.

and disease complications. An HBI < 5 was considered as clinical remission and an HBI ≥5 as clinically active. In total, 36 patients with CD were in remission and 17 had active disease at the time of blood withdrawal.

Correspondingly, we used the partial Mayo score, which accounts for stool frequency, rectal bleeding, and a physician's global assessment, to evaluate clinical disease activity among patients with UC. A partial Mayo score of ≤2 was considered as clinical remission and a partial Mayo score of ≥3 was considered as clinically active disease. Altogether, 39 patients with UC were found to be in remission and 4 had active disease. Finally, 45 age- and sex-matched healthy blood donors without any history of chronic gastrointestinal disease were recruited as healthy control patients (HC).

All individuals gave their informed written consent to participate in this study, before blood samples were collected. The regional ethical board in Uppsala, Sweden approved the study (Dnr 2010/313).

Sample Collection and Cell Isolation

Peripheral blood (36 mL) was collected in heparin tubes and processed within an hour from blood withdrawal. Peripheral blood mononuclear cells were isolated from whole blood by centrifugation, using Lymphoprep (Axis Shield) as the gradient medium. After centrifugation, lymphocytes were washed in phosphate-buffered saline (PBS), counted and frozen in a fetal calf serum (FCS) 10% dimethylsulfoxide solution at –180°C.

Flow Cytometry

Frozen samples were thawed at 37°C water, pipetted drop by drop into Iscove's Modified Dulbecco's Medium (IMDM) 10% FCS medium, and washed by centrifugation. Three million cells per sample were stained with antibodies (Table S1) for 30 minutes at 4°C, washed with PBS, fixed with 2% paraformaldehyde for 7 minutes, washed again with PBS, and resuspended in fluorescence-actived cell-sorting (FACS) buffer (PBS, containing 2 mM EDTA and 2% FCS). When stained for the intracellular cytokine IL-13, after surface staining, cells were fixed with 2% paraformaldehyde for 7 minutes at room temperature (RT), and washed and permeabilized with BD Perm 2 buffer (BD Biosciences) for 10 minutes at RT. Next, the cells were incubated for 30 minutes with anticytokine antibodies (Table S1) at RT, washed again with PBS, and resuspended in FACS buffer (PBS, containing 2 mM EDTA and 2% FCS). All samples were acquired on a Fortessa LSR II (BD Biosciences) cytometer using FACS Diva Software version 7 (BD Biosciences). All data were analyzed using Flowjo software version 9 (TreeStar). The full gating strategy is shown in Fig. 1. Fluorescence minus one controls are shown in Fig. S1.

In Vitro Cultures

Thawed peripheral mononuclear blood cells were cultured in IMDM medium supplemented with 2% normal human serum and Yssel's supplement (made in house) in a 96-well plate. Cells were stimulated overnight with IL-2 (10 U/mL), IL-25, IL-33 plus TSLP (all 50 ng/mL) followed by stimulation with phorbol 12-myristate 13-acetate and 0.5 uM/L ionomycin for 3 hours, with Golgi Plug (1:10; BD Biosciences) and Golgi Stop (1:15; BD Biosciences), and stained for intracellular IL-13 (Table S1).

Dimensionality Reduction

Postprocessing of the flow cytometry data was performed with R software (version 3.6.1, The R Foundation of Statistical Computing). Stochastic neighbor embedding analysis was implemented to reduce the multidimensionality of the flow cytometry data to two dimensions (Rtsne package),[25] as described in Hengst et al.[26]

Statistics

To compare differences between the three groups, we used the Kruskal-Wallis test, followed by pairwise comparisons whenever a significant difference was observed. Correlations were calculated via the Spearman correlation test. The *P* value threshold for statistical significance was set to 0.05. Statistical analyses were performed using the software Prism 7 from GraphPad Prism.

RESULTS

Multidimensional Flow Cytometry Characterization of Circulating ILCs in IBD

We investigated ILC frequencies via flow cytometry by using a well-established gating strategy (Figs. 1 and S1).[6,27] We defined ILCs as CD45+CD3- lymphocytes lacking lineage markers (Table S1) and expressing CD127. The Lin-CD127+ ILCs were further subdivided into CD117-CRTH2- cells containing ILC1,[28,29] CD117+ ILC progenitors (referred to as ILCp),[12] and CRTH2+ILC2 (referred to as ILC2).[30] The NK cells were gated as non-ILCs expressing intermediate or high levels of CD56 (CD56dim and CD56bright NK cells; Fig. 1). We included CD4 as a marker of a specific ILC1 subset previously reported in multiple sclerosis,[29] CD45RA as a marker of naïve ILCp,[12] and NKp44/CD56 as markers of activated ILCp[31] or ILC3/NK cell-committed ILCp.[32] We also analyzed two IBD-relevant[33,34] surface proteins previously shown to be expressed by ILCs on either the protein (HLA-DR[35,36]) or the transcriptional level (SLAMF1).[37] Because multicolor flow cytometry provides extensive data in multiple dimensions, we performed stochastic neighbor embedding analysis to graphically visualize multiple dimensions in a simple two dimensional plot (Figs. 2-4). This approach allowed us to compare cytometry data between groups of samples (UC, CD, and HC)
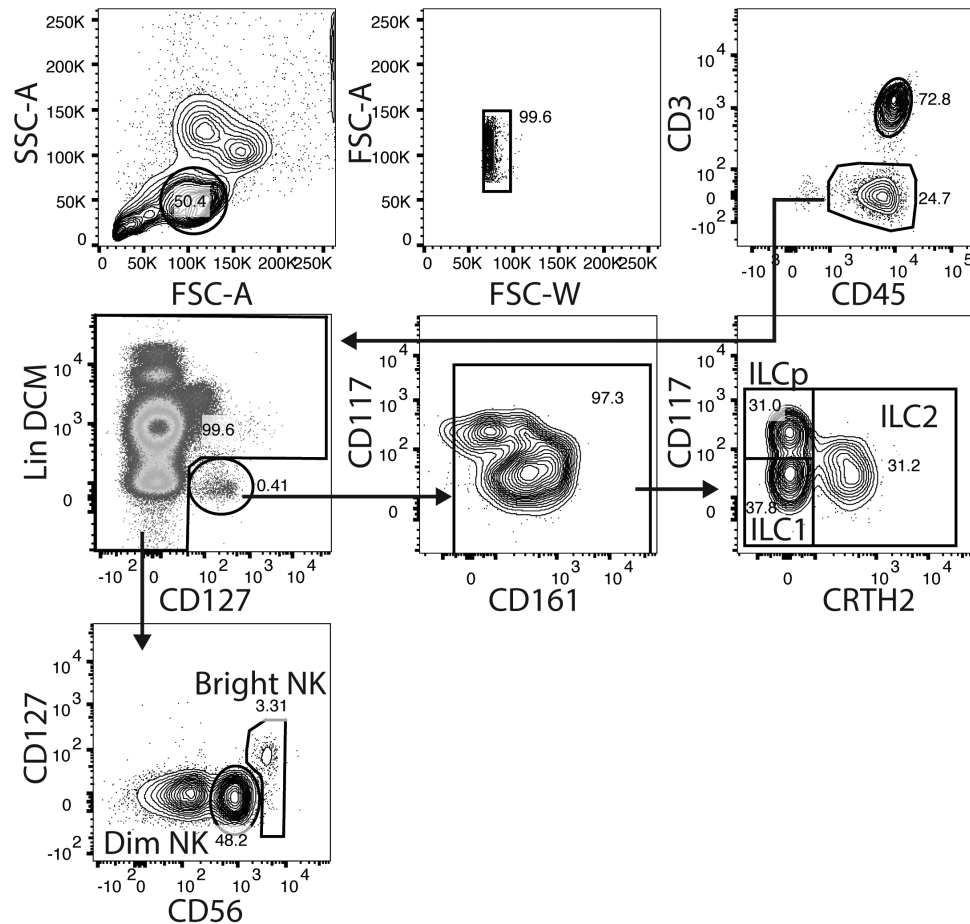
FIGURE 1. Flow cytometry gating strategy for analysis of ILCs in peripheral blood mononuclear cells; sample from a representative HC. Numbers represent percentages of cells in respective gates. Lin DCM: lineage markers plus dead cell marker. Lineage cocktail contained CD1a, CD14, CD19, CD34, CD94, CD123, TCRαβ, TCRγδ, BDCA2, and FcεR1.

by assigning them electronic barcodes. For statistical verification of the graphically apparent differences, we also performed traditional unpaired nonparametric statistical evaluations between the groups (Fig. 5 and Tables S2-S4).

Dysregulation of Circulating ILCs in CD and UC

We detected several alterations in CD127+ ILCs in CD/UC samples (Figs. 2-5). Possibly mirroring the systemic inflammation in CD, we revealed multiple alterations in the circulating ILC compartment in patients with CD as compared with HC (Figs. 2 and 5). We observed the increased expression of two proteins associated with ILC activation (NKp44 and CD56[31,38,39]), ILCp commitment to the ILC3/NK cell lineage (CD56[32]), and ILCp in CD as compared with HC (Figs. 2 and 5B). The frequency of CD56+ and NKp44+ ILCp was correlated among the patients with CD (Fig. 5B), indicating a strong coregulation of these markers on ILCp. In parallel with the upregulation of putative activation markers on ILCp in CD, we also detected decreased frequencies of CD45RA+ ILCp in patients with CD vs patients with UC (Figs. 3 and 5C). This finding indicates a switch from naïve to activated

ILCp in CD as compared with UC, with the latter predominantly composed of patients in remission. Activation of ILCp in CD was supported by the observation that CD45RA+ ILCp frequencies and ILC1 frequencies were negatively correlated to CD56+ ILCp (Fig. 5C). We could not detect significant differences in NK cell frequencies between patients with CD/UC and HC (Fig. S2A).

Statistical analysis revealed an increase in HLA-DR expression in ILC1 and ILC2 of patients with CD as compared with HC (Fig. 5A). We also observed the increased expression of SLAMF1 within the ILC2 compartment of patients with CD and patients with UC as compared with that of HC (Figs. 2, 4, 5D). Notably, SLAMF1 was not differentially expressed on any other subset (ILC1, ILCp, NK, or CD3+ T cells) in patients with IBD vs HC (data not shown). Previously, SLAMF1 has only been reported on the transcriptional level in ILC2,[37] and the role of SLAMF1+ ILC2 remains unknown. To assess the role for IBD-related medications in increasing the frequencies of SLAMF1+ ILC2 in IBD we stratified the patients with IBD on the basis of usage of immunomodulatory drugs. While we detected similar SLAMF1+ ILC2 frequencies in patients with
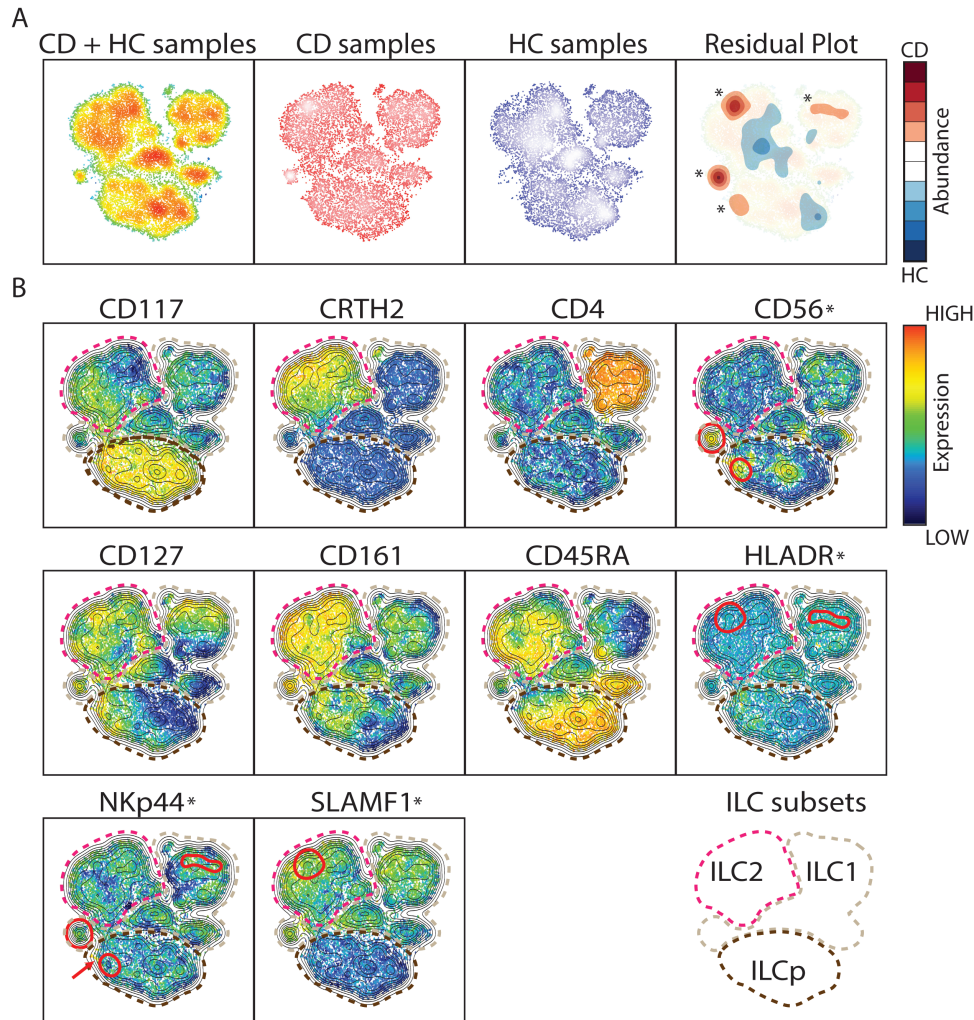
FIGURE 2. High-dimensionality reduction analysis of ILC (gated as lymphocytes, singlets, and CD45$^+$CD3$^-$Lin$^-$CD127$^+$ as depicted in Fig. 1) from peripheral blood of patients with CD and HC. A, Barnes Hut SNE maps of combined CD + HC and separated CD and HC samples. Residual plot showing the differences between CD and HC maps. Analysis is based on data from 28 CD and 28 HC samples, with 500 events per sample. B, Relative expression intensities (combined CD + HC samples) of the 10 parameters used in the SNE analysis. Phenotypes within red circles were confirmed to be statistically more common in CD samples, and vice versa for blue circles and HC samples. The arrow in the NKp44 plot points out the few high-NKp44 cells. SNE indicates stochastic neighbor embedding.

untreated and treated CD and UC, we observed higher frequencies of SLAMF1$^+$ ILC2 in treated vs untreated CD and UC patients combined (Fig. 5E). However, treatment did not completely explain the increased frequencies in IBD because we continued to observe higher frequencies of SLAMF1$^+$ ILC2 in untreated patients with IBD vs HC.

Association of Disease Activity With SLAMF1$^+$ ILC2 Frequencies in Patients With Active CD

We next asked whether the disturbances in circulating ILCs that we detected in patients with CD and with UC were related to disease activity. Our analysis revealed that SLAMF1$^+$ ILC2 frequencies were slightly higher in the blood of patients with active CD than in that of patients with CD in remission (not statistically significant; Fig. 6A).

However, within the group of patients with active CD, we observed that SLAMF1$^+$ ILC2 frequencies were inversely correlated to HBI score (Fig. 6B). However, we did not observe any correlation between partial Mayo scores, which were generally low in our cohort, and SLAMF1$^+$ ILC2 frequencies in patients with UC (data not shown). This finding suggests that SLAMF1$^+$ ILC2 are involved in reduced disease activity, specifically among patients with CD with HBI ≥ 5.

SLAMF1$^+$ ILC2 Express High Levels of CRTH2, CD161, and GATA3

To better understand the potential role of SLAMF1$^+$ ILC2 in IBD, we further characterized SLAMF1$^+$ ILC2 on the basis of the expression of three well-characterized ILC2-associated proteins (Figs. 7A-C). As compared with
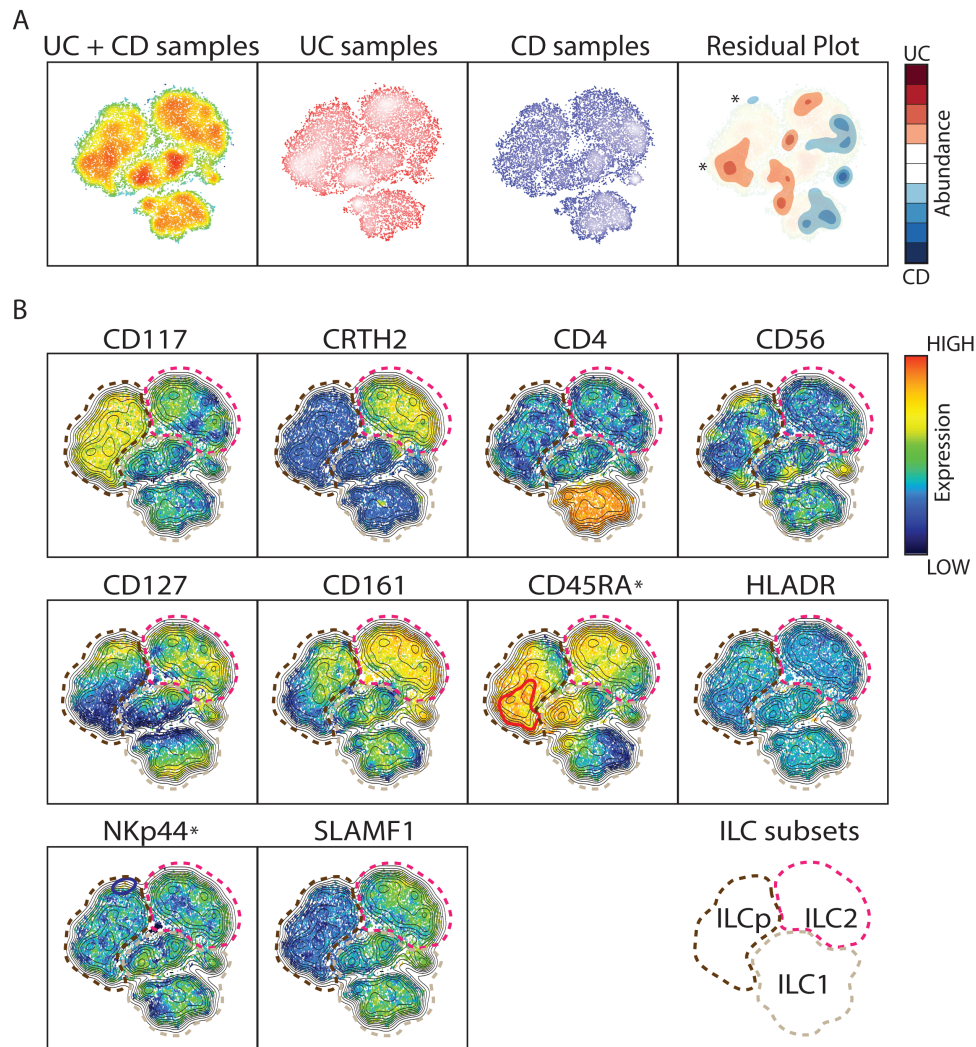
FIGURE 3. High-dimensionality reduction analysis of ILC (gated as lymphocytes, singlets, and CD45+CD3-Lin-CD127+ as depicted in Fig. 1) from peripheral blood of patients with UC and with CD. A, Barnes Hut SNE maps of combined UC + CD and separated UC and CD samples. Residual plot showing the differences between UC and CD maps. Analysis is based on data from 28 UC and 28 CD samples, with 500 events per sample. B, Relative expression intensities (combined UC + CD samples) of the 10 parameters used in the SNE analysis. Phenotypes within red circles were confirmed to be statistically more common in UC samples, and vice versa for blue circles and CD samples. SNE indicates stochastic neighbor embedding.

SLAMF1- ILC2, SLAMF1+ ILC2 showed higher expression of the prototypical ILC2 surface marker and prostaglandin D_2 receptor CRTH2. Although not significantly different ($P = 0.056$), the master transcription factor of ILC2, GATA3, tended to follow the same trend as CRTH2. Both of these proteins are essential for ILC2 activity, including the secretion of immune- and tissue-modulatory cytokines.[30,40] SLAMF1+ ILC2 also expressed higher levels of CD161, a C-type lectin and phenotypic marker of ILC2[30] whose function in ILC2 is currently unknown. To assess the activation status of SLAMF1+ ILC2 in HC and CD, we analyzed CD25 and CD69 expression. Although CD69 generally had low expression, CD25 showed widespread expression in ILC2, but there was no difference in the expression of these activation markers on SLAMF1+ ILC2 and SLAMF1- ILC2 (Fig. S3). We also showed that SLAMF1+ ILC2, similar to SLAMF1- ILC2, can produce IL-13 upon IL-2, IL-25, IL-33 plus TSLP stimulation (Fig. S3), confirming their lineage identity as ILC2.

DISCUSSION

We and others have previously reported the dysregulated composition of ILC1, ILC2, and ILC3 in intestinal tissues from patients with CD and/or UC.[13,41-44] Specifically, IBD is associated with decreased frequencies of putative tissue–protective NKp44+ ILC3[6,13,44] and increased frequencies of potentially proinflammatory IFN-γ-producing ILC1[6,13,44,45] in the intestinal tissues. An increase in ILC2 frequencies has also been reported
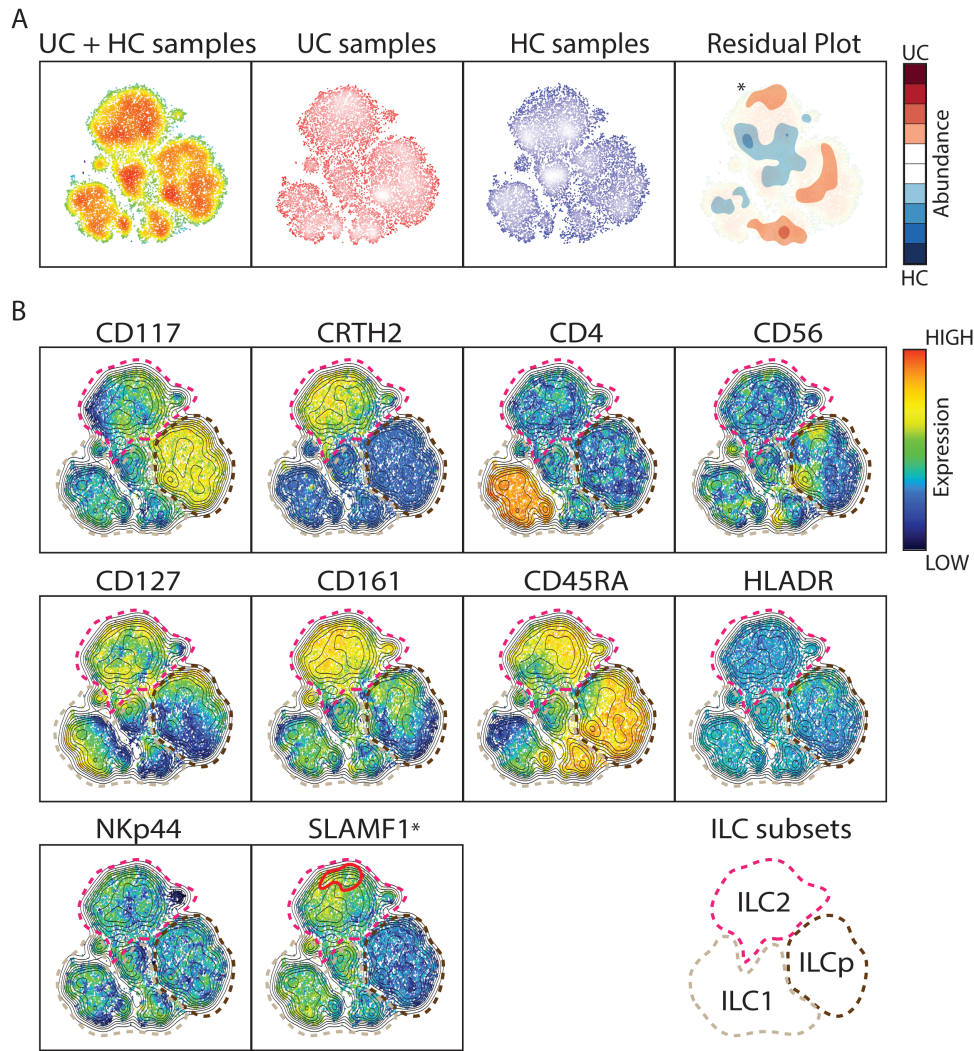
FIGURE 4. High-dimensionality reduction analysis of ILC (gated as lymphocytes, singlets, and CD45$^+$CD3$^-$Lin$^-$CD127$^+$ as depicted in Fig. 1) from peripheral blood of patients with UC and HC. A, Barnes Hut SNE maps of combined UC + HC and separated UC and HC samples. Residual plot showing the differences between UC and HC maps. Analysis is based on data from 28 UC and 28 HC samples, with 500 events per sample. B, Relative expression intensities (combined UC + HC samples) of the 10 parameters used in the SNE analysis. Phenotypes within red circles were confirmed to be statistically more common in UC samples. SNE indicates stochastic neighbor embedding.

previously in patients with CD[13,44,46] and with UC,[13,44] which could imply a role for these cells in IBD, as shown in a mouse model of type-2 driven intestinal inflammation.[47]

Interestingly, we observed that ILC disturbances in IBD are restricted to the inflamed intestinal mucosa because they were not seen in the noninflamed mucosa or blood of the same patients with IBD.[13] However, a recent study found increased ILC1 and decreased ILC2 and NKp44$^-$ ILC3 in the blood of patients with IBD (UC + CD).[44] In the present study, we confirmed our previous findings of unaltered frequencies of the basic ILC subgroups ILC1, ILC2, and ILCp in the blood of patients with IBD vs that of HC. Notably, we extended these observations by assessing a set of activation markers on ILCs. This

assessment revealed the dysregulation of ILCs in the blood of patients with CD and UC as compared to that of HC, pointing toward an activation of circulating ILC during IBD. More specifically, we observed an increased expression of the activation marker NKp44 on ILCp from patients with CD as compared to UC and HC. Similarly, CD56 expression was increased on ILCp from patients with CD as compared to HC. Furthermore, CD45RA$^+$ ILCp were reduced in patients with CD when compared to the UC cohort. Research has shown that CD45RA is expressed by the majority of blood ILCs but not tissue ILCs,[12] indicating that CD45RA serves as a marker for naïve/immature ILCs similar to CD45RA expression on naïve T cells. The inverse correlation between CD45RA$^+$ ILCp and CD56$^+$ ILCp
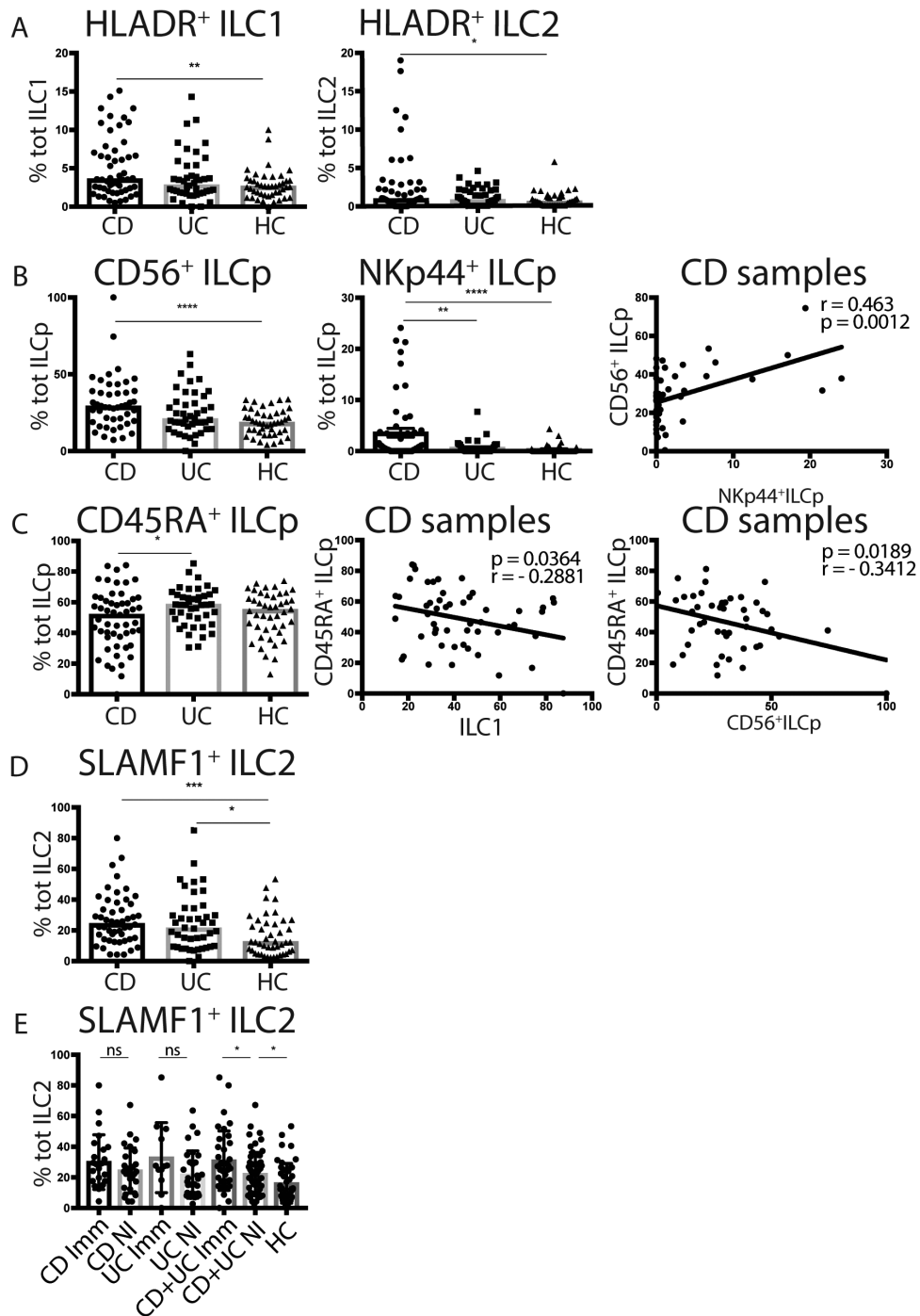
FIGURE 5. Dot plots showing the distribution in frequencies of ILC populations that are statistically different between any of the groups compared. A, HLA-DR⁺ ILC1 and ILC2 as frequency of total ILC1 or ILC2, respectively. B, CD56⁺ and NKp44⁺ ILCp as frequency of total ILCp and their correlation in CD samples. C, CD45RA⁺ ILCp as a frequency of total ILCp and its correlation to ILC1/CD56⁺ILCp. D-E, SLAMF1⁺ ILC2 as a frequency of ILC2 in HC and patients with CD and with UC with (Imm) or without (NI) immunomodulatory treatment. Mann-Whitney test: *$P < 0.05$, **$P \leq 0.01$, and ***$P < 0.001$, ****$P \leq 0.0001$.

A

FIGURE 6. A, Dot plot showing the percentage of SLAMF1^{+} ILC2 in CD remission vs active CD samples. B, Correlation between HBI score and percentage of SLAMF1^{+} ILC2 among patients with active CD. *$P < 0.05$. The *r* indicates the Pearson correlation coefficient.
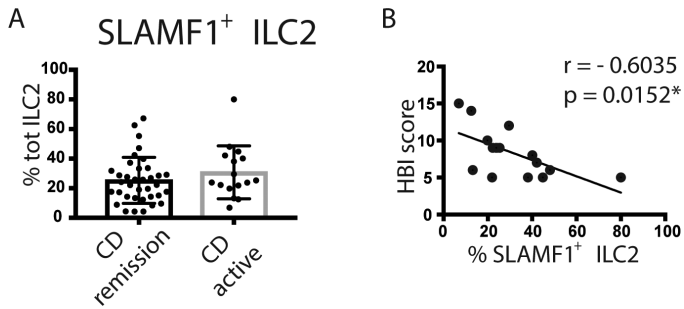
in patients with CD therefore points toward the maturation of ILCp in patients with high CD56^{+} ILCp frequencies.

NKp44 on ILCp may be indicative of enhanced inflammation status in patients with CD. Although ILCs in the blood of healthy donors lacked NKp44, circulating NKp44^{+} ILCp were reported in a smaller CD cohort (n = 7)[48] and more recently in patients with CD treated using biologics.[44] Furthermore, increased frequencies of circulating NKp44-expressing ILCp were also associated with a reduced risk of intestinal graft-versus-host disease after hematopoetic stem cell transfer in patients with leukemia.[31] Because NKp44 expression is related to IL-22 production, known to regulate epithelial homeostasis,[49] this finding suggests an intestinal tissue–protective role of NKp44^{+}ILCp. Indeed, Munneke et al[31] detected chemokine receptors on circulating NKp44^{+}ILCp in patients with graft-versus-host disease, indicating that these cells may be recruited to sites of inflammation. Furthermore, another activation marker increased in the CD samples was HLA-DR, which may reflect the proinflammatory environment in the blood of patients with IBD[50] because HLA-DR expression on human ILCs is NFkB-dependent.[36]

Interestingly, few significant differences were observed between patients with UC and HC, possibly because of the lower systemic inflammation in UC as compared to CD. An alternative explanation could be the generally low disease activity of the patients with UC, which was a limitation of our study. This reason still remains to be determined. However, supporting the latter explanation, we found that SLAMF1^{+} ILC2 was increased in patients with CD and with UC as compared with HC. This finding was explained by the IBD diagnosis but potentially also by the usage of immunomodulatory drugs in IBD because we observed lower SLAMF1^{+} ILC2 frequencies in patients with untreated IBD than in those with treated IBD but still higher SLAMF1^{+} ILC2 frequencies in patients with untreated IBD than in HC. In patients with clinically active CD (HBI ≥ 5),

frequencies of SLAMF1^{+} ILC2 negatively correlated with disease activity. These findings may suggest a protective role of SLAMF1^{+} ILC2 in CD and possibly UC. ILC2 have been assigned a tissue-repairing role in various organs such as the liver,[51] lung,[52] and gut,[13] although in humans ILC2 frequencies in the gut are very low yet accumulate in the mucosa of patients with UC and those with CD as compared with those of HC.[13] SLAMF1 has been shown to be regulated by several transcription factors, including NFkB,[53] and we recently found SLAMF1 to be associated to a mature phenotype, given its coexpression with IL-13 transcripts in lung ILC2 (Mazzurana et al. Cell Research. Accepted manuscript). CD161 or NKR-P1A (encoded by KLRB1) is an immunoreceptor tyrosine-based inhibition motif-signaling inhibitory receptor on NK cells whose function is unknown in ILC2. GATA3 is the defining transcription factor of ILC2, and it has been shown to increase its expression with a mature phenotype, also defined by IL-13 production.[9] Hence, the increase in SLAMF1^{+} ILC2 with a pronounced ILC2 phenotype in patients with active CD with lower HBI scores could suggest a role for these cells in tissue healing. This hypothesis deserves further exploration.

This study is associated with a number of strengths but also some weaknesses. The use of dimensionality reduction allowed us to take an unbiased approach to analyzing the data, which was confirmed by statistical analysis of FACS counts. The fact that assessment of disease activity was based purely on clinically established indices and not on endoscopic data limited the study because clinical disease activity is poorly correlated with endoscopic activity, especially in CD. In spite of this limitation, we observed a correlation between disease activity and SLAMF1^{+} ILC2 in active CD. The inclusion of patients with a relatively long disease duration, instead of incident patients, may have introduced bias because of disease history, including previous and ongoing medications.

CONCLUSIONS

We identified multiple disturbances in the circulating ILC compartment in patients with CD, likely reflecting their systemic immune activation. The ILC alterations included a reduction in the frequency of the precursors of mature ILCs (CD45RA^{+} ILCp) and an increase in the frequency of possibly activated ILC phenotypes including HLA-DR^{+} ILC1, HLA-DR^{+} ILC2, CD56^{+} ILCp, NKp44^{+} ILCp, and SLAMF1^{+} ILC2. We suggest that the latter is a mature ILC2 population because it produces IL-13 and expresses high levels of CRTH2, CD161, and GATA3. Increased frequencies of SLAMF1^{+} ILC2 were associated with lower disease activity among patients with active CD. Future studies should be aimed at exploring the role
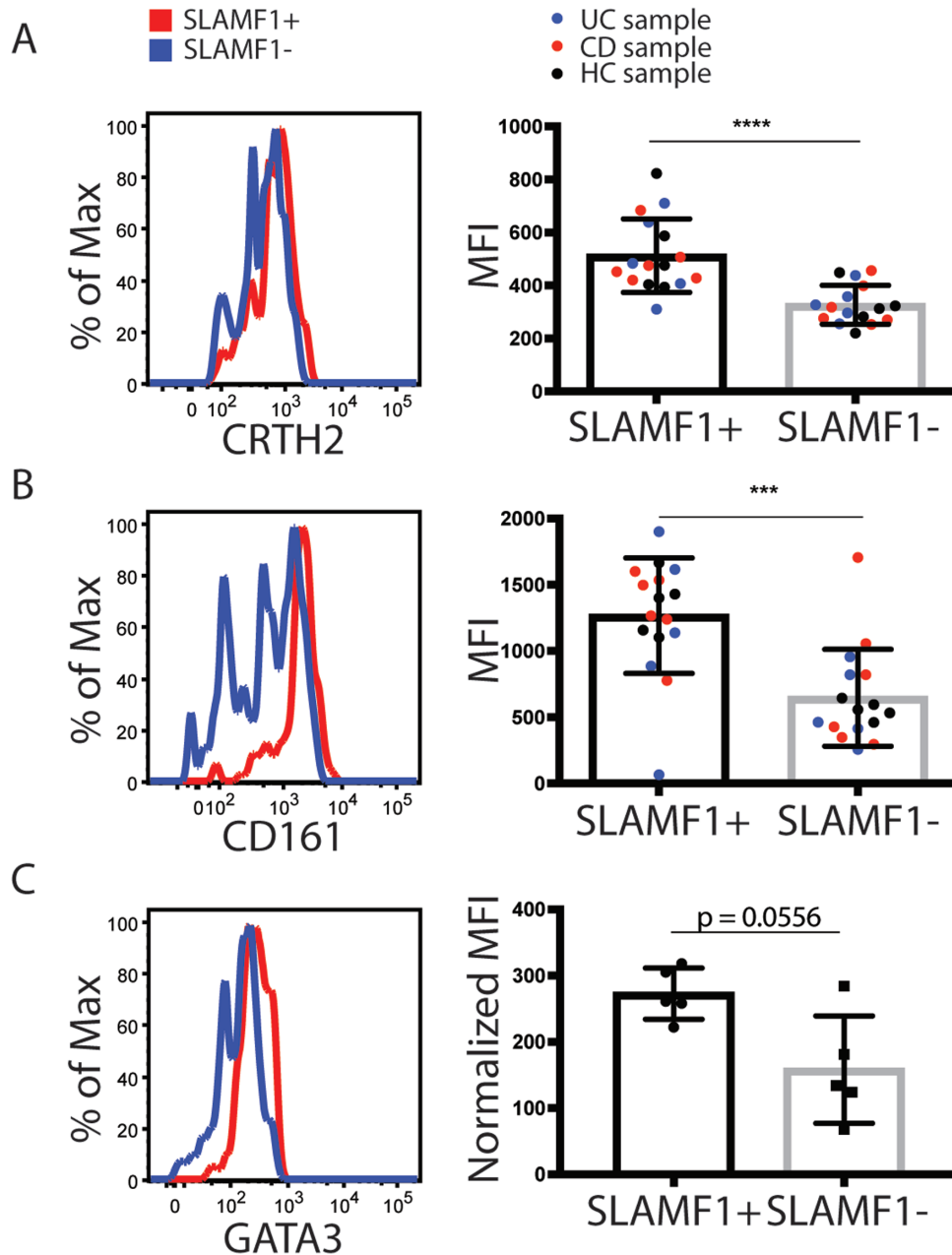
FIGURE 7. Representative histogram overlays of SLAMF1$^+$ and SLAMF1$^-$ ILC2 and mean fluorescent intensities of (A) CRTH2, (B) CD161, and (C) GATA3 of SLAMF1$^+$ and SLAMF1$^-$ ILC2. Dots colored by sample group. Mann-Whitney test: ***$P \leq 0.0001$ and ****$P \leq 0.0001$.

of SLAMF1$^+$ ILC2 in intestinal healing and explore its potential as a therapeutic target.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

Table S1. Antibodies.
Table S2. CD vs HC statistics.

Table S3. UC vs HC statistics.
Table S4. CD vs UC statistics.
Figure S1. Full stained samples vs FMO stainings for markers of interest. FMO indicates fluorescence minus one.
Figure S2. Percentage of CD56^{bright} and CD56^{dim} NK cells of CD45$^+$Lin$^-$ non-ILCs.
Figure S3. A, Percentage of activation (CD25/CD69, top) and migration (α4β7, bottom) markers expressed on ex vivo isolated SLAMF1$^+$ and SLAMF1$^-$ ILC2. B, FACS plots (top) show-

ing expression of IL-13 and SLAMF1 on CD127$^+$ ILC gated from peripheral blood mononuclear cells stimulated with IL-2, IL-25, and IL-33 plus TSLP. Data shown are representative of 3 healthy blood donors. Bar graph (bottom) indicates the percentage of IL-13 expression in SLAMF1$^+$ and SLAMF1$^-$ ILCs. FMO indicates fluorescence minus one.

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