

INTESTINAL DISEASE

Single-cell profiling of environmental enteropathy reveals signatures of epithelial remodeling and immune activation

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Environmental enteropathy (EE) is a subclinical condition of the small intestine that is highly prevalent in low- and middle-income countries. It is thought to be a key contributing factor to childhood malnutrition, growth stunting, and diminished oral vaccine responses. Although EE has been shown to be the by-product of a recurrent enteric infection, its full pathophysiology remains unclear. Here, we mapped the cellular and molecular correlates of EE by performing high-throughput, single-cell RNA-sequencing on 33 small intestinal biopsies from 11 adults with EE in Lusaka, Zambia (eight HIV-negative and three HIV-positive), six adults without EE in Boston, United States, and two adults in Durban, South Africa, which we complemented with published data from three additional individuals from the same clinical site. We analyzed previously defined bulk-transcriptomic signatures of reduced villus height and decreased microbial translocation in EE and showed that these signatures may be driven by an increased abundance of surface mucosal cells—a gastric-like subset previously implicated in epithelial repair in the gastrointestinal tract. In addition, we determined cell subsets whose fractional abundances associate with EE severity, small intestinal region, and HIV infection. Furthermore, by comparing duodenal EE samples with those from three control cohorts, we identified dysregulated WNT and MAPK signaling in the EE epithelium and increased proinflammatory cytokine gene expression in a T cell subset highly expressing a transcriptional signature of tissue-resident memory cells in the EE cohort. Together, our work elucidates epithelial and immune correlates of EE and nominates cellular and molecular targets for intervention.

INTRODUCTION

Environmental enteropathy (EE) is a subclinical condition of the small intestine that is driven by an exposure to environmental enteropathogens (1). Also referred to as environmental enteric dysfunction, EE affects millions of children and adults around the world and is associated with stunted growth, neurocognitive impairment, reduced oral vaccine efficacy, and increased risk of metabolic syndrome (2–4). Water, sanitation, and hygiene interventions for preventing EE have proven ineffective, and ongoing work is assessing alternative therapeutic interventions (5). However, development of effective treatments

has been hindered by a limited understanding of the underlying pathophysiology of EE.

Pathologically, EE in the proximal small intestine is characterized by reduced villus height, greater crypt depth, and increased microbial translocation (1, 2, 6). However, in a study of Zambian children with EE and nonresponsive growth stunting over time, reduced villus height was associated with decreased microbial translocation (7). In agreement, a bulk transcriptomic study of Zambian children with enteropathy stratified participants by biopsy villus height and by measured microbial translocation and found a notable similarity between the genes up-regulated

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in biopsies with reduced villus height and the genes up-regulated in biopsies with reduced microbial translocation. These studies suggest that EE is an adaptive response to potentially lethal enteropathogen exposure that results in reduced absorptive capacity and impaired growth.

Histological analyses of intestinal tissue from patients with EE have revealed increased abundance of lymphocytes, reduced goblet cell numbers, and altered Paneth cell morphology (6). Low plasma concentrations of tryptophan in children with growth stunting (8) and the amelioration of villus blunting in Zambian adults with EE given amino acid supplementation suggest that amino acid deficiency plays a role in EE (9). Bulk transcriptomic studies of EE duodenal biopsies have revealed increased expression of nicotinamide adenine dinucleotide phosphate oxidases, chemokines, mucins, matrix metalloproteases, interferon (IFN)-stimulated genes, and antimicrobial genes including lipocalin-2 (*LCN2*), dual oxidase 2 (*DUOX2*), and dual oxidase maturation factor 2 (*DUOX2A2*) (10, 11). Whereas previous work has lacked the single-cell resolution required to localize these changes to specific epithelial and immune cell subsets, application of single-cell RNA-sequencing (scRNA-seq) could help to comprehensively resolve the cellular and molecular changes that underlie EE pathophysiology (12).

Here, we applied the Seq-Well S³ platform for massively parallel scRNA-seq (13) to characterize the expression profile of small intestinal biopsies from 11 adults from a community in Zambia where EE is known to be ubiquitous (14). Across these individuals, we profiled 27 biopsies spanning three small intestinal regions, patients who are HIV-positive and HIV-negative, and a range of EE severity scores. In addition, by comparing EE biopsies with those from control groups from South Africa and United States, we found that EE was associated with transcriptional signatures of up-regulated WNT and down-regulated mitogen-activated protein kinase (MAPK) signaling within the epithelium, as well as increased proinflammatory cytokine gene expression in a T cell subset highly expressing a transcriptional signature of tissue-resident memory cells. Together, our data provide insight into the epithelial and immune correlates of EE, suggesting several therapeutic targets for further investigation.

RESULTS

scRNA-seq of the proximal small intestine with and without EE

We collected 27 small intestinal biopsies from 11 Zambian volunteers with varying degrees of EE severity (eight were HIV-negative and three were HIV-positive) (Fig. 1A and table S3). For all 11, we profiled the duodenal bulb and distal duodenum; for a subset, we also collected jejunal samples (tables S1 and S2). Because of the widespread prevalence of EE in Zambia and a lack of existing screening methods to identify patients without EE, we could not obtain control biopsies from participants without EE in Zambia. Thus, as controls, we profiled samples from five adults recruited from a gastrointestinal unit in Durban, South Africa (two of which we profiled and three for which data were publicly available) (15, 16), and two cohorts of patients in Boston, USA, where EE can safely be assumed not to occur (tables S1 and S2). We note that EE is often contextualized to health by either comparing intermediate EE with severe EE (17) or comparing patients with EE to control cohorts in the United States or the United Kingdom (6). The validity of these international comparisons is supported by the environmental nature of EE and the resolution of EE in Peace Corps volunteers upon repatriation to the United States (18).

In total, we analyzed 26,556 high-quality single-cell transcriptomes across 38 samples from the Zambian, U.S., and South African cohorts (Fig. 1B). After data preprocessing, uniform manifold approximation and projection (UMAP) visualization of the entire dataset revealed differences in cellular distribution by patient cohort, intestinal region, and HIV status (Fig. 1C). To identify cellular subsets, we applied an existing pipeline for iterative clustering of cell subsets to the Zambian and U.S. datasets (19). Next, we integrated these data with the South African data to account for potential batch effects (20). These analyses revealed that the expected major cell types were represented in almost all biopsies and that epithelial cells were the most abundant major cell type (Fig. 1D). Along with more standard quality control (QC) metrics (figs. S1 to S4), this indicated consistent sample quality. In total, we identified 48 detailed cellular subsets that varied in abundance across samples (Fig. 1E, figs. S1 to S4, and table S4) (12, 21, 22).

Surface mucosal cells expressing *DUOX2* in the EE epithelium

When identifying cell subsets, we noticed a similarity between the marker genes of surface mucosal cells (a cell subset most commonly found in the distal stomach) and existing gene signatures of reduced villus height and decreased plasma lipopolysaccharide (LPS) concentrations in EE (fig. S5A) (17, 23). Relative to all other cell subsets, surface mucosal cells were significantly enriched for both gene signatures ($P < 10^{-15}$, Wilcoxon test) (Fig. 2, A and B, and fig. S5, B and C). In addition, the genes in these signatures overlapped with surface mucosal cell marker genes and three antimicrobial genes (*DUOX2*, *DUOX2A2*, and *LCN2*) recently identified as histological markers of EE (Fig. 2, C and D) (11, 23). Thus, our data suggest that these bulk gene signatures may have been driven by an increase in surface mucosal cell abundance. In our data, the vast majority of surface mucosal cells came from duodenal bulb samples of patients with EE (fig. S2C). In these tissues, surface mucosal cell fractional abundances were significantly correlated ($P < 0.05$, permutation test) with those of the stem cycling subset and the Ent *ANXA2* *PTMA* subset, which highly expressed marker genes of dedifferentiating enterocytes (*PTMA*) (24) and wound-associated epithelial cells (*ANXA2*) (Fig. 2E) (25). To further examine potential relationships between these cell subsets that cooccurred with surface mucosal cells, we inferred differentiation trajectories for the epithelial cells in our dataset by generating a partition-based graph abstraction (PAGA) map of the connectivity between epithelial cell subsets (Fig. 2F) (26). The wound healing-like epithelial subsets and surface mucosal cells all lay in between mature enterocyte and stem cell subsets in the inferred differentiation hierarchy. Running the RNA velocity package *Velocyto* produced similar results (fig. S5, D and E) (27). In addition, an immunohistochemical staining revealed that *DUOX2* localized to the villus tip in a blunted villi (Fig. 2G and fig. S6). Together, these results suggest that surface mucosal cells occur at the villus tip in EE and are associated with the presence of intermediate wound healing-like cell populations. Furthermore, we observed that surface mucosal cells uniquely expressed *MUC5AC*, a marker of *Helicobacter pylori* infection (fig. S5A and table S4) (28). Applying the metagenomic classification tool *Kraken2*, we found that, relative to the control cohort samples, six samples from four participants in the EE cohort contained significantly higher counts of *H. pylori* mapping reads (adjusted $P < 0.05$, Student's *t* test; fig. S5). These samples were predominantly from the duodenal bulb (fig. S5G). Thus, the presence of surface mucosal cells in EE may be associated with *H. pylori* infection.

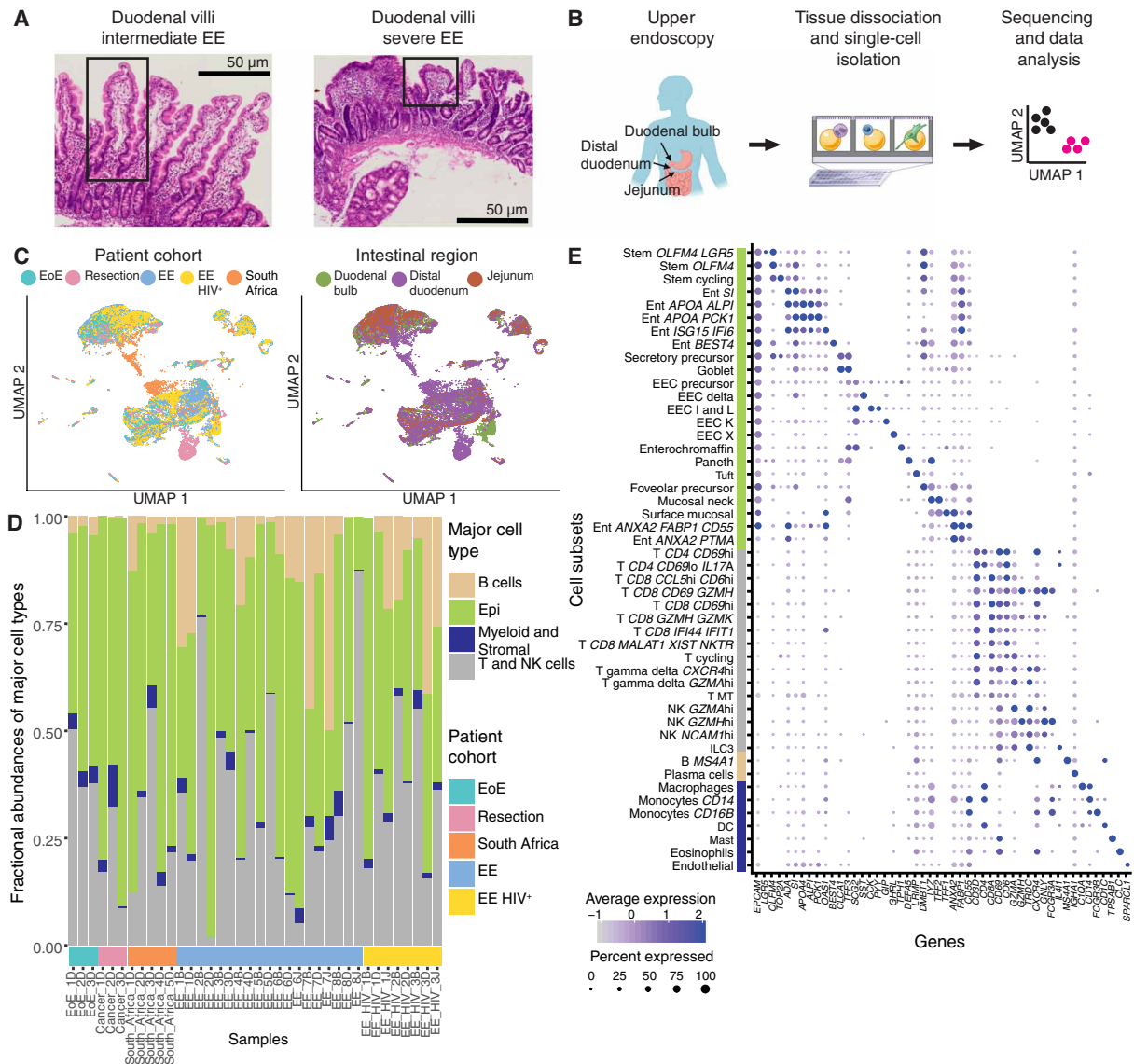


Fig. 1. scRNA-seq of the small intestine with and without EE. (A) H&E imaging of the small intestine with intermediate and severe EE. Intestinal villi with normal morphology (left image) and with severe blunting (right image) are highlighted in boxes. (B) Experimental workflow: Small intestinal biopsies from the duodenal bulb, distal duodenum, and jejunum were obtained via endoscopy or tissue resection, dissociated into cells, loaded onto a Seq-Well S³ array, processed for single-cell sequencing, and analyzed. (C) UMAP visualization by patient cohort and intestinal region for all 26,556 high-quality cells from 38 samples and 22 patients. (D) Fractional abundances of major cell types among all single cells analyzed per sample. NK, natural killer. (E) Expression of marker genes for final cell subsets. Dot size represents the fraction of a cell subset (rows) expressing a given gene (columns). Dot hue represents the scaled average expression by gene column. For clarity, dots for genes expressed in 5% or less of cells within a given subset are not shown.

Cellular correlates of intestinal region, disease severity, and HIV infection in EE

We next identified cell subsets whose fractional abundance shifted across intestinal region within HIV-negative patients with EE (Fig. 3A). Duodenal bulb samples were enriched for surface mucosal cells, mucosal neck cells, and enterocytes highly expressing *ANXA2*, *FABP1*, and *CD55*, as well as three T cell subsets expressing markers of immune activation (*IL17A*, *CXCR4*, and *GZMA*) (29, 30). Distal duodenal samples were enriched for enterocytes, goblet cells, and stem cells highly expressing *OLFM4*.

We then mapped the correlates of histologically determined EE severity in 11 biopsies (Fig. 3, B and C, Supplementary Materials

and Methods, and table S5) (6). In the epithelium, greater severity was associated with lower fractional abundances of the mature enterocyte and stem *OLFM4* subsets, as well as higher fractional abundances of immature enterocytes. In the immune compartment, greater severity associated with a higher abundance of two T cell subsets expressing markers associated with inflammation in the intestine (*GZMA* and *CD6*) (29) and one T cell subset with high expression of *MALAT1* and the lowest median number of unique molecular identifiers (UMIs) of all T cell subsets, which together suggest that this subset may represent low-quality preapoptotic cells (fig. S2D) (31). In line with past findings, plasma cell abundances decreased with EE severity (32). These results suggest that severe EE

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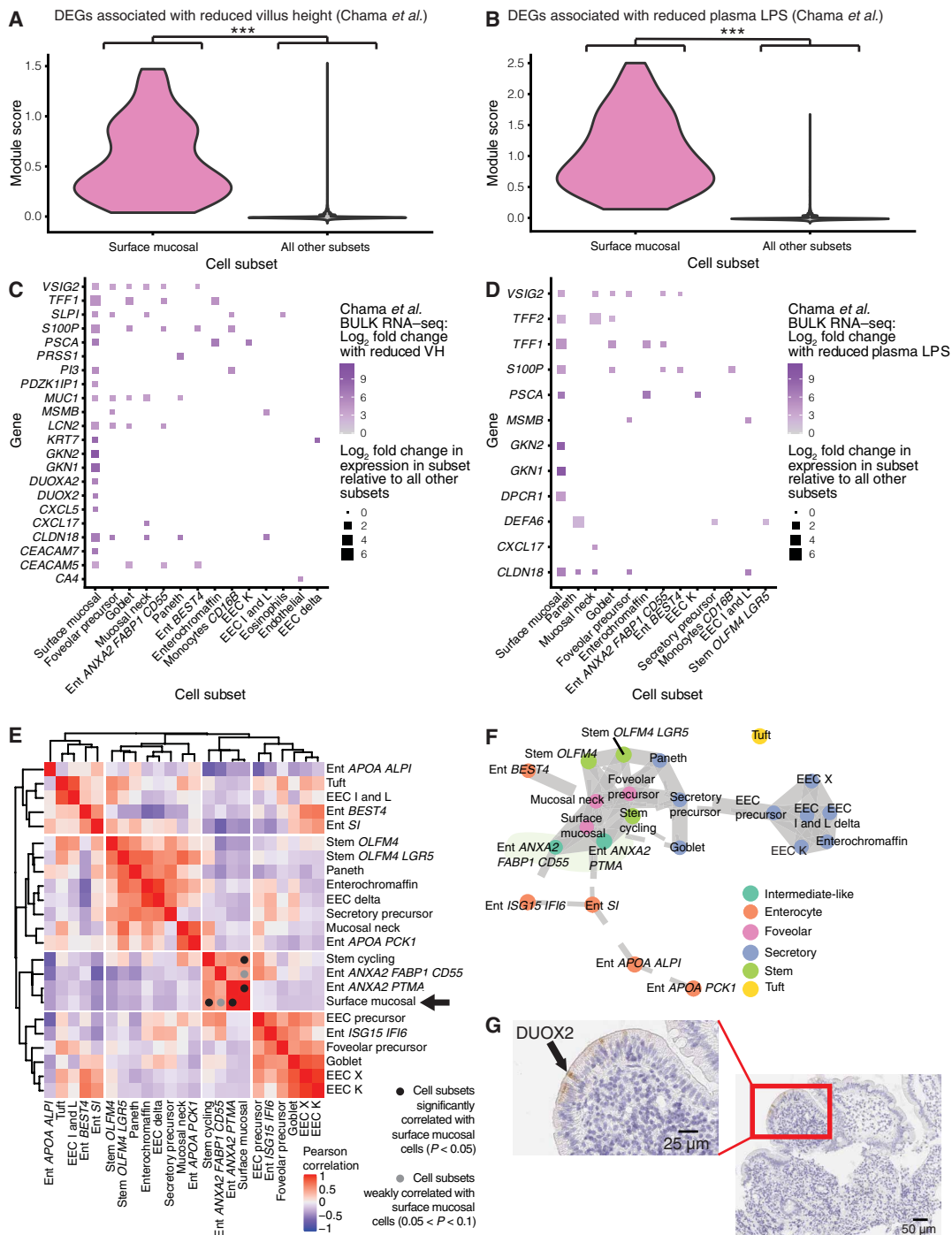


Fig. 2. Surface mucosal cells uniquely express *DUOX2* and are associated with a wound healing-like phenotype. (A) Violin plot of a module score generated from genes up-regulated in EE samples with reduced villus height (VH) in Chama *et al.* (17) ($***P < 0.001$, Wilcoxon test). (B) Violin plot of a module score generated from genes up-regulated in EE samples with decreased plasma LPS concentrations in Chama *et al.* (17) ($***P < 0.001$, Wilcoxon test). (C) Dot plot of cell subset marker genes that overlapped with the genes used to generate the module scores in (A). (D) Dot plot of cell subset marker genes that overlapped with the genes used to generate the module scores in (B). (E) Hierarchically clustered heatmap of the Pearson correlations between the fractional abundances of epithelial cells within duodenal bulb samples from patients with EE. Cell subsets that significantly correlated with surface mucosal cells are highlighted with a black circle ($P < 0.05$, permutation testing). Cell subsets weakly correlated with surface mucosal cells are highlighted with a gray circle ($0.05 < P < 0.1$, permutation testing). (F) PAGA trajectory visualization of epithelial subsets. (G) H&E (purple) and immunohistochemical staining for *DUOX2* protein (brown) on an EE biopsy from the duodenal bulb.

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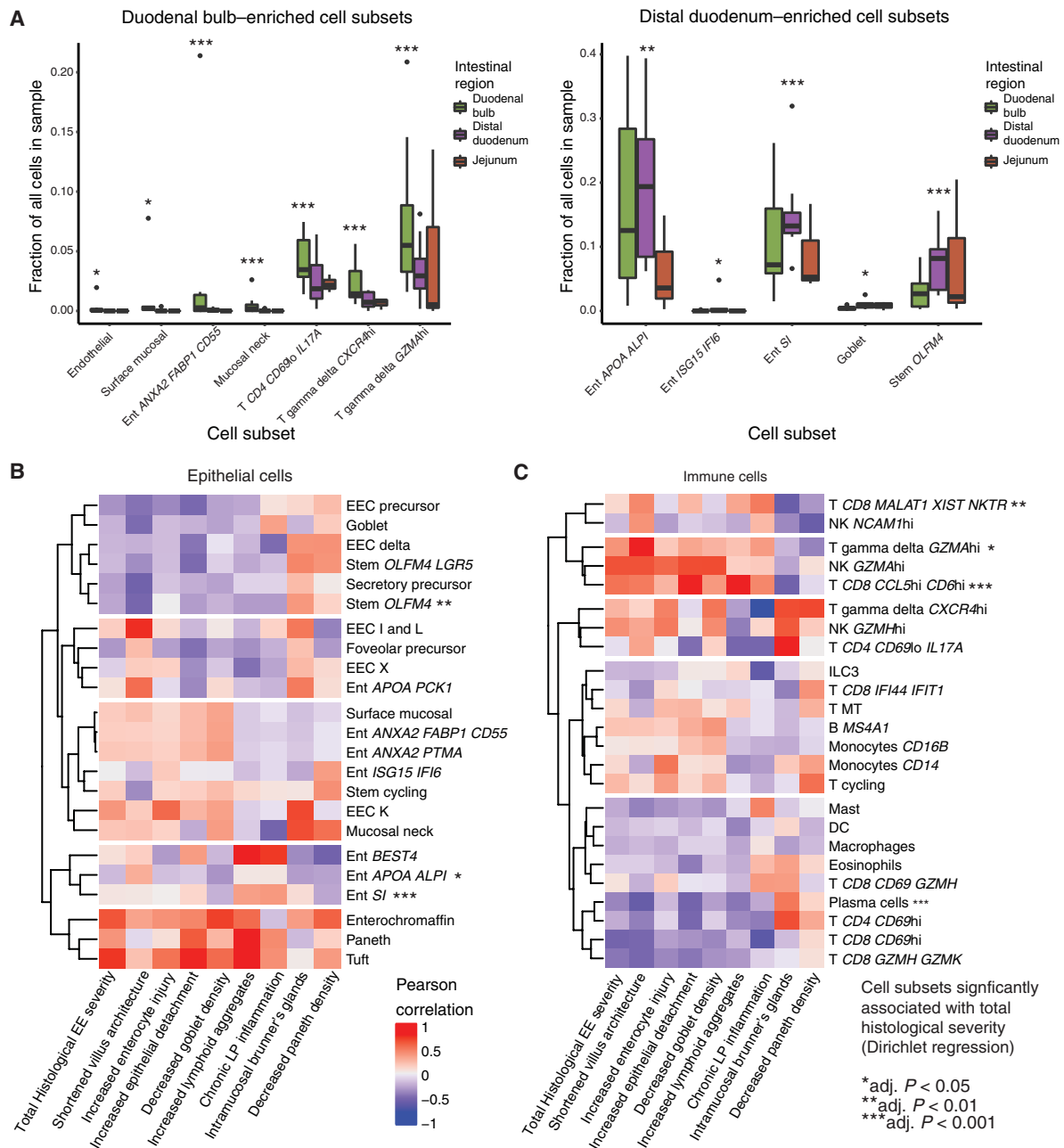


Fig. 3. Cell subsets associated with intestinal region and histologically determined EE severity. (A) Cell subsets enriched in duodenal bulb and distal duodenal samples from HIV-negative patients with EE (*adjusted $P < 0.05$, **adjusted $P < 0.01$, and ***adjusted $P < 0.001$, Fisher's exact test). (B) Hierarchically clustered heatmap of HIV-negative EE epithelial cell subset relative abundance Pearson correlations with component scores of the total EE histological severity score (*adjusted $P < 0.05$, **adjusted $P < 0.01$, and ***adjusted $P < 0.001$, Dirichlet regression). (C) Hierarchically clustered heatmap of HIV-negative EE immune cell subset relative abundance Pearson correlations with component scores of the total EE histological severity score (*adjusted $P < 0.05$, **adjusted $P < 0.01$, and ***adjusted $P < 0.001$, Dirichlet regression).

is associated with an intermediate-like epithelial phenotype and inflammatory lymphocyte subsets.

Next, we sought to characterize the impact of antiretroviral-treated HIV infection on EE pathology. We found that HIV-positive samples displayed higher EE severity than HIV-negative samples ($P = 0.034$, Wilcoxon test) (fig. S7A). Examining the shifts in cell subset fractional abundances with HIV infection, we found known features of HIV

biology including decreased fractional abundances of $CD4^{hi}$ T cells and increased fractional abundances of $\gamma\delta$ T cells highly expressing the HIV co-receptor *CXCR4* (fig. S7, B and C) (15). In addition, within duodenal bulb samples, HIV pathology associated with increased fractional abundances of enterocytes highly expressing *ANXA2*, *FABP1*, and *CD55*, suggesting that HIV pathology may contribute to the presence of this wound healing–like subset within the duodenal bulb (fig. S7D).

Epithelial correlates of EE

We next sought to identify features that distinguished HIV-negative EE distal duodenal samples from matched samples from participants in South Africa and United States. Although histology was not available for the South African dataset, hematoxylin and eosin (H&E) staining of a duodenal biopsy from a separate patient at the same clinical site revealed features of EE, including villus blunting, goblet cell depletion, and Paneth cell depletion, but no signs of inflammation (fig. S8A). To investigate whether samples from this site displayed features of EE, we performed a pairwise comparison of the fractional abundances of all cell subsets among the three geographical locations in this study (fig. S8, B to D). Relative to the U.S. cohorts, both the Zambian and South African cohorts displayed two characteristic features of EE: reduced goblet cell and increased plasma cell fractional abundances (6). However, plasma cells and T cell subsets expressing markers of inflammation (*IL17A* and *GZMA*) were increased in fractional abundance in the Zambia EE cohort relative to the South African cohort (29), suggesting that not all features of EE were present in the South African samples. Thus, we took two approaches to identifying the distinguishing attributes of confirmed EE in the Zambian cohort. First, we compared the Zambian cohort to all control cohorts. Then, we compared the Zambian cohort with confirmed EE to only the U.S. cohorts in case our previous analysis was confounded by potential features of EE in the South African cohort.

Comparing the fractional abundances of epithelial cells from patients with confirmed EE with those from all other cohorts, we found an enrichment of stem *OLFM4* cells, foveolar precursor cells, and enterocytes coexpressing *APOA4* and *ALPI* in EE, as well as reduced fractional abundance of enteroendocrine (EEC) K cells (Fig. 4A). Differential expression analysis between epithelial cells in EE and control cohorts revealed compartment-wide up-regulation of genes (*PIGR* and *CCL25*) involved in antibody transport and lymphocyte recruitment, among others (Fig. 4, B and C, and table S6) (33, 34). EE epithelial cells also highly expressed *CTNNB1*, a key component of WNT/ β -catenin signaling. In agreement, we inferred pathway activity based on pathway-responsive genes (PROGENY) annotated in the PROGENY database and found increased WNT signaling in all three stem cell subsets and decreased MAPK signaling in cycling stem cells and stem *OLFM4* cells in EE (Fig. 4D) (35). To help corroborate and extend these findings, we immunohistochemically stained Zambian EE and U.S. control samples for β -catenin. We found that β -catenin was stained at higher intensity in the EE epithelium (fig. S9). In addition, tuft cells in EE highly expressed *ALOX5AP*, which is involved in inflammation via leukotriene biosynthesis (36), and *SERPINA1*, which encodes α -1 antitrypsin, a biomarker of epithelial damage in EE (Fig. 4C) (5). Last, comparison of our data to past intestinal scRNA-seq datasets revealed no evidence for “colonification” of the small intestine in EE and showed limited overlap between the genes up-regulated in EE and ulcerative colitis (fig. S10).

Immune correlates of EE

Next, we compared cell proportions of immune cells between the Zambian cohort with confirmed EE and all control cohorts. This revealed that EE samples were enriched for *CD8^{hi}* T cells highly expressing *MALAT1* and $\gamma\delta$ T cells highly expressing *GZMA* (Fig. 5A). Consistent with previous findings, plasma cells were increased in EE relative to non-EE cohorts (fig. S10C) (32). Conducting differential expression analyses, we found that the majority of significant gene expression changes in EE immune cells occurred within the

T cell compartment (adjusted $P < 0.05$, Wilcoxon test; Fig. 5B and fig. S11, A to C). Of all T cell subsets, T *CD8 CD69^{hi}* cells displayed the most differentially expressed genes between EE and controls, including up-regulation of effector-like genes (*IFNG*, *CCL5*, and *IL32*) in EE and down-regulation of genes (*IL7R* and *CXCR4*) promoting memory T cell formation after infection (Fig. 5C and table S6) (37, 38). Because *CD69* is a potential marker for T cell tissue residency, we scored all T cell subsets on a gene signature of tissue-resident memory T cells and found that the T *CD8 CD69^{hi}* subset scored the highest (adjusted $P = 4.98 \times 10^{-78}$, one-sided Wilcoxon test; Fig. 5D) (39). Scoring all T *CD8 CD69^{hi}* cells on T cell activation signatures, we found that, relative to controls, cells in this subset from EE scored higher for signatures of cytotoxicity and cytokine production (Fig. 5E) (40). In agreement, immunohistochemical staining revealed more cells positive for granzyme B in EE samples relative to controls (fig. S12). Nominating putative ligand-receptor interactions between cellular subsets with the NicheNet, algorithm and database for inferring intercellular communication, we found potentially increased IFN γ signaling in the EE epithelium stemming from IFN γ production by T cells, especially the T *CD8 CD69^{hi}* cells (fig. S13) (41). Together, our data reveal immune correlates of EE that may contribute to pathogenesis and reduced oral vaccine efficacy.

Evidence of reduced epithelial proliferation in EE relative to the U.S. cohorts

Last, we compared only the Zambian EE and U.S. cohorts. Differential expression analysis revealed that EE samples displayed compartment-wide down-regulation of genes (*KLF4*, *ATF3*, *FOS*, and *JUN*) involved in epithelial proliferation, interleukin-22 (IL-22) signaling, and goblet cell development (Fig. 6A) (42–44). Consistently, the EE samples displayed lower fractional abundances of goblet cells and type 3 innate lymphoid cells ILC3s (producers of IL-22), as well as higher fractional abundances of $\gamma\delta$ T cells (negative regulators of IL-22 production in mice fed a low-protein diet) (fig. S8B) (45, 46). Furthermore, gene set enrichment analysis (GSEA) revealed an enrichment of the reactome signature for response of Eukaryotic translation initiation factor 2-alpha kinase 4 (EIFAAK4) and general control non-repressible 2 (GCN2) to amino acid deficiency in the epithelial cells (Fig. 6B and table S8). In addition, goblet cells from patients with confirmed EE up-regulated markers for lower crypt goblet cells, suggesting that EE goblet cells have a more immature phenotype (Fig. 6C) (47). Furthermore, EE stem cells scored significantly lower on a gene signature of cycling human cells and displayed lower PROGENY scores for the proliferative epidermal growth factor receptor (EGFR), MAPK, and phosphatidylinositol 3-kinase (PI3K) pathways (adjusted $P < 0.05$, Wilcoxon test; Fig. 6, D and E, and table S8) (48). We also inferred transcription factor activity by scoring cells for genes annotated as being downstream of a given transcription factor in the DoRothEA database (49). This revealed the reduced activation of activating transcription factor 2 (ATF2) and ATF4 broadly across the epithelium, consistent with reduced IL-22 signaling (fig. S14A and table S9). In summary, our results suggest that, relative to the U.S. controls, the EE epithelium is characterized by reduced proliferation, IL-22 signaling, and goblet cell development. However, within the EE cohort, EE severity scores correlated with cycling scores in stem cells (fig. S1B). This suggested that although patients with EE as a whole display evidence of decreased epithelial proliferation relative to U.S. controls, more severe EE leads to relatively higher epithelial proliferation than less severe EE.

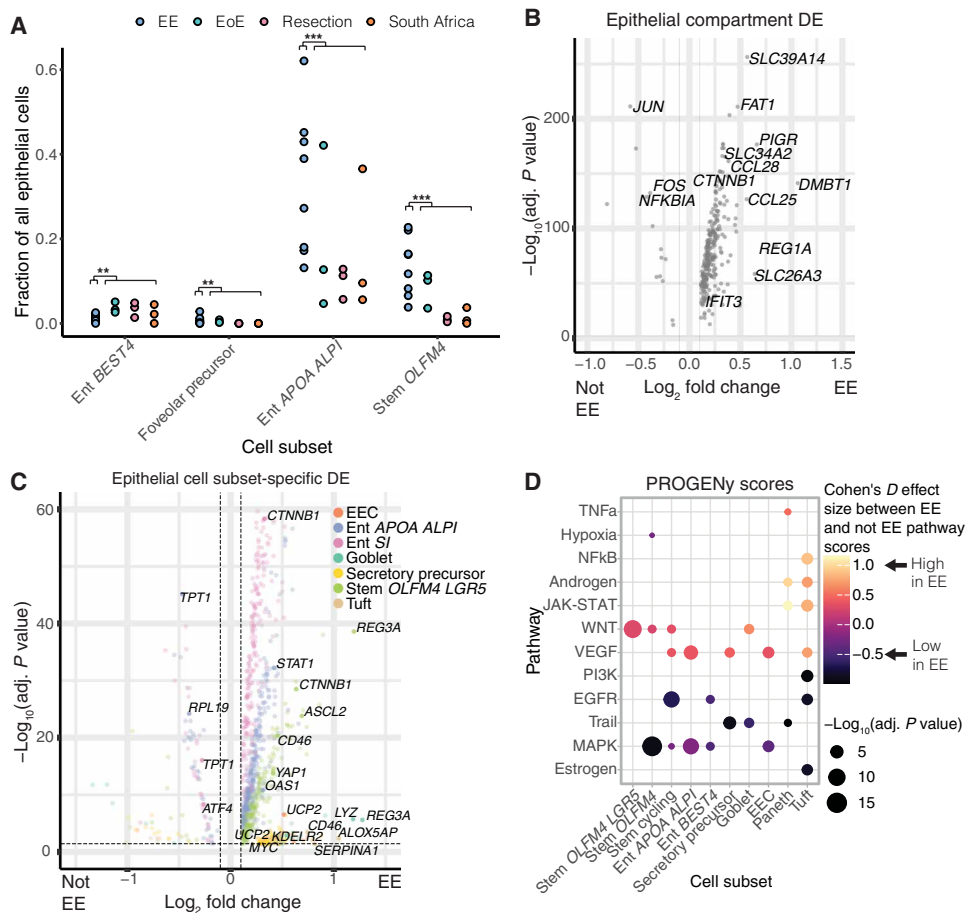


Fig. 4. The epithelium of EE is characterized by increased WNT signaling and decreased MAPK signaling. (A) Cell subsets with significant shifts in relative abundances between EE and all control cohorts. (*adjusted $P < 0.05$, **adjusted $P < 0.01$, and ***adjusted $P < 0.001$, Fisher's exact test). (B) Genes differentially expressed in the epithelial compartment in EE relative to all control cohorts. Horizontal and vertical lines respectively refer to an adjusted P value threshold of 0.01 and a log fold change threshold of 0.1. (C) Genes differentially expressed in EE relative to all control cohorts within specific cellular subsets. Horizontal and vertical dashed lines respectively refer to an adjusted P value threshold of 0.01 and a log fold change threshold of 0.1. (D) PROGENy pathway prediction scores for epithelial cells in EE relative to controls. TNF α , tumor necrosis factor- α ; NFkB, nuclear factor κ B; JAK-STAT, Janus kinase-signal transducers and activators of transcription; VEGF, vascular endothelial growth factor.

DISCUSSION

Here, we profiled EE with the Seq-Well S³ platform for scRNA-seq. We thereby identified a cell subset, surface mucosal cells, which highly expressed *DUOX2* and whose gene expression pattern matched existing bulk gene signatures of reduced villus height and reduced plasma LPS concentrations in EE. In addition, our dataset revealed variations in cell subset fractional abundance by small intestinal region, HIV infection, and EE severity, as well as epithelial and immune subsets differing between EE and control samples. Together, our work recontextualizes past bulk transcriptomic studies of EE and maps the cellular correlates of EE pathology.

The presence of surface mucosal cells highly expressing *DUOX2* in EE may reflect remodeling of the epithelium into an intermediate wound healing-like state. Dedifferentiation of mature cells could facilitate repair of the epithelial barrier and reduce microbial translocation at the expense of reducing surface area and absorptive capacity, explaining why reduced nutrient absorption in EE is associated

with decreased microbial translocation (7). This process may be due in part to *H. pylori*: Because *H. pylori* gastritis has been shown often to be associated with duodenal colonization in children (50), *H. pylori* infection may explain why previous studies have found high expression of *DUOX2* transcripts in the distal duodenum of some children with EE, whereas our study found *DUOX2* expressing surface mucosal cells in the duodenal bulb (which is closest to the stomach where most *H. pylori* infection occurs) but not the distal duodenum of adults with EE.

Comparison to all control cohorts revealed the epithelial and immune cell correlates of EE. Increased abundances of immature epithelial cell subsets, increased WNT/ β -catenin signaling, and decreased MAPK signaling suggested that the EE epithelium is biased toward an immature phenotype. Furthermore, Tuft cells up-regulated genes involved in promoting and responding to inflammation (51). In addition, we found lower relative abundance of T cells expressing a transcriptional signature of tissue-resident memory T cells, but those cells had elevated expression of inflammatory cytokines (including IFN γ) in EE, suggesting that although these cells are present in a lower abundance in EE, they may be chronically activated. This, in turn, may lead to immune exhaustion and impaired responses to new immune stimuli, which may contribute a hindered response to oral vaccines (52). Although IFN γ is often viewed as a proinflammatory cytokine in acute inflammation, numerous studies have demonstrated that in chronic inflammation, IFN γ can produce tolerogenic effects, which would be consistent

with reduced oral vaccine efficacy in EE (53).

In addition, we compared EE to only the U.S. cohorts to account for potential confounding features of moderate EE in the South African cohort. Relative to the U.S. cohorts, the EE epithelium was characterized by decreased epithelial proliferation and changes in cell subset fractional abundances, consistent with decreased IL-22 signaling (45, 46, 54). This is in line with previous work showing decreased abundances of transcripts from proliferative pathways in the feces of Malawian children with EE (55) and work showing that during *Cryptosporidium* infection, protein malnutrition leads to reduced turnover of intestinal epithelial cells (56). Consistently, reduced epithelial proliferation in EE relative to the U.S. cohorts was associated with GSEA enrichment of a response to amino acid deficiency and reduced goblet cell abundances, whose differentiation can be induced by tryptophan (57). This agrees with a previous study demonstrating decreased tryptophan metabolism in Pakistani children

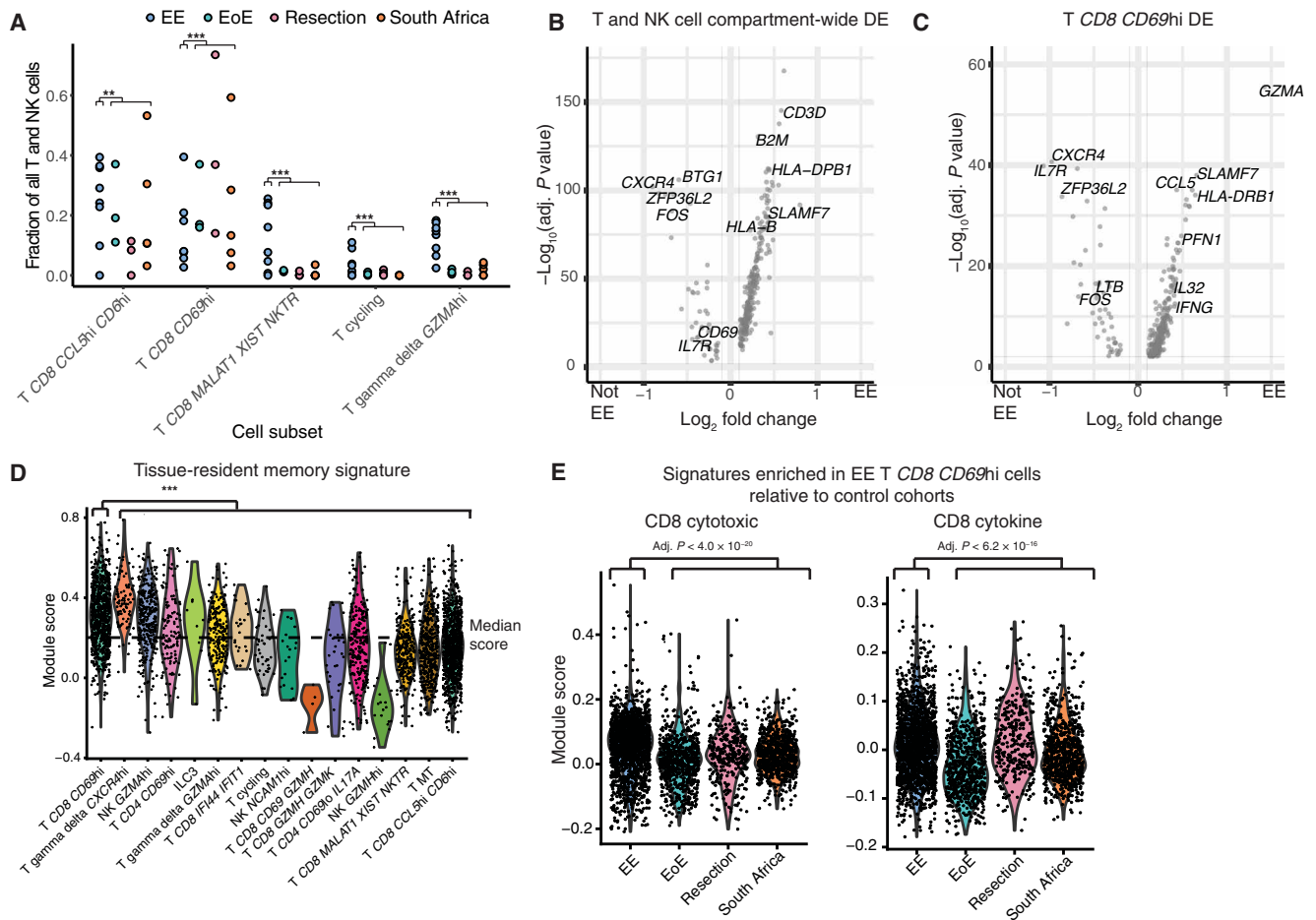


Fig. 5. EE is associated with a shift toward activated T cell phenotypes. (A) Cell subsets with significant shifts in relative abundances between EE and all control cohorts (*adjusted $P < 0.05$, **adjusted $P < 0.01$, and ***adjusted $P < 0.001$, Fisher's exact test). (B) Genes differentially expressed in the T and NK cell compartment in EE relative to all control cohorts. Horizontal and vertical lines respectively refer to an adjusted P value threshold of 0.01 and a log fold change threshold of 0.1. (C) Genes differentially expressed in EE relative to all control cohorts within specific cellular subsets. Horizontal and vertical lines respectively refer to an adjusted P value threshold of 0.01 and a log fold change threshold of 0.1. (D) Module scores for a tissue-resident memory T cell signature in EE relative to all control cohorts. (E) T cell activation signatures enriched in T $CD8 CD69^{hi}$ cells from patients with EE relative to all control cohorts. Proliferation: adjusted $P = 3.2 \times 10^{-04}$, Cohen's D effect size = 0.32; CD8 cytotoxic: adjusted $P = 6.2 \times 10^{-25}$, Cohen's D effect size = 0.95; CD8 cytokine: adjusted $P = 2.3 \times 10^{-23}$, Cohen's D effect size = 0.90.

with EE and with data showing that amino acid supplementation ameliorates villus blunting in adults with EE (9, 11). Thus, amino acid deficiency may lead to hypoproliferative signaling in the EE epithelium characterized by reduced stem cell proliferation and goblet cell abundance. One intriguing aspect of this hypoproliferative signaling is that it stands in stark contrast to the hyperproliferative signaling observed in Crohn's disease, which is of particular interest, because limited evidence suggests lower rates of inflammatory bowel disease in countries where EE is endemic (15, 49).

However, when comparing within the EE cohort, more severe EE was positively associated with stem cell proliferation. This may be due to the interplay of malnutrition and infection. The reduced dietary quality in the population of Zambian adults we studied may impose a proliferative constraint on enteropathy, leading to reduced stem cell turnover relative to intestinal homeostasis. However, individuals with more severe EE may have more proliferation than others in response to infective and inflammatory drivers. This discrepancy between our within- and across-country analyses is congruent with past work, which found similar discrepancies, highlighting the necessity

of comparing to an out-group to fully understand the pathophysiology of EE (32, 58). Follow-up mechanistic studies are needed to clarify the role that malnutrition and infection play in epithelial proliferation in EE.

It is important to recognize that our study has several inherent limitations. We were not able to include a non-EE control group of age-matched adults in Lusaka, Zambia. Thus, we cannot rule out the possibility that the observed differences between patients with EE and the U.S. and South African cohorts are due to unobserved variables that differed between these patient populations, especially the high burden of enteropathogens in tropical settings. In addition, our findings are primarily correlative due to the associative nature of measuring mRNA expression and the difficulties associated with mechanistic follow-up validation in humans. This is further limited by the lack of available tissue for histological analysis of samples from the South African cohort. Because we saw few stromal cells in our scRNA-seq dataset, our tissue dissociation was likely biased against this subset, and future work will be needed to characterize these cells in EE. In addition, the inflamed small intestinal epithelium

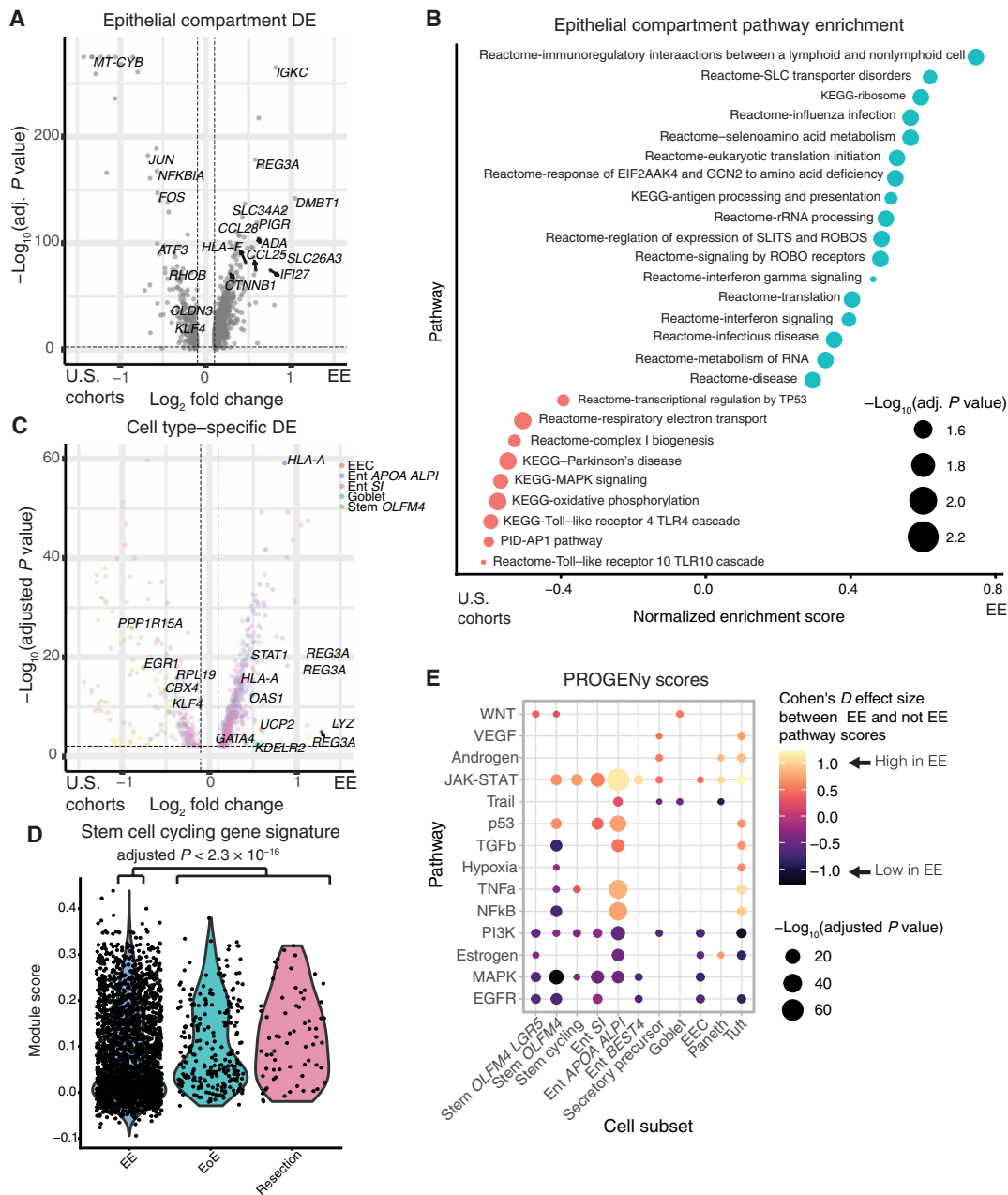


Fig. 6. Evidence of reduced proliferation in the EE epithelium relative to U.S. cohorts. (A) Genes differentially expressed in the epithelial compartment in EE relative to the U.S. cohorts. Horizontal and vertical dashed lines respectively refer to an adjusted *P* value threshold of 0.01 and a log fold change threshold of 0.1. (B) Gene set enrichment analysis of genes up-regulated in epithelial compartment cells in EE relative to the U.S. cohorts. (C) Genes differentially expressed in EE relative to the U.S. cohorts within specific cellular subsets. Horizontal and vertical dashed lines respectively refer to an adjusted *P* value threshold of 0.01 and a log fold change threshold of 0.1. (D) Module scores for cell cycle genes in EE and U.S. cohorts in all cells from stem cell subsets. (E) PROGENy pathway prediction scores for epithelial cells in EE relative to U.S. cohorts.

and lamina propria are highly heterogeneous environments containing several relatively rare cell types such as Paneth cells and a variety of immune subsets that we did not have sufficient power to analyze in great detail. Furthermore, the HIV status of the participants from the U.S. cohorts was not determined. In addition, we did not screen patients with EE for Celiac disease and cannot completely rule out the possibility that some patients may have had Celiac disease; however, we note that, in Zambia, the staple diet is maize (a gluten-free

food) and that past studies of EE in Zambian adults have found no evidence of Celiac disease (59). In addition, pediatric EE may differ from EE in adults, which calls for future studies in pediatric cohorts. Last, because EE is an endemic condition in low- and middle-income countries across the globe, it will be necessary to validate our results in cohorts with EE from geographic locations other than Zambia.

Examining our work as a whole, a potential picture of EE pathogenesis emerges. Relative amino acid deficiency due to a low-quality

diet may lead to reduced epithelial proliferation and differentiation toward goblet cells, which would diminish antimicrobial mucosal defense, leading to increased pathogen-mediated damage of the enterocyte. This may then lead to epithelial remodeling toward an intermediate wound healing-like phenotype associated with the presence of surface mucosal cells. In addition, enteropathogen-mediated damage would further exaggerate the pathogen-induced IFN γ response and may explain the observed proinflammatory polarization of CD8 CD69^{hi} T cells in our data. Together, these findings nominate several therapeutic axes for inducing healthy epithelial proliferation and immune efficacy in EE.

MATERIALS AND METHODS

Study design

Adult volunteers were recruited from a disadvantaged community in Lusaka, Zambia, in which we have carried out previous studies of EE (7, 10). All volunteers gave a written, fully informed consent. The study was approved by the University of Zambia Biomedical Research Ethics Committee (reference 006-11-15, dated 12 January 2016). From July to August 2018, volunteers underwent endoscopy with a Pentax 2990i gastroscope or a Pentax VSB2990i enteroscope in the endoscopy unit of the University Teaching Hospital, Lusaka, under sedation with diazepam and pethidine. Duodenal tissue was collected from patients with eosinophilic esophagitis (EoE) undergoing surveillance gastroscopy at Massachusetts General Hospital (MGH), Boston. Informed consent was obtained from patients with EoE under a protocol approved by MGH. Resection samples were obtained from patients undergoing duodenal resection for pancreatic cancer (but in whom no local spread was apparent) in accordance with MGH Institutional Review Board guidance under Mass General Brigham Protocol 2010P000632. Informed consent was obtained from participants recruited into this study at the Inkosi Albert Luthuli Central Hospital in Durban, South Africa. No randomization or blinding was done in this study. No power analyses were conducted because of the observational nature of this study and the lack of preexisting scRNA-seq datasets of EE. The number of samples used is presented in the figures, supplementary figures, and supplementary tables.

Biopsy handling, immunohistochemical staining, and tissue digestion

Biopsies from the patients with EE were collected into normal saline, then orientated under a dissecting microscope, fixed in buffered formal saline, and processed to 3- μ m sections for H&E staining. These sections were scanned using an Olympus VS120 scanning microscope, measured for average villus height and crypt depth, and scored for EE severity using a recently published methodology (6). Duodenal bulb samples from patients with EE were stained for DUOX2 protein, and distal duodenal samples from patients with EE and from normal tissue obtained from Mass General Brigham were stained for β -catenin and granzyme B protein; for more details, see Supplementary Materials and Methods. Biopsies from patients with EE, EoE, and resection and from South African individuals were dissociated into single-cell suspensions using a modified version of a previously published protocol (21). For further detail, see Supplementary Materials and Methods.

scRNA-seq with Seq-Well S³

Please refer to Supplementary Materials and Methods for further detail. Briefly, the epithelial and lamina propria layers of the biopsies

were dissociated into single-cell suspensions. Then, single cells were loaded onto a functionalized polydimethylsiloxane array preloaded with uniquely barcoded mRNA capture beads (ChemGenes, MACOSKO-2011-10), and sequencing libraries were obtained and sequenced on an Illumina NextSeq. Sequencing read alignment and demultiplexing was performed on the cumulus platform using snapshot 6 of the Drop-seq pipeline (48), resulting in a cell barcode by UMI digital gene expression matrix. QC was performed to remove low-quality cell barcodes and doublet cells. Bam files from sequencing were classified with Kraken2 to find metagenomic mapping reads. To identify cell subsets, we adopted an existing pipeline for automated iterative clustering of single-cell data that has been shown to identify batch effects without collapsing distinct rare cell types (19). We applied this pipeline to the data from the EE and U.S. cohorts. To correct for batch effects between data collected in different laboratories, we integrated our data with the South African dataset (20). All subsets were scored on gene signatures of reduced villus height and decreased and plasma LPS signatures from Chama *et al.* (17) using the AddModuleScore function in Seurat, and a Wilcoxon test was used to assess significance. Epithelial trajectories were inferred with PAGA (26). RNA velocity trajectories were inferred with Velocity (27).

Analyses examining variation within samples from the EE cohort

To assess the epithelial subsets associated with surface mucosal cells in duodenal bulb samples from patients with EE, we calculated the Pearson correlation between the fractional abundances of all epithelial subsets within these subsets. We then hierarchically clustered the resulting correlations with the ComplexHeatMap R package. Changes in the relative abundances of cell subsets by differing HIV infection status and small intestinal region were detected by a leave-one-out approach to avoid identifying patient-specific effects using a Fisher's exact test. Cell subsets that significantly associated with histological EE severity were identified by running Dirichlet regression. Further details are provided in Supplementary Materials and Methods.

Comparison of distal duodenal samples from HIV-negative EE and control cohorts

Distal duodenal samples from HIV-negative patients with EE were compared with matched samples from control cohorts. We sought to identify biological features driven by variation in EE biology relative to the control cohorts (as opposed to identifying biology that distinguished only one cohort from EE). Thus, in all subsequent analyses, we required that results pass the following two criteria: (i) result that is significant when comparing EE versus all control cohorts and (ii) result that displayed the same direction of change between EE and each control cohort. Further details are provided in Supplementary Materials and Methods.

Statistical analysis

The statistical test used for each comparison is denoted in the corresponding figure legend. Tests were conducted in R, and a Benjamini-Hochberg adjusted *P* value of 0.05 was used for significance.

SUPPLEMENTARY MATERIALS

www.science.org/doi/10.1126/scitranslmed.abi8633
Materials and Methods
Figs. S1 to S14

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[View/request a protocol for this paper from Bio-protocol.](#)

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Single-cell profiling of environmental enteropathy reveals signatures of epithelial remodeling and immune activation

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Profiling environmental enteropathy

Single-cell RNA sequencing (scRNA-seq) has been shown to be a powerful tool for understanding the pathophysiology of numerous disorders. Here, Kummerlowe *et al.* applied scRNA-seq on small intestine biopsies from patients with environmental enteropathy (EE), an intestinal subclinical condition prevalent in low-income countries, caused by exposure to environmental enteropathogens. The authors identified common pathways associated with the disease- and cell-type contribution to EE severity. Samples from patients with EE showed increased proinflammatory molecules and alterations in WNT- and MAPK-associated signaling, suggesting that targeting these pathways could be effective for treating EE.

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