Single-cell analyses of Crohn’s disease tissues reveal intestinal intraepithelial T cells heterogeneity and altered subset distributions

Natalia Jaeger  
*Washington University School of Medicine in St. Louis*

Ramya Gamini  
*Pfizer Worldwide Research, Development and Medical*

Marina Cella  
*Washington University School of Medicine in St. Louis*

Jorge L. Schettini  
*Pfizer Worldwide Research, Development and Medical*

Mattia Bugatti  
*University of Brescia*

*See next page for additional authors*

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

Please let us know how this document benefits you.

**Recommended Citation**

Jaeger, Natalia; Gamini, Ramya; Cella, Marina; Schettini, Jorge L.; Bugatti, Mattia; Zhao, Shanrong; Rosadini, Charles V.; Esaulova, Ekaterina; Di Luccia, Blanda; Kinnett, Baylee; Vermi, William; Artyomov, Maxim N.; Wynn, Thomas A.; Xavier, Ramnik J.; Jelinsky, Scott A.; and Colonna, Marco, "Single-cell analyses of Crohn's disease tissues reveal intestinal intraepithelial T cells heterogeneity and altered subset distributions." *Nature Communications*. 12, 1. 1921 (2021).  
[https://digitalcommons.wustl.edu/open_access_pubs/10356](https://digitalcommons.wustl.edu/open_access_pubs/10356)

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Authors
Natalia Jaeger, Ramya Gamini, Marina Celli, Jorge L. Schettini, Mattia Bugatti, Shanrong Zhao, Charles V. Rosadini, Ekaterina Esauleva, Blanda Di Luccia, Baylee Kinnett, William Vermi, Maxim N. Artyomov, Thomas A. Wynn, Ramnik J. Xavier, Scott A. Jelinsky, and Marco Colonna
Single-cell analyses of Crohn’s disease tissues reveal intestinal intraepithelial T cells heterogeneity and altered subset distributions

Natalia Jaeger1,8, Ramya Gamini2,8, Marina Cella1,8, Jorge L. Schettini2,6, Mattia Bugatti3, Shanrong Zhao2,7, Charles V. Rosadini2, Ekaterina Esaulova1, Blanda Di Luccia1, Baylee Kinnett4, William Vermi3, Maxim N. Artyomov1, Thomas A. Wynn2, Ramnik J. Xavier5, Scott A. Jelinsky2 & Marco Colonna1✉

Crohn’s disease (CD) is a chronic transmural inflammation of intestinal segments caused by dysregulated interaction between microbiome and gut immune system. Here, we profile, via multiple single-cell technologies, T cells purified from the intestinal epithelium and lamina propria (LP) from terminal ileum resections of adult severe CD cases. We find that intraepithelial lymphocytes (IEL) contain several unique T cell subsets, including NKp30+γδT cells expressing RORγt and producing IL-26 upon NKp30 engagement. Further analyses comparing tissues from non-inflamed and inflamed regions of patients with CD versus healthy controls show increased activated TH17 but decreased CD8+T, γδT, TFH and Treg cells in inflamed tissues. Similar analyses of LP find increased CD8+T, as well as reduced CD4+T cells with an elevated T17 over Treg/TFH ratio. Our analyses of CD tissues thus suggest a potential link, pending additional validations, between transmural inflammation, reduced IEL γδT cells and altered spatial distribution of IEL and LP T cell subsets.

https://doi.org/10.1038/s41467-021-22164-6

1Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO, USA. 2Pfizer Worldwide Research, Development and Medical, Cambridge, MA, USA. 3Department of Pathology, University of Brescia, Brescia, Italy. 4Division of Gastroenterology, Washington University School of Medicine, St Louis, MO, USA. 5Immunology Program, Infectious Disease and Microbiome Program, Broad Institute of MIT, Cambridge, MA, USA. 6Present address: Global Gene Therapy, Takeda Pharmaceuticals, Cambridge, MA, USA. 7Present address: AbSci Inc, Vancouver, WA, USA. 8These authors contributed equally: Natalia Jaeger, Ramya Gamini, Marina Cella. ✉Email: mcolonna@wustl.edu
Inflammatory bowel disease (IBD) encompasses intermittent chronic inflammatory disorders of the gastrointestinal tract that significantly impair the quality of life in affected individuals and can result in comorbidities and complications requiring repeated surgery. IBD includes two major subtypes, Crohn’s disease (CD) and ulcerative colitis (UC). CD inflammation spans across all layers of the gut, while tissue damage in UC is confined to the mucosa. Both genetic and environmental factors contribute to IBD by generating abnormal interactions between the commensal microbiome and the mucosal immune system that result in uncontrolled intestinal inflammation. The conventional therapy with anti-inflammatory and immunomodulatory drugs has been recently integrated with biologicals that can effectively target cytokines, such as TNF-α, IL-12 and IL-23, or inflammatory cell recruitment with α4β7 blockers. However, IBD still poses significant therapeutic challenges.

High-dimensional single-cell profiling approaches, such as single-cell RNA sequencing (scRNA-seq) and mass cytometry, have been recently performed on intestinal specimens from patients with CD or UC and controls. These studies provided unbiased analyses of cell lineages and their functional states in IBD, deconvoluted pathways underlying IBD pathogenesis and supplied biomarkers predicting the course of disease and the response to therapy. While these studies have analyzed whole mucosal biopsies or the lamina propria (LP) selectively, few studies have analyzed intraepithelial lymphocytes (IEL) purified from CD specimens. IEL comprise a quite diverse and complex repertoire of TCRαβ+ and TCRγδ+ T cells, which are strategically located at the interface between the luminal environment and the intestinal barrier, contributing to intestinal homeostasis and mucosal protection.

Here, we examine T cells from 90 intestinal specimens of CD and controls by either scRNA-seq, multi-parameter flow cytometry or CyTOF, with CD specimens derived from surgical resections of the terminal ileum of adult patients with severe CD. Comparing IEL T cell profiles with those of T cells purified from the LP, our data not only provide an unbiased view of T cell lineages diversity and functional states in the intestinal mucosa under both healthy and CD conditions, but also identify an altered spatial distribution of T cell subsets between the IEL and the LP compartments that potentially correlates with transmural inflammation, although this remains to be validated with larger patient cohorts.

**Results**

scRNA-seq analysis identifies multiple IEL T cell subsets. IEL were prepared from surgical resections of the terminal ileum of CD patients, which included both macroscopically inflamed tissue (II) and adjacent non-inflamed tissue (NI) (Supplementary Fig. 1a–c). Most CD cases required surgical treatment because of severity and resistance to medical therapy (patient information: Supplementary Data 1–3). Ileal resections from patients undergoing surgery for colonic polyposis or cancer were used as controls. In an initial survey, we defined the baseline heterogeneity of IEL T cells by scRNA-seq of about 15,000 cells sorted from two CD patients and two controls (sorting strategy Supplementary Fig. 1d). Unsupervised clustering by UMAP of gene expression data from both CD and control samples identified ten cell clusters (Fig. 1a, b). Differential expression of marker genes was used to annotate the different cell types and states. Three T cell clusters expressed CD8α (0, 1, and 6) and five expressed CD4 (2, 3, 4, 5, and 8) (Fig. 1c). Among the CD8+ clusters, cluster 6 expressed genes indicating a canonical effector phenotype, including KLRG1 (Fig. 1d), GZMB, GZMK, PRFI, IFNG, and FCRL6 (Supplementary Fig. 2a, b). Cluster 6 also expressed high level of KLF2 (Supplementary Fig. 2c), a transcription factor that promotes lymphocytes circulation. In contrast, cluster 1 and cluster 0 shared expression of ITGAE, the receptor for E-cadherin, which is indicative of tissue residency, and CD160, a receptor for HVEM expressed on epithelial cells (Fig. 1d). Expression of ENTPD1, which encodes the activation marker CD39, distinguished cluster 1 from cluster 0 (Fig. 1d). The expression of γδTCR, NK cell receptors, cytotoxic mediators, and chemokines (Supplementary Fig. 2a, b) in a subset of cells within these two clusters suggested that additional subpopulations may be present (see below).

Within CD4+ cells, clusters 2 and 4 showed a Th17 transcriptional profile, featuring RORC, CCR6 (Fig. 1d), RORA, LTβ, CCL20, and KLRE1 (Supplementary Fig. 2a–c). Both subsets expressed CD40LG (Supplementary Fig. 2b), which sustains Th17 differentiation in vitro and in vivo. Moreover, cluster 2 expressed genes indicative of immediate effector function, including (a) cytokine genes IL17A and IL26 (Supplementary Fig. 2a); (b) IL23R, which endows responsiveness to IL-23 and IL27 (Supplementary Fig. 2e); (c) ENTPD1 (Fig. 1d); (d) CXCR6 (Supplementary Fig. 2b), which promotes retention in the intestinal epithelium; (e) CCLA and GZMB (Supplementary Fig. 2a), known mediators of Th17 pathogenicity. Cells in cluster 3 corresponded to naive CD4+ T cells expressing CCR7 (Fig. 1d), SELL, TCF7, LEF1, as well as KLF2 (Supplementary Fig. 2b, c). This cluster also contained a few naive CD8+ T cells. Clusters 5 and 8 were enriched for genes related to Th1 and Treg lineages. These genes encoded cell surface receptors, such as IL6ST (for GP130) (Fig. 1d), PDCD1 (for PD-1), TIGIT, ICOS, TNRPS1 (for OX40), and IL6R (Supplementary Fig. 2b), as well as transcription factors, such as TOX2 (Fig. 1d), MAF and BATE (Supplementary Fig. 2c). The left portion of cluster 5 expressed CXCL13 (Fig. 1d), BCL6 and CXCR5 (Supplementary Fig. 2b, c), pointing to a Th1 phenotype. The right portion of cluster 5 expressed the Treg markers FOXP3, IL2RA, ENTPD1 (Fig. 1d), CTLA4, and IL10 (Supplementary Fig. 2a, b). In addition, the Treg side of cluster 5 expressed PRDM1 (Supplementary Fig. 2b), which facilitates Treg over Th1 differentiation. Cluster 8 uniquely expressed BTLA (Fig. 1d) and CD200 (Supplementary Fig. 2b), which are markers of terminal Th1 differentiation. Cluster 7 included CD4 and CD8 MKI67+ proliferating cells (Fig. 1c, d), while cluster 9 (Fig. 1b) represented a small B cell contamination. Altogether, our survey of human IEL compartment shows the presence of circulating and resident CD8+ T cell subsets, as well as CD4+ T cell subsets belonging to the Th17, Th1, and Treg lineages.

**Flow cytometry of IEL corroborates T cell diversity.** To validate scRNA-seq data we analyzed IEL of CD and control patients by flow cytometry (gating strategy: Supplementary Fig. 3a, b patient information Supplementary Data 4). Cells were clustered using viSNE analysis in Cytobank. To facilitate comparison of flow cytometry with scRNA-seq data, t-SNE clusters were labeled with the same numbers of the corresponding scRNA-seq cluster followed by an asterisk. We adopted CD103 as a marker of tissue-resident CD8+ T cells (Fig. 2a, b). Most CD103- non-resident cells expressed KLRG1+ (cluster 6*) (Fig. 2b), consistent with the KLRG1+ T effector cells identified by scRNA-seq (cluster 6) (see Fig. 1d). CD103+ cells were split into CD39+ and CD39- clusters (1* and 0*), which corresponded to scRNA-seq clusters 1 and 0. Flow cytometry also identified a unique cluster of CD103~ KLRG1- cells (U) that lacked all tested markers and thus could not match any subset identified by scRNA-seq (Fig. 2a).

Among CD4+ T cells, Th17 were marked by CD161 (Fig. 2c, d) and spanned clusters 2* and 4*, which were distinguished...
IEL show an aberrant representation of T cell subsets in CD.

To examine the impact of CD on IEL T cells, we quantified percentages of CD8⁺, CD4⁺, and γδ T cells in a large cohort of patients (Controls: n=33–34; CD NI: n=19–20; CD II: n=19) by flow cytometry (Fig. 3a–c, Supplementary Data 5 and Source Data 1). CD8⁺ T cells were significantly decreased in CD patients at the inflamed site (Fig. 3a), a finding that was further supported by immunohistochemistry data (Supplementary Fig. 3c–f). CD8⁺ T cell attrition was paralleled by a significant increase in CD4⁺ T cells (Fig. 3b). In addition, γδ T cells were significantly reduced in the inflamed tissue of CD patients, as compared to controls (Fig. 3c). We further examined whether CD impacted specific T cell subsets in a fraction of the larger cohort of patients described above (Controls: n=15; CD NI n=9; CD II n=9). Quantification of resident CD103⁺ CD8⁺ T cells and non-resident CD103⁻ KLRG1⁺ CD8⁺ T cells did not reveal major differences between CD and controls (Fig. 3d–f), indicating global rather than subset-specific reduction of CD8⁺ T cells in CD. In contrast, quantification of different CD4⁺ T cell clusters showed a significant increase of CD39⁺ CD4⁺ T cells, CD39⁺CCR6⁺ Tfh17 cells (Fig. 3g, h) and CD39⁺CD4⁺ Tfh17 within the non-resident CD103⁻ population (Fig. 3i) in CD patients, suggesting that these cells may have recently settled into the tissue. In addition, BTLA⁺ and TIGIT⁺ CD4⁺ T cells were decreased in CD patients (Fig. 3j, k), indicating reduced representation of Tfh1.
significant decrease in the relative proportion of CD25hi CD4+ T cells in CD, which may correspond to Tregs (Fig. 3l). Altogether, these results suggested that the IEL compartment of CD patients exhibits an aberrant T cell landscape. Notably, some CD-associated alterations were evident in the non-inflamed tissue. The non-inflamed tissue may exhibit early cellular changes reflecting ongoing disease without any macroscopic sign of inflammation and may predict risk of recurring inflammation.

Reclustering reveals T cell subsets with unique features. To further dissect T cell heterogeneity within the major IEL populations, we reclustered scRNA-seq data of three pairs of clusters (see Fig. 1a). These included tissue-resident CD8+ T cells from a representative donor showing expression of CD103, CD39, and KLRG1. This revealed the relationship with clusters observed by scRNA-seq. Asterisk illustrates the different experimental approach. U; undefined. b t-SNE clustering of IEL CD8+ T cells from three representative donors showing expression of CD161, CD39, GP130, TIGIT, BTLA, PD-1, ICOS, and CD25.

Reclustering reveals T cell subsets with unique features. To further dissect T cell heterogeneity within the major IEL populations, we reclustered scRNA-seq data of three pairs of clusters (see Fig. 1a). These included tissue-resident CD8+ T cells from a representative donor showing expression of CD103, CD39, and KLRG1. As opposed to clusters 0–10 and 0–13, this cluster did not exhibit CD39 or NK cell receptors but expressed the epithelial membrane protein 3 (EMP3) (Fig. 4b and Supplementary Fig. 4a). The small cluster 0–14 contained CD8+ T cells marked by CD40LG and a network of p53 regulators, such as EIF5A, MDM4, and SET (Fig. 4b and Supplementary Fig. 4a). CD8+ T cell clusters 0–14, 0–13, and 0–12 produced the S100A family members A4, A6, and A10, which have antimicrobial functions (Supplementary Fig. 4a).

We also re-analyzed the cluster pair 5 and 8 including TFH and Treg. This analysis identified six clusters (Fig. 4c). Clusters 5–81 and 5–84 represented two distinct clusters of TFH, both of which expressed the chemokine CXCL13 and the TFH master transcription factor BCL6 (Fig. 4d and Supplementary Fig. 4b). However, 5–85 uniquely expressed CXCR5, TNFSF8, and the G-protein-coupled receptor P2RY8. Conversely, cluster 5–83 expressed CYSLTR1 (Fig. 4d and Supplementary Fig. 4b). Cluster 5–84 also expressed BTLA, as well as unique genes, such as amyloid precursor protein (APP), MS4A6A, and SERPINE2 (Supplementary Fig. 4b). Cluster 5–83 also expressed BTLA, as well as unique genes, such as amyloid precursor protein (APP), MS4A6A, and SERPINE2 (Supplementary Fig. 4b). Cluster 5–83 also expressed BTLA, as well as unique genes, such as amyloid precursor protein (APP), MS4A6A, and SERPINE2 (Supplementary Fig. 4b). Cluster 5–83 also expressed BTLA, as well as unique genes, such as amyloid precursor protein (APP), MS4A6A, and SERPINE2 (Supplementary Fig. 4b).
shared expression of PRDM1 with Treg, but showed residual expression of CXCR5 and CXCL13; this subset may reflect a T<sub>FH1</sub>-Treg plasticity reported both in human and mouse<sup>35</sup>. Cluster 5–8 encompassed cells expressing the γδ<sub>T</sub>TCR transcripts TRCG1 and TRCG2, as well as CD8A, CCL5, and the cytotoxic mediators GZMA and GZMB (Supplementary Fig. 4b). These cells may be γδ<sub>T</sub> cells or CD8<sup>+</sup> αβ<sub>T</sub> cells expressing TRCG1 and TRCG2 transcripts<sup>32</sup>. Noteworthy, a few cells in this cluster expressed FOXP3. Because of a lack of specific markers, cluster 5–8 could not be explicitly identified.

We finally re-analyzed the CD4<sup>+</sup> T cell cluster pair 2–4, because it contained a small group of CD8A<sup>+</sup> cells among a large population of T<sub>H17</sub> cells (see Fig. 1c). Reclustering of the 2–4 cluster pair generated four distinct subsets (Fig. 4e). Cluster 2–4<sup>0</sup> corresponded to activated cytokine-secreting IL23R<sup>+</sup> T<sub>H17</sub>, whereas cluster 2–4<sup>+</sup> expressed CXCR4, GPR183, and CXCR3, which indicate quiescent T<sub>H17</sub><sup>23</sup> (Fig. 4f and Supplementary Fig. 4c). Cluster 2–4<sup>−</sup> belonged to the γδ<sub>T</sub> cell lineage, as indicated by TRCG2 and CD8A expression. These cells expressed RORC, IL17A, and IL26, explaining the initial co-clustering with T<sub>H17</sub> cells and suggesting that they may represent a discrete subpopulation of γδ<sub>T</sub> cells with a type 17 polarization (Fig. 4f). This γδ<sub>T</sub> T cell subset was also marked by NCR3 (Fig. 4f). To validate this γδ<sub>T</sub> T cell subset, we stained IEL with a mAb for...
TCRVδ2, followed by an antibody that recognizes all γδ TCRs, and a mAb for NKp30 (patient information in Supplementary Data 6). Among Vδ2− γδ+ T cells, about 25–50% of the cells expressed NKp30 in different individuals (n = 9), suggesting that NKp30+ γδ T cells represent approximately half of the TCRVδ2− Vδ1+ γδ T cells (Fig. 4g, h and Supplementary Fig. 4d). All of these cells expressed CD39 (Fig. 4h). In contrast, NKp30 was not expressed by terminal ileum CD8+ TCRγδ− T cells (Fig. 4i and Supplementary Fig. 4d), contrary to what shown for colonic CD8+ T cells of UC patients10.

To validate the functional relevance of NKp30 on this unique γδ T cell subset, we sorted all γδ T cells from control patients and tested the expression of RORγt on NKp30+ vs. NKp30− cells. As shown in Fig. 4j, NKp30+ γδ T cells expressed higher level of RORγt than NKp30−. We further expanded NKp30+ γδ T cells in vitro to perform functional assays. Expanded γδ T cells
maintained high levels of NKp30 expression (Fig. 4k) and produced IL-26 upon engagement of NKp30 with a cognate antibody (Fig. 4l). Because of the reported antibacterial properties of IL-26, our results suggest that this discrete γδ T cell subset may have a protective role in intestinal homeostasis.

T cell clusters in LP are differentially activated and represented compared to IEL. We next surveyed the baseline heterogeneity of CD3+ cells in the LP by scRNA-seq of 29,247 cells sorted from the same CD and control samples analyzed for IEL (Supplementary Fig. 1a–c, e; patient information in Supplementary Data 1). Unsupervised clustering of scRNA-seq data from LP T cells by UMAP identified nine clusters (Fig. 5a, b). Three T cell clusters expressed CD8A (1, 2, and 8), six expressed CD4 (0, 3, 4, 5, 6, and 7) (Fig. 5c), while one (9) expressed both and was enriched in cell cycle genes, such as MKI67, STMN1, TUBB, and TUBA1B (Fig. 5b, d). Within CD8A+ clusters, cluster 1 showed the hallmarks of effector CD8+ T cells: KLRG1 (Fig. 5d), EOMES, TBX21, CCL4, CCL3, CCL5, IFNG, PRF1, GZMB, GZMA, GZMH, and GZMK (Supplementary Fig. 5a, b). This cluster was reminiscent of IEL cluster 6. Cluster 2 and 8 expressed the HVEM receptor CD160 and ITGA1 (Fig. 5d and Supplementary Fig. 5c), and the chemokines XCL1 and XCL2 (Supplementary Fig. 5b). Cluster 8 expressed the NK cell receptors KLRC1, KIR2DL4, KLRC2, KLRC1, TRDC, and ENTPD1 (Fig. 5b, d and Supplementary Fig. 5b). Overall, LP clusters 2 and 8 paralleled IEL clusters 0 and 1, respectively.

Among CD4 T cells, cluster 0 corresponded to Th17 based on expression of CCR6, RORA (Fig. 5d), KLRB1, LTB, and CCL20 (Supplementary Fig. 5b, c). Only few cells on the top of the cluster were enriched in genes indicative of effector function, such as IL17A, IL2RA, and TOX2.
Severe CD modifies the T cell landscape in the LP. To validate scRNA-seq analysis of LP and quantify differences related to disease status, we performed mass cytometry on fresh LP T cells of CD (NI: n = 9; II n = 6) and control patients (n = 8) (patient information: Supplementary Data 7). Dimensionality reduction by viSNE analysis enabled to solve T cell heterogeneity in 20 different clusters. Notably, mass cytometry identified more clusters than scRNA-seq analysis, suggesting that this technique may better distinguish cell subsets that express shared markers at different levels. Eight clusters expressed CD8 (15, 8, 17, 16, 2, 4, 18, and 12), ten clusters expressed CD4 (14, 5, 13, 10, 9, 7, 6, 3, 1, and 20), a small cluster of γδ T cells (19) expressed neither CD4 or CD8, and a tiny cluster (11) remained undefined (Fig. 6a, b). CD103 distinguished tissue-resident CD8+ and CD4+ T cells from circulating cells. CD103− non-resident CD8+ T cells included clusters 15 and 8, which corresponded to naïve CD45RAhi and memory CD45RO+ CD8+ T cells, respectively. CD103+ resident CD8+ T cell clusters 18, 2, 4, and 12 were separated based on expression of CD161 (16 and 2), CD39 (16), CD94 and NKG2A (12), and CCR4 (18) (Fig. 6a, b). Cluster 17, which mapped close to clusters 15 and 8 between circulating and tissue-resident cells, exhibited low/intermediate levels of CD103 (Fig. 6b), suggesting that it may represent recently immigrated CD8+ T cells settling into the tissue.

Within CD4+ T cells, CD103+ clusters 1, 3, 6, and 20, corresponded to circulating memory T17 cells expressing CD161, CD45RO, and CD127 (Fig. 6a, b). The four clusters were distinguished based on the expression of CD39, CCR4 (6), CD27 (6 and 3), and CD226 (1 and 20) (Fig. 6a, b). The expression of CD226 may be suggestive of pathogenicity, as CD226 has been implicated in autoimmunity46. Clusters 5 and 14 represented tissue-resident memory T17 because of the expression of CD103, CD45RO, and CD161. As in IEL, these cells were split in CD39+ and CD39− subsets. Cluster 7 consisted of Treg as indicated by high CD25 and CD39 expression (Fig. 6b), while cluster 9 included TFH cells based on high TIGIT expression41 (Fig. 6b). Clusters 13 represented naïve CD45RAhi CD45RO− CD4+ T cells, while cluster 10 was marked by expression of CD45RO and loss of CD45RA, indicative of a memory phenotype (Fig. 6a, b). Finally, cluster 20 was distinguished based on CD56 expression (Fig. 6b), which is typical of cytokine-induced killer cells.

Quantification of different LP clusters in CD and control patients revealed an opposite trend than what observed in IEL, with a significant increase in CD8+ T cells paralleled by a decrease in CD4+ T cells (Fig. 6c, d; fresh samples triangles). These data were confirmed by flow cytometry in a different cohort of frozen samples from control (n = 9) and CD (n = 11) patients and compared to the CyTOF data (Fig. 6c, d; frozen samples circles; patient information: Supplementary Data 7 and Source Data 2). Among CD8+ T cells, CD103+ resident cells were increased in CD compared to controls (Fig. 6e), as evidenced by expansion of clusters 2, 4, 12, and 16 (Fig. 6f-i). Although total CD4+ T cells were reduced in CD, T17 clusters of 5 and 14 were significantly increased at the non-inflamed site of CD lesions (Fig. 6f). On the contrary, Treg (7) and Tfh (9) were decreased (Fig. 6k, l). The increase in T17 cells (CD4+CCR6+CD161+), and decrease of Tfh (TIGIT+) was further confirmed by running CITRUS analysis (Supplementary Fig. 7a–c). Taken together, our data suggested that inflammatory T17 and CD8+ T cells are increased in the LP of CD, paralleled by an attrition of CD4+ T cells with regulatory properties, consistent with deepened gut wall inflammation.

Discussion

Through a high-resolution analysis of human intestinal IEL T cells in controls and severe adult CD, our study defined a vast heterogeneity of T cell lineages in the IEL compartment, including various subsets of CD8+, γδT, and CD4+ T cells. Compared to controls, CD was associated with major abnormalities in the composition of IEL T cells, which included: (a) an increase in inflammatory CD39+ T17; (b) a decrease in Treg, which might exacerbate inflammation; (c) a decrease in Tfh, which may explain the impaired mucosal IgA production previously reported in CD42, and (d) a global reduction of CD8+ T cells and γδ T cells. These changes in the IEL compartment were coupled with increased CD8+ T cells and T17, as well as reduced Tfh and Treg in the LP, likely reflecting the deepening of inflammation and overt transmural damage1,2. Overall, despite some limitations of our study due to cohort heterogeneity of CD patients, our results offer insight into T cell correlates of transmural inflammation and relapsing/recurrent disease.

The remarkable heterogeneity of human T cells identified in the IEL compartment reflected the presence of different T cell lineages, as well as various stages of differentiation, activation, and tissue residency within each lineage. T17 cells included quiescent cells and cells capable of immediate effector functions, which were readily distinguished based on the mutually exclusive expression of CCR4 and CD39, among other distinctive markers. IEL CD39+ T17 may be a two-edged sword. On one hand, they exhibit pathogenic features, such as expression of GZMB and CCL4, suggesting that they may be cytotoxic against epithelial cells and recruit inflammatory cell types that promote tissue destruction. On the other hand, production of IL-17 and IL-26...
may enhance barrier function, providing protection. Given that CD39 has been shown to sustain T<sub>FH</sub> survival by degrading proapoptotic ATP released in the intestinal environment<sup>43</sup>, CD39 may sustain survival of the TH17 cells. Further increasing diversity, TH17 expressed different levels of CD103<sup>+</sup>, indicating the presence of TH17 at different stages of migration ranging from circulating to tissue-resident.

**Fig. 6** Quantification of CD8 and CD4 T cell clusters by CyTOF analysis in LP of control and CD patients. a Schematic t-SNE of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from LP of all donors concatenated together (n = 18) controls (N), n = 8; CD, non-inflamed site (NI), n = 9; CD, inflamed site (II), n = 6. Total of 23 samples. b t-SNE of the indicated markers in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. c, d Quantification of total CD8<sup>+</sup> (c), total CD4<sup>+</sup> (d) in LP of controls and CD patients by CyTOF (triangles = fresh samples) and FACS (circles = frozen samples). e, d Control (N), n = 17 (8 fresh, 9 frozen); CD, non-inflamed site (NI) n = 18 (9 fresh, 10 frozen); CD, inflamed site (II), n = 14 (6 fresh, 8 frozen). e-i Quantification of total CD8<sup>+</sup> T<sub>RM</sub> (e) and CD8<sup>+</sup> clusters 2 (f), 4 (g), 12 (h), and 16 (i) in LP of controls and CD patients by CyTOF. j-l Quantification of the CD4<sup>+</sup> clusters 5 and 14 (j), 7 (k), and 9 (l) in LP of controls and CD patients by CyTOF. e-i Controls (N), n = 8; CD, non-inflamed site (NI), n = 9; CD, inflamed site (II), n = 6. Circles and triangles on the boxplots show data collected for each individual donor. Data were median and interquartile range. Significance was calculated using an ordinary, one-way ANOVA, multiple comparisons test with Prism v8 software. c **P = 0.0014; d *P = 0.028; e P = 0.0139; f P = 0.0178; h *P = 0.0178; i *P = 0.0219; j N vs. NI *P = 0.0156, NI vs. II *P = 0.0465; k **P = 0.0014; l **P = 0.0283. T<sub>RM</sub> tissue-resident memory T cell. Source data are provided as a Source Data file (Source Data 2).
markers: P2RY8+ TFH expressed CXCR5 and TNFSF8 (for CD30L); CysLT1R+ TFH expressed BTLA and CD200. P2RY8 mediates the retention of TFH and B cells in the germinal center, while BTLA restrains TFH germinal center responses. TNFSF8 gene polymorphisms have been associated with risk of CD. Although IEL TFH did not show mRNA for the B cell stimulatory cytokine IL-21, P2RY8+, and CysLT1R+ TFH subsets may control mucosal B cell responses through other mechanisms, such as BTLA–HVEM and TNFSF8–TNFRSF18 interactions.

IEL CD8+ T cells were also quite heterogeneous, including canonical CD103+KLRC1+ cytotoxic CD8+ T cells and multiple subsets of tissue-resident CD8+ T cells, which shared the expression of CD103 and CD160 that may secure retention of T cells through binding to E-cadherin and HVEM on epithelial cells, respectively. Moreover, CD160 may shape the function of CD8+ T cells by inducing IFN-γ. Tissue-resident CD8+ T cells included a subset of IL-7R+TCF7+ cells producing the DC chemotactants XCL1 and XCL2, which may recruit DCs from the LP. Other resident CD8+ T cell subsets were prone to effector functions; one subset expressed IFN-γ and the Tc1 NK receptor KLRC1 specific for HLA-E; another expressed cytotoxicity mediators, lacked NK receptors but expressed the membrane protein EMP3, which has yet unknown function in immune responses. Finally, a group of resident CD8+ T cells expressed EGF5A, MDM4, and SET, which control p53 activity, suggesting evasion from apoptosis and senescence. Many tissue-resident CD8+ T cells expressed S100A family members, which may contribute to antimicrobial functions.

One remarkable result of this study is the identification of at least two subsets of γδ T cells within the IEL. One subset expressed Tigit17 markers, such as RORC, IL-23R, IL-22, and IL-26. RORγt+ γδ T cells have been extensively described in mouse but not in human. Distinctive features of these γδ T cells included the expression of TCRV61, CD39, and NKP30, an activating cell surface receptor specific for B7H6. Importantly, NKP30+ γδ T cells expressed higher levels of RORγt ex vivo as compared to NKP30+ γδ T cells and engagement of NKP30 resulted in IL-26 production, suggesting that these cells may have a protective function during homeostasis. Some traits of these γδ T cells, such as NKP30 expression and IL-26 production, were recently reported in a subset of CD8 T cells expanded in colon of UC patients. Whether these colonic cells are bona fide CD8 T cells or γδ T cells expressing CD8, as we find in the small intestine, remains to be established. Another γδ T cell subset expressed the canonical γδ T cell transcription factor ID3, this subset expressed NK cell receptors and produced CSF1 and PDGFD, pointing to a potential crosstalk with macrophages and epithelial cells. While subsets of human γδ secreting CSF1 have been reported, PDGFD secretion by γδ T cells has not been described before. In addition, a rare cell subset expressing γ-constant region of γδTCR and CD8A expressed FOXP3. Future studies will be required to precisely identify these cells and to test their regulatory function.

Notably, our cohort of severe adult CD showed a reduction of IEL CD8+ T cells and γδ T cells. Consistently, a population of CD39+CD8+ T cells and γδ T cells has been recently reported to decrease in colonic mucosa biopsies of pediatric CD patients. This population overlaps with the γδ and CD8+ T cell subsets expressing NK cell receptors and CD39 reported here. While we observed a global reduction rather than a selective loss of specific subsets of CD8 and γδ T cells, this discrepancy may depend on differences in patient ages (children vs. adults), sampling location (colon vs. ileum), type of specimen (biopsies vs. surgical resections) or degree of disease.

Finally, analysis of LP T cell transcriptomes identified unique functional features not immediately related to the classical T cell functional modules. A subset of LP CD4+ T cells expressed heat-shock induced stress-pathway genes and cytokines, such as TNF and IL-2. A T cell subset expressing heat-shock proteins was also among five IEL populations recently reported in CD. Intriguingly, a recent study showed that febrile temperature in mice induces Tigit17 differentiation and augments pathogenicity through heat-shock response genes. Together, these observations highlight the involvement of heat-shock response in the differentiation of T cells in the intestine. Expression of GPX1 in Tregs of the LP revealed the activation of anti-oxidative pathways in intestinal T cells, which may be particularly relevant to CD, as GPX1 gene polymorphisms have been associated with risk of CD. These results will prompt future studies to determine the impact of these unique functions in CD pathogenesis.

Methods
Preparation of single-cell suspension from intestinal samples. Single-cell suspension was prepared as previously described. Briefly, mucosal tissue from terminal ileum was separated from the muscular layer and serosa and cut into 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 5 mM ethylenediaminetetraacetic acid (EDTA). Cells were filtered through 100-μm cell strainers and diithiothreitol (DTT) 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. Cells were collected, sorted, and processed for scRNA-seq or collected and stained for CyTOF. From a set of patients cells were processed and frozen for later CyTOF or flow cytometry analysis. 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 the Institutional Review Boards of Washington University. All ileum samples were provided as surgical waste with no identifiers attached on written informed consent to the Digestive Disease Research Cores Center at Washington University. The demographic data provided in this study will not allow patient identification.

Immunohistochemistry. Formalin-fixed paraffin embedded tissue blocks used for this study were retrieved from the tissue bank of the Department of Pathology (ASIT, Spedali Civili di Brescia, Brescia, Italy). Four-micron thick tissue sections were used for immunohistochemical staining. Sections were incubated with anti-human CD4 (clone 4B12 1:50 Thermo Scientific) and anti-human CD8 antibody (clone C8-144B Agilent 1:50) and the reaction was revealed using Novolink Polymer (Leica Microsystems). For double staining, after completing the first immune reaction, the second was visualized using Mach 4 MR-AP (Biocare Medical), followed by Ferangi Blue. Finally, the slides were counterstained with Meyer’s Haematoxylin.

Antibodies. Information on the antibodies used for flow cytometry and sorting is available in Supplementary Data 8.

Flow cytometry and sorting. Cells were sorted on BD FACs Aria II and flow cytometry analyses were performed on BD Symphony A3 instrument. Data were analyzed by FlowJo software v10.7.1 (TreeStar).

scRNA-seq and data analysis. T cells were sorted from processed IEL and LP as CD45+ lymphocyte gate, singlets, and alive CD3+ CD19+ expression. Sorted cells were sequenced using 10X Genomics platform with chemistry version 2. Cell Ranger pipeline was used to process Chromium single cell RNA-seq output to align reads and generate cell expression matrices. Briefly, short-sequencing reads were aligned to the GRCh38 reference genome and Ensembl transcriptome by STAR. The uniquely aligned reads were used to quantitate gene expression levels for all Ensembl genes. We filtered out low-quality cells from the dataset if the number of genes detected was <500 or >3,000, or the percentage of expression reads was >50%. We filtered on condition that the number of expression reads usually consumed a large fraction of reads in our dataset, and their relative abundance varied significantly from sample to sample. Such genes were not interesting in our research, and thus were excluded for downstream data analysis. Additionally, all genes that were not detected in at least 1% of all our single cells were discarded. Average UMI were 2960 and 3020 for IEL and LP T cells, respectively.

scRNA-seq downstream analysis. Downstream analyses were performed using Seurat R software package version 3.0 (http://satijalab.org/seurat/). After removing
unwanted cells and genes from the dataset, raw UMIs in each cell were first scaled by library size and then log-transformed. To improve downstream dimensionality reduction and clustering, we first regressed out unwanted source of variation arising from the number of detected molecules. Then highly variable genes were identified and selected for PCA reduction of high-dimensional data. Cells in this reduced space were harmonized to adjust for batch effects coming from multiple donors including both normal and CD using the Harmony tool implemented in Seurat v3. The low dimensional corrected Harmony embeddings were used for downstream analyses. Graph-based clustering was performed on the reduced data for clustering analysis with Seurat v3. The resolution in the FindClusters function in Seurat was set to 0.6 and the clustering results were shown in a UMAP plot. For different cell types, cells were grouped based on top markers. MAST in Seurat v3 provided with this paper.

CyTOF acquisition and analysis. All antibodies information is available in Supplementary Data 9. Cells were washed with Cy-FACS buffer (CyPBS, Rockland, MB-008; 0.1% BSA, Sigma, A3059; 0.02% Sodium Azide, Sigma, 71289, 2 mM EDTA, Hoefer, GR123-100) stained on ice for an hour. After two washes cells were stained with cisplatin (Enzo Life Sciences, NC0503617) for 1 min, washed again twice, in 4% PFA (Electroscopy Microscopy Sciences, 15710) for 15 min, spun down and re-suspended in Interleukar-1r125 (Fluidigm, 201192A) overnight. Cells were washed and counted and analyzed on a CyTOF 2 mass cytometer (Fluidigm). Samples were manually gated using CytoBank. Background, dead cells (Cisplatin stained with cisplatin (Enzo Life Sciences, NC0503617) for 1 min, washed again twice, EDTA, Hoefer, GR123-100) stained on ice for an hour. After two washes cells were sorted from IEL of control patients and cell surface stained for NKp30, CD39, FoxP3 staining kit.

Statistical Analysis. Statistical analysis was performed using Graphpad Prism 8.4.3 (GraphPad Software, La Jolla, CA) or R version 3.6.2 (2019), as indicated in the figure legends. Data were presented as median and interquartile range. Unless otherwise noted, statistically significant differences between groups were determined by ordinary one-way ANOVA. In all figures, the following symbols were used to designate significance: *P < 0.05, **P < 0.01, ***P < 0.001.

Data availability

The scRNA-seq have been deposited in GEO under the GSE157477. Source data are provided with this paper.

Received: 30 October 2020; Accepted: 3 March 2021; Published online: 26 March 2021

References


Acknowledgements

We thank the Genome Technology Access Center at the McDonnell Genome Institute at Washington University for scRNA-seq on the 10X platform. The center is supported by the Washington University Institute of Clinical and Translational Sciences grant UL1TR002345 from the National Center for Advancing Translational Sciences (NCATS) of the National Institutes of Health (NIH). We thank E. Lantelme and D. Brinza and the Pathology and Immunology Flow Cytometry Core for cell sorting. We thank D. Bender, R. Lin, and S. Oh and the Center for Human Immunology at Washington University for help in processing the samples for mass cytometry and for advice on analysis. We thank the Washington University Digestive Disease Research Core (NIDDK P30 DK052574) for support. Author R.G. thanks Eric Fauman for supporting the completion of this work. N.J. was partly supported by the Lucille P. Markey Special Emphasis Pathway in Human Pathobiology. This work was in part supported by grants U01 AI095542, RO1 DE025884, RO1 AI134035, RO1 DK124699, and U19 AI42733 (to MCo).

Author contributions

N.J., R.G., and M.Co. designed, performed, analyzed, and interpreted experiments. J.L.S. helped with CyTOF and Citrus analysis. S.Z. designed the pipeline for scRNA-seq data analysis. BK collected patients’ data. B.D.L. helped in processing tissue. E.E. and M.N.A. helped with scRNA-seq analysis. W.V. and M.B. performed immunohistochemistry, C.V.R., T.A.W., and R.J.X. provided critical insights to the study. S.A.J. and M.Co. supervised the study. N.J., M.Ce., S.A.J., and M.Co. wrote the manuscript and all the authors contributed editing and suggestions.

Competing interests

R.G., S.Z., J.S., C.V.R., T.A.W., and S.A.J. are Pfizer employees. M.Co. received funding from Pfizer to study ILC biology in Inflammatory Bowel Disease. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22164-6.

Correspondence and requests for materials should be addressed to M.C.

Peer review information *Nature Communications* thanks the anonymous reviewer(s) for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/. 

© The Author(s) 2021