



Original Article

# $\alpha$ E $\beta$ 7 Integrin Identifies Subsets of Pro-Inflammatory Colonic CD4<sup>+</sup> T Lymphocytes in Ulcerative Colitis

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Conference presentation: Earlier versions of this work were presented at European Crohn's and Colitis Organisation [ECCO], Digestive Diseases Week [DDW] and British Society of Gastroenterology [BSG] conferences in 2014: Lamb CA, Mansfield JC, Tew GW, *et al.* Ulcerative colitis: The  $\alpha$ E $\beta$ 7 integrin is associated with a high frequency of Th17, Th1 and Th17/Th1 CD4 lymphocytes.

*J Crohns Colitis* 2014;8 Suppl 1:S18–19.

*Gastroenterology* 2014;146 Suppl 1:S826.

*Gut* 2014;63 Suppl 1:A71.

## Abstract

**Background and Aims:** The  $\alpha$ E $\beta$ 7 integrin is crucial for retention of T lymphocytes at mucosal surfaces through its interaction with E-cadherin. Pathogenic or protective functions of these cells during human intestinal inflammation, such as ulcerative colitis [UC], have not previously been defined, with understanding largely derived from animal model data. Defining this phenotype in human samples is important for understanding UC pathogenesis and is of translational importance for therapeutic targeting of  $\alpha$ E $\beta$ 7–E-cadherin interactions.

**Methods:**  $\alpha\text{E}\beta 7^+$  and  $\alpha\text{E}\beta 7^-$  colonic T cell localization, inflammatory cytokine production and expression of regulatory T cell-associated markers were evaluated in cohorts of control subjects and patients with active UC by immunohistochemistry, flow cytometry and real-time PCR of FACS-purified cell populations.

**Results:**  $\text{CD}4^+\alpha\text{E}\beta 7^+$  T lymphocytes from both healthy controls and UC patients had lower expression of regulatory T cell-associated genes, including FOXP3, IL-10, CTLA-4 and ICOS in comparison with  $\text{CD}4^+\alpha\text{E}\beta 7^-$  T lymphocytes. In UC,  $\text{CD}4^+\alpha\text{E}\beta 7^+$  lymphocytes expressed higher levels of IFN $\gamma$  and TNF $\alpha$  in comparison with  $\text{CD}4^+\alpha\text{E}\beta 7^-$  lymphocytes. Additionally the  $\text{CD}4^+\alpha\text{E}\beta 7^+$  subset was enriched for Th17 cells and the recently described Th17/Th1 subset co-expressing both IL-17A and IFN $\gamma$ , both of which were found at higher frequencies in UC compared to control.

**Conclusion:**  $\alpha\text{E}\beta 7$  integrin expression on human colonic  $\text{CD}4^+$ T cells was associated with increased production of pro-inflammatory Th1, Th17 and Th17/Th1 cytokines, with reduced expression of regulatory T cell-associated markers. These data suggest colonic  $\text{CD}4^+\alpha\text{E}\beta 7^+$  T cells are pro-inflammatory and may play a role in UC pathobiology.

**Key Words:** ulcerative colitis; inflammatory bowel disease;  $\alpha\text{E}\beta 7$  integrin; CD103; etrolizumab; intraepithelial lymphocytes; Th1; Th17; Th17/1; regulatory T cell; Treg; cytokine;  $\text{CD}4^+$  T cell; mucosal immunology; effector T cell; helper T cell

## 1. Introduction

Expression of the  $\beta 7$  integrin on lymphocytes mediates critical functions in mucosal immunity. This subunit is only present in two heterodimeric integrins,  $\alpha 4\beta 7$  and  $\alpha\text{E}\beta 7$ .<sup>1</sup> Therapeutic targeting of  $\alpha 4\beta 7$ :MAdCAM-1-mediated lymphocyte trafficking to the gut in inflammatory bowel disease [IBD] has been clinically validated. Treatment with natalizumab, a humanized monoclonal antibody directed against the  $\alpha 4$  integrin subunit, demonstrated clinical efficacy in Crohn's disease,<sup>2,3</sup> but was also associated with progressive multifocal leukoencephalopathy,<sup>4</sup> presumably resulting from impaired  $\alpha 4\beta 1$  integrin-mediated lymphocyte homing to the central nervous system and/or mobilization of  $\text{CD}34^+$  JC virus-containing stem cells from bone marrow.<sup>5</sup> More recently, vedolizumab, a humanized monoclonal antibody specific for the  $\alpha 4\beta 7$  integrin heterodimer, demonstrated efficacy in both Crohn's disease and UC.<sup>6,7</sup>

In addition to the  $\alpha 4\beta 7$  integrin heterodimer,  $\beta 7$  integrin can also pair with  $\alpha\text{E}$  [CD103]. The resulting  $\alpha\text{E}\beta 7$  integrin heterodimer is predominantly expressed on mucosal lymphocytes and dendritic cells,<sup>8</sup> where it plays a role in cellular retention through adhesive interactions with E-cadherin expressed on the surface of epithelial cells.<sup>9</sup> Transcription of the  $\alpha\text{E}$  integrin subunit is induced by transforming growth factor [TGF] $\beta 1$ ,<sup>10</sup> which is highly expressed in mucosal tissues by epithelial cells,<sup>11</sup> suggesting that differentiation to and maintenance of an  $\alpha\text{E}\beta 7^+$  phenotype occurs in proximity to the epithelial compartment. In support of this, >85% of intraepithelial lymphocytes [IELs] and ~40% of lamina propria lymphocytes [LPLs] in the small intestine are  $\alpha\text{E}\beta 7^+$ .<sup>12</sup>  $\alpha\text{E}$ -deficient mice have reduced numbers of IELs and LPLs in the intestine, while Peyer's patches [PPs] and splenic lymphocyte populations remain intact.<sup>13</sup> Additionally,  $\beta 7$ -deficient mice lack both  $\alpha 4\beta 7$  and  $\alpha\text{E}\beta 7$  integrin expression and have severely impaired gut-tropic lymphocyte migration, leading to poorly developed PPs with diminished cellularity.<sup>14</sup>

Although  $\alpha\text{E}\beta 7^+$  cells have been studied in mouse models,<sup>15-22</sup> there are only limited data on these cells in the human gut, and the role of these cells in human colitis is not understood. Several studies in mice have highlighted  $\alpha\text{E}$  expression as a marker of distinct and highly suppressive regulatory T cell [TReg] populations, although suppressive activity is also observed in  $\alpha\text{E}^-$  populations.<sup>15-18</sup> Highlighting the potential for  $\alpha\text{E}$  to mark effector populations in murine studies,  $\alpha\text{E}$  blockade can be beneficial in mouse models of

colitis,<sup>19</sup> graft-versus-host disease-associated intestinal inflammation<sup>20</sup> and solid organ transplant.<sup>21,22</sup> The role that  $\alpha\text{E}\beta 7^+$  T cells play in the normal and inflamed human intestine is still unclear.

Etrolizumab is a humanized monoclonal antibody specific for the  $\beta 7$  integrin subunit and therefore targets both intestinal lymphocyte recruitment and retention by binding, respectively, the  $\alpha 4\beta 7$  and  $\alpha\text{E}\beta 7$  integrins. This is in contrast to the mechanism of action of natalizumab and vedolizumab that target lymphocyte recruitment alone by binding  $\alpha 4$ - and  $\alpha 4\beta 7$ -mediated homing, respectively. The additional blockade of  $\alpha\text{E}\beta 7$  integrin represents a unique potential mechanism of action, and this therapeutic approach has shown efficacy in moderate to severely active UC.<sup>23,24</sup> The relative impact of etrolizumab-mediated  $\alpha 4\beta 7$  vs  $\alpha\text{E}\beta 7$  integrin blockade on patient response is at present unclear. However, a retrospective analysis of Phase II data in UC observed increased clinical remission among patients with higher expression levels of  $\alpha\text{E}$  at baseline in comparison to patients with lower levels of  $\alpha\text{E}$  at baseline, suggesting that  $\alpha\text{E}^+$  cells may be predictive for response to drug therapy.<sup>24,25</sup> Understanding the role of  $\alpha\text{E}\beta 7^+$  T cells in humans is essential to safely and most effectively use etrolizumab therapy in the correct patients and to the best advantage.

To better understand the role of these cells in intestinal inflammation, we evaluated expression of inflammatory and regulatory markers in  $\alpha\text{E}\beta 7^+$  and  $\alpha\text{E}\beta 7^-$  colonic T lymphocytes in patients with active UC in comparison to healthy control subjects. This analysis of  $\alpha\text{E}\beta 7^+$  and  $\alpha\text{E}\beta 7^-$  colonic T lymphocytes will help to understand the role of the  $\alpha\text{E}$  integrin in disease pathogenesis, as well as offering insight into the mechanism of etrolizumab and the potential role of this integrin as a biomarker for therapeutic stratification in IBD.

## 2. Methods

### 2.1. Participants and disease stratification

Patients with active UC on stable therapy and healthy control subjects [Table 1] were enrolled in prospective tissue repositories at Newcastle University and King's College London [KCL], UK. Specimens from the Newcastle cohort were investigated for protein expression by immunohistochemistry and flow cytometry, and specimens from the KCL cohort were investigated for gene expression by quantitative polymerase chain reaction [qPCR]. The aim of this approach was to confirm cellular phenotypic associations by multiple scientific

**Table 1.** Summary of patient demographics.

|   | Control | UC             |
|---|---------|----------------|
| Patients [n]  | 35      | 27             |
| Mayo endoscopy subscore [n]:  |         |                |
| 0   |         | 0              |
| 1   |         | 6              |
| 2   |         | 16             |
| 3   |         | 5              |
| Gender: male/female [n]   | 19/16   | 17/10          |
| Mean age at biopsy, years [range]                                       | 55      | 45 [19–73]     |
|   |         | [21–77]        |
| Mean duration of disease, years [range]                                 |         | 9.8 [0.2–33]   |
| Concomitant IBD medications [n]   |         |                |
| Anti-TNF [infliximab, adalimumab]                                       |         | 0              |
| Anti-leukocyte trafficking [etrolizumab, vedolizumab, natalizumab]      |         | 0              |
| Immunomodulators [azathioprine, mercaptopurine, methotrexate, MMF]      |         | 8 [5, 0, 2, 1] |
| Systemic corticosteroid [oral prednisolone, intravenous hydrocortisone] |         | 6 [5, 1]       |
| Topical corticosteroid  |         | 4              |
| 5ASA  |         | 20             |
| Topical 5ASA  |         | 2              |
| No IBD meds   |         | 3              |

modalities and in distinct geographical patient populations. Enrolled healthy control subjects had normal colonic mucosa undergoing routine endoscopy [e.g. for investigation of iron deficiency anaemia or for polyp surveillance]. All patients were naïve to therapies targeting integrins [etrolizumab, vedolizumab and natalizumab], and tumour necrosis factor [TNF] $\alpha$  [infliximab and adalimumab]. UC patients were considered to have active disease if they had a Mayo endoscopic subscore<sup>26</sup> of 1 or more; UC patients with endoscopic subscores of 0 were not included in this study. In total, 35 control subjects and 27 UC patients provided tissue for research. These studies were performed according to the principles of the *Declaration of Helsinki*. Written informed consent was obtained in accordance with research and ethics committee [REC] approval [Newcastle and North Tyneside 1 REC 10/H0906/41, Newcastle and North Tyneside 2 REC 22/02 and Wandsworth REC 07/H0803/237].

## 2.2. Tissue handling

Colonic mucosal biopsies were obtained at the time of colonoscopy or flexible sigmoidoscopy, and collected from the distal colon to avoid anatomical variations in lymphocyte phenotype or frequency. Between four and eight biopsies were collected per patient with a yield of approximately 75 000–100 000 T cells from each biopsy. Specimens for immunohistochemistry [IHC] analysis were fixed immediately in neutral buffered formalin prior to paraffin embedding. Fresh tissue was stored in Hank's balanced salt solution [HBSS] with or without calcium or magnesium [Gibco, Carlsbad, CA, USA] on ice for up to 2 h until enzymatic dissociation.

## 2.3. Colonic biopsy dissociation and lymphocyte enrichment

Biopsies were washed in HBSS containing 5 mM 1,4-dithiothreitol [DTT, Sigma-Aldrich, St Louis, MO, USA] prior to enzymatic tissue dissociation using 10% complete RPMI containing 1.5 mg/ml Collagenase VIII [Sigma-Aldrich] and 50 microg/ml DNase I [Roche, Penzberg, Germany]. The resultant cell suspension was

filtered through a 40- $\mu$ m cell strainer and washed with FACS buffer to collect any residual cells prior to centrifugation and staining.

## 2.4. Cell surface and intracellular protein quantification by flow cytometry

Viable cells were identified using a Live/Dead fixable aqua dead cell stain kit [Invitrogen, Grand Island, NY, USA] according to the manufacturer's instructions. For identification of cell surface proteins, cells were suspended in 2% fetal calf serum PBS and incubated for 10 min with human FcR blocking reagent [Miltenyi Biotec, Bergisch Gladbach, Germany], prior to the addition of optimized concentrations of fluorochrome-tagged anti- $\alpha$ E, - $\beta$ 7, - $\alpha$ 4 $\beta$ 7, -CD45, -CD3, -CD4, -CD8a, -CD161 or isotype control antibodies and incubated for 20 min [Supplementary Table 1]. Gating strategies to identify lymphocyte populations are shown in Supplementary Fig 1.

For identification of intracellular proteins, *ex-vivo* cells were left unstimulated or stimulated at 37 °C in 5% CO<sub>2</sub> for 5 h in the presence of 10 ng/ml phorbol 12-myristate 13-acetate [PMA] and 1  $\mu$ g/ml ionomycin, with the addition of 10 ng/ml brefeldin A [all Sigma Aldrich] after 1 h. Cells were fixed for 30 min in 4% paraformaldehyde fixation buffer [Biolegend, San Diego, CA, USA], prior to washing with and suspending in permeabilization buffer [eBioscience, San Diego, CA, USA], and then stained for 30 min with optimized concentrations of anti-IL-17A, -IFN $\gamma$ , -TNF $\alpha$  or isotype control antibodies [Supplementary Table 1].

Data were acquired using a BD FACSCanto II flow cytometer using FACSDiva software v6 [Becton Dickinson, Franklin Lakes, NJ, USA]. Analysis was performed using FlowJo software v9.4.7 [TreeStar, Ashland, OR, USA]. Cell population gates were established based on levels of background fluorescence in isotype control stained, or unstimulated samples.

## 2.5. qPCR analysis of colonic T cell subsets

T lymphocytes isolated from colonic biopsies and identified by flow cytometry as previously described<sup>25</sup> were sorted using a Becton Dickinson FACS Aria II [SORP] cell sorter into four groups [CD4+ $\alpha$ E $\beta$ 7+, CD4+ $\alpha$ E $\beta$ 7-, CD8+ $\alpha$ E $\beta$ 7+ and CD8+ $\alpha$ E $\beta$ 7-] directly into RLT lysis buffer [Qiagen, Hilden, Germany] and stored at -80 °C prior to RNA extraction. cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit [Life Technologies, Carlsbad, CA, USA], and quantitative real time PCR analysis was performed using the BioMark HD System [Fluidigm, South San Francisco, CA, USA] using Taqman gene expression of specified genes [Life Technologies]. All gene expression values were normalized to GAPDH expression using the  $\Delta$ CT method.

## 2.6. Immunohistochemistry staining and quantification

IHC was performed on formalin-fixed and paraffin-embedded colonic biopsies. Blocks were cut into 4- $\mu$ m sections and stained using a BenchMark Ultra autostainer [Ventana Medical Systems, Tucson, AZ, USA] with optimized concentrations of anti- $\alpha$ E integrin, FOXP3, CD3, CD4 and CD8 antibodies [Supplementary Table 2]. Slides were developed with Alkaline Phosphatase Red [Ventana Medical Systems] and Vina Green [Biocare Medical, Concord, CA, USA] chromogen kits.

Whole slide images were acquired with a Nanozoomer 2.0-HT automated slide-scanning platform [Hamamatsu, Hamamatsu City, Japan] at 200 $\times$  final magnification. Scanned images were analysed in the Matlab software package vR2012b [Mathworks, Natick, MA, USA].

Images from IHC slides were captured on an Olympus BX61 microscope [Olympus, Center Valley, PA, USA] outfitted with a Nuance Multispectral Imaging System [Perkin Elmer, Waltham, MA,

USA]. A spectral library was created based on single colour pixel selection from each individual chromogen, allowing unmixing of multiple spatially co-localized chromogens. For each slide, a series of images was collected at a final magnification of 200x with exposure of 160 ms and red, green and haematoxylin were unmixed. Using the spectrally unmixed images as a guide, areas were manually selected on the whole-slide scans specific to either single chromogen or co-localized chromogen. These areas were used to train a Support Vector Machine [SVM] on the RGB colour profile specific to those areas. Application of this SVM to the entire slide image resulted in three binary masks, one for each single or co-localized chromogen. Individual cell borders were segmented as described,<sup>27</sup> and then scored IHC positive according to the greatest contingent of staining present within its borders, with co-localized area given twice the weight of either single chromogen, as well as at least  $\sim 10 \mu\text{m}^2$  of staining present.

Crypt epithelial areas were identified using a combination of an SVM and Genetic Programming [GP]. First, a training set of representative areas was generated manually and assigned a binary classification [positive for crypt lumen, or negative for regions to exclude]. Then, an SVM was trained using RGB<sup>28</sup> and texture values from these selections. GP was used to determine a sequence of simple morphological operators that maximized the solution accuracy for both positive and negative selections. The resulting SVM and GP algorithm was then applied to the entire set of images. Post-processing, any individual samples with less than  $15\,000 \mu\text{m}^2$  of total crypt area, or approximately three small crypts, were removed from the analysis. The layer of cell nuclei immediately adjacent to the crypt lumen was identified by dilating the lumen areas approximately one cell diameter, and reconstructing the overlapping cells.

## 2.7. Statistics

Statistical analysis was performed using Prism5 [GraphPad software, San Diego, CA, USA] and JMP11 [SAS, Cary, NC, USA]. The distributions of the samples were assessed for normality using the D'Agostino–Pearson omnibus test. Tukey box plots depict IHC and flow cytometry data. Intra-individual and inter-individual flow cytometry data were compared using paired and unpaired Student's *t*-tests, respectively. Interleaved scatter plots depict qPCR data, and dotted lines on graphs represent the lower limit of detection for genes of interest. The Mann–Whitney U test was used to compare gene expression data for  $\alpha\text{E}^+$  vs  $\alpha\text{E}^-$  cell populations. No adjustment for multiple comparisons was performed, except where noted in the text. Statistical significance [ $p < 0.05$ ] was defined based on nominal *p*-values, and different levels were noted on graphs as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

## 3. Results

### 3.1. UC is associated with an expansion of $\alpha\text{E}^-$ FOXP3+ cells in the lamina propria

Human studies have previously demonstrated an influx of TReg to the intestinal mucosa in active IBD by a combination of flow cytometry, IHC and qPCR.<sup>29</sup> We first sought to determine if T cells were the predominant leukocyte subset expressing  $\alpha\text{E}$  during active UC, and whether there was any association of  $\alpha\text{E}$  integrin with markers of regulatory T cell function.

Flow cytometry demonstrated a significant increase in total CD45+ leukocytes and CD3+ T cells in active UC in comparison to healthy subjects [Figure 1A&B] without a difference in CD4:CD8 ratio [Figure 1C]. Of all  $\alpha\text{E}^+$  leukocytes, CD3+ T cells represented the dominant leukocyte subset [Figure 1D]. In control subjects nearly 90% of  $\alpha\text{E}^+$  leukocytes were T cells, significantly rising to nearly 95%

in UC. The frequency of  $\alpha\beta7$  integrin expression was higher on  $\alpha\text{E}^+$  relative to  $\alpha\text{E}^-$  T cells in both UC patients and controls [Figure 1E]. In addition, not all  $\alpha\text{E}^+$  T cells expressed  $\alpha\beta7$  as up to 40% of  $\alpha\text{E}^+$  T cells in patients with active UC did not express  $\alpha\beta7$  [Figure 1E].

We next examined the expression of  $\alpha\text{E}$  integrin and the regulatory transcription factor FOXP3 using dual colour IHC. Consistent with preferential localization of  $\alpha\text{E}^+$  cells to anatomical sites rich in E-cadherin,  $\alpha\text{E}$  was predominantly observed on epithelial-associated CD3+, CD4+ and CD8+ cells with a high proportion of cells showing co-staining for T cell markers and  $\alpha\text{E}$  [Supplementary Figure 2A–C]. Very few  $\alpha\text{E}^+\text{FOXP3}^+$  cells were observed in either the epithelium or the LP, with a small but significant increase in epithelial-associated cells [Figure 2A&B]. In contrast to the relative scarcity of  $\alpha\text{E}^+\text{FOXP3}^+$  cells, there was a marked expansion of FOXP3+ cells lacking the  $\alpha\text{E}$  integrin in the LP of patients with active UC [Figure 2C]. The majority of  $\alpha\text{E}^+$  cells were observed to be FOXP3–, with preferential localization to the epithelium [Figure 2D].

### 3.2. Human colonic CD4+ $\alpha\text{E}^-$ T cells display enhanced levels of TReg-associated genes compared with CD4+ $\alpha\text{E}^+$ cells

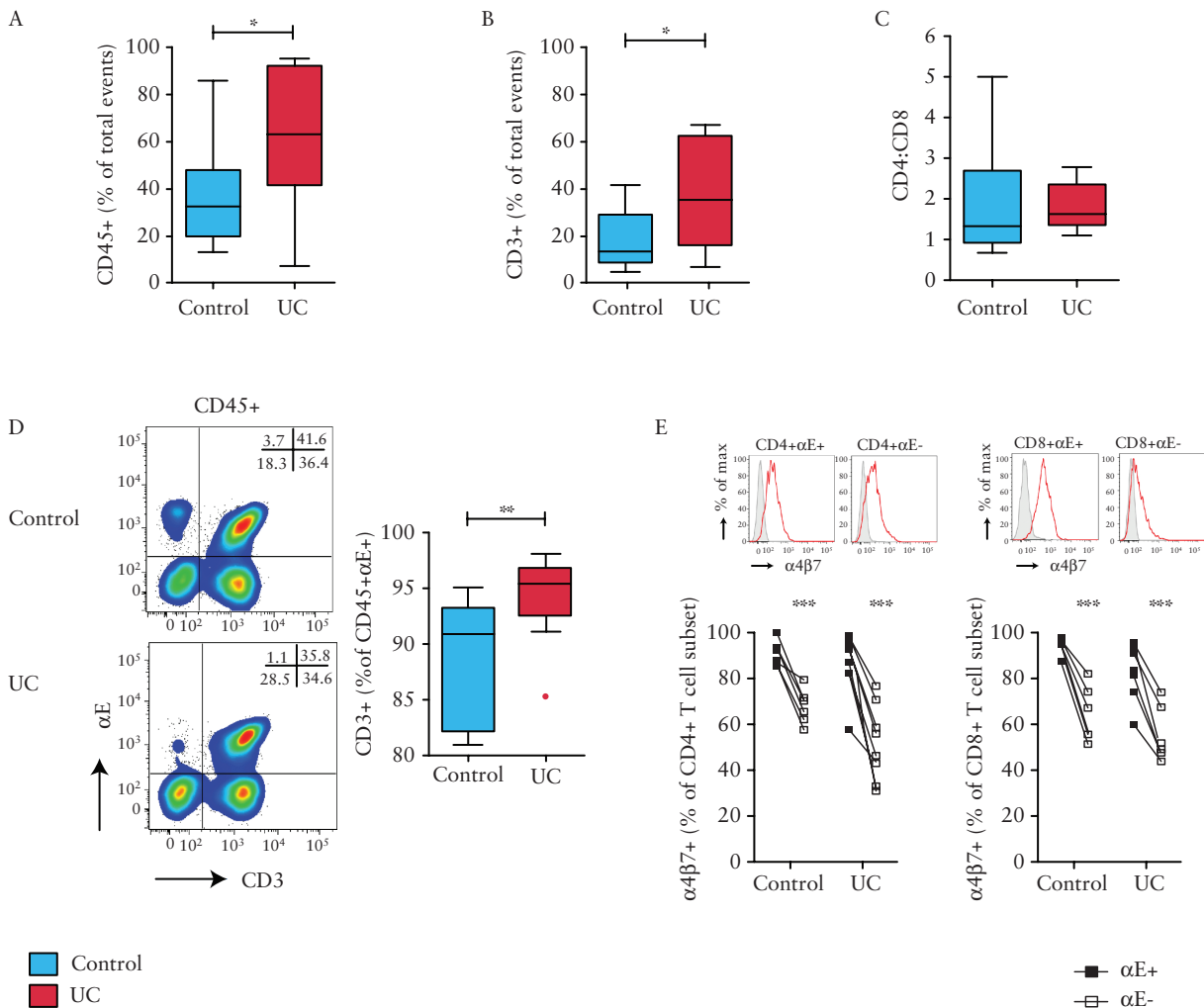
An expansion of FOXP3+ cells in the LP during inflammation may be indicative of infiltrating/expanding TReg populations. However, activated effector T cells can express FOXP3<sup>30</sup> and so the transcription factor is not specific enough by itself to identify TReg. Human TReg have been shown to have high expression of IL-10, CTLA4, ICOS and GARP in addition to FOXP3.<sup>31–33</sup> Therefore, gene expression of these TReg-associated markers was evaluated in purified  $\alpha\text{E}^+$  and  $\alpha\text{E}^-$  subsets of both CD4+ and CD8+ TCR $\alpha\beta$ + T cells. Consistent with the IHC analysis, CD4+ $\alpha\text{E}^-$  T lymphocytes consistently expressed higher levels of TReg-associated genes in comparison to CD4+ $\alpha\text{E}^+$  cells. Both in control subjects and more strikingly in UC patients, sorted CD4+ $\alpha\text{E}^-$  T cells had significantly higher levels of FOXP3, IL-10, CTLA4 and ICOS in comparison with CD4+ $\alpha\text{E}^+$  T cells [Figure 2E&F]. When correction was made for multiple comparisons using the Benjamini–Hochberg false detection rate adjustment of 10%, FOXP3 remained significantly elevated in CD4+ $\alpha\text{E}^-$  vs CD4+ $\alpha\text{E}^+$  T cells in both UC and control subjects. No difference in expression of TReg-associated genes was noted between CD8+ $\alpha\text{E}^+$  and CD8+ $\alpha\text{E}^-$  lymphocytes in either group [Supplementary Figure 3A].

### 3.3. Expression of the $\alpha\text{E}$ integrin by colonic CD4+ T cells is associated with Th17 differentiation

These findings suggest that, in contrast to the mouse,  $\alpha\text{E}$  is not a marker of TReg in the human colon. We next investigated if  $\alpha\text{E}$  was associated with a pro-inflammatory T cell phenotype.

The RORC gene encodes a member of the nuclear hormone receptor family of transcription factors, and is an important component of Th17 differentiation which has been associated with IBD in a number of studies.<sup>34,35</sup> Relative gene expression levels of FOXP3, RORC and IL17A in CD4+ $\alpha\text{E}^+$  and CD4+ $\alpha\text{E}^-$  T cells were therefore examined in our active UC cohort. In contrast to CD4+ $\alpha\text{E}^+$  lymphocytes, CD4+ $\alpha\text{E}^-$  T lymphocytes had lower levels of RORC and IL17A, along with higher levels of FOXP3 expression [Figure 3A&B], suggesting that these CD4 subsets consist of T cells in unique states of differentiation.

To confirm the association of Th17 phenotype with  $\alpha\text{E}$  integrin expression, cellular cytokine production was examined by flow cytometry. Analysis of CD4+ $\alpha\text{E}^+$  colonic lymphocytes revealed a 2- to 3-fold increased frequency of IL-17A+ cells in comparison with CD4+ $\alpha\text{E}^-$  lymphocytes [Figure 3C]. Furthermore, the frequency of IL-17A was twice as high in active UC CD4+ $\alpha\text{E}^+$  cells compared to



**Figure 1.** Ulcerative colitis is associated with an influx of mucosal leukocytes. Cells isolated from colonic biopsies sampled from control subjects [ $n = 14$ ] and UC patients [ $n = 10$ ] were evaluated by flow cytometry. Frequency of [A] CD45+ leukocytes, and [B] CD3+ T lymphocytes in the colonic mucosa is shown, along with [C] CD4:CD8 ratio of all CD3+ T cells and [D] representative  $\alpha E$  vs CD3 FACS plots of CD45+ leukocytes, with summary graph of the proportion of CD45+ $\alpha E$  leukocytes that were T cells. [E] In a subset of patients, surface expression of  $\alpha 4\beta 7$  dimer on  $\alpha E$ + and  $\alpha E$ - T cells from control subjects [ $n = 6$ ] and UC patients [ $n = 8$ ] was examined.

control subject CD4+ $\alpha E$ + lymphocytes. Consistent with previous research showing that IL-17-producing T cells are enriched in the CD161+ population, the majority of CD4 cells expressing either IL-17A or  $\alpha E$  also expressed surface CD161 [Figure 3D&E].<sup>36</sup>

In the CD8 compartment,  $\alpha E$ + cells from patients with active UC demonstrated a higher gene expression of IL-17A compared with CD8+ $\alpha E$ - T cells, while no difference in gene expression was noted in control subjects [Supplementary Figure 3B]. However, FACS analysis of colonic *ex vivo* stimulated CD8+ T cells demonstrated very low frequency of IL-17A cytokine-producing cells irrespective of disease status or  $\alpha E$  expression [Supplementary Figure 4A].

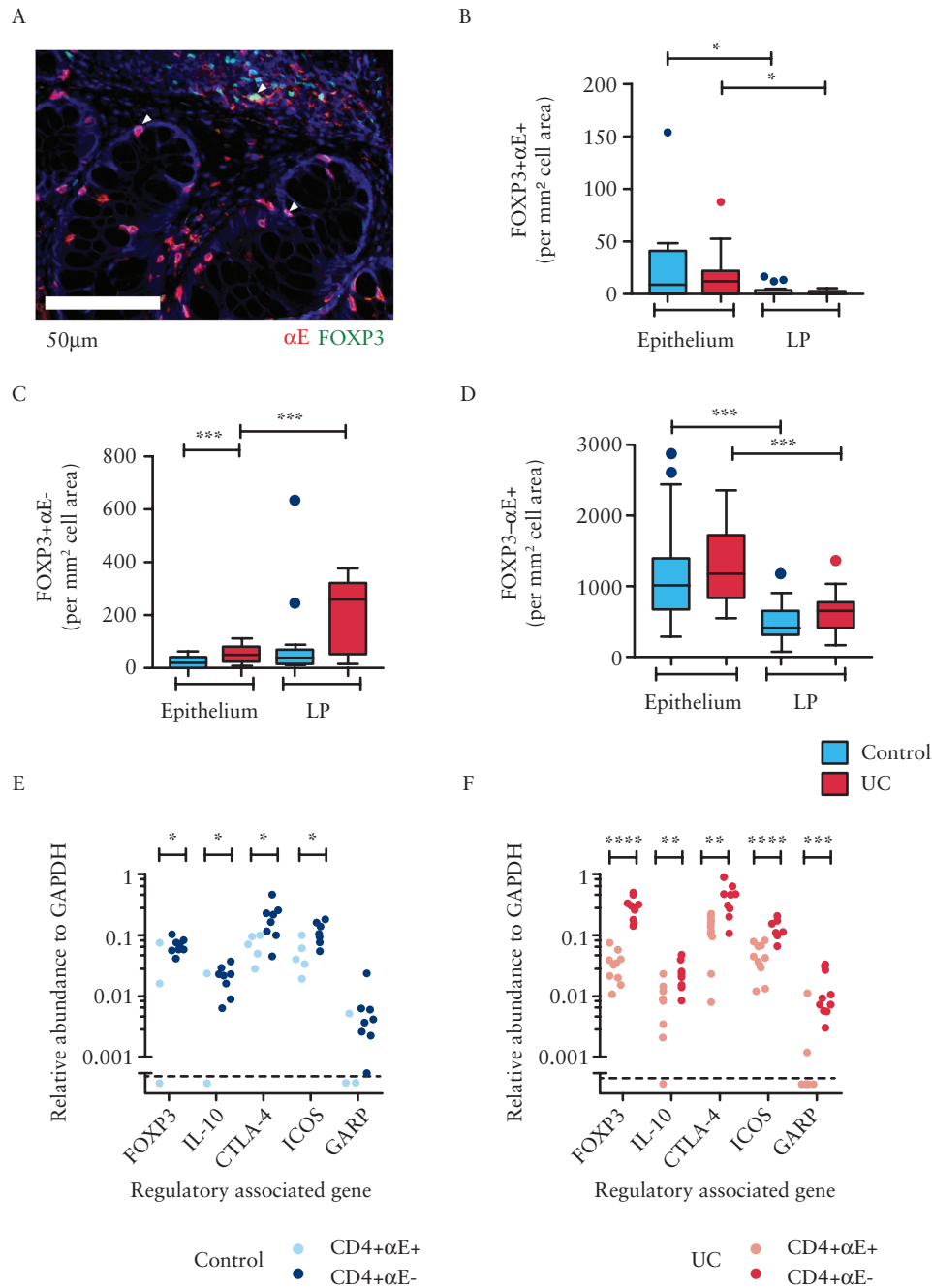
### 3.4. Higher production of Th1 cytokines is observed in CD4+ $\alpha E$ + relative to CD4+ $\alpha E$ - colonic T cells

Given the association between Th17 differentiation and  $\alpha E$  integrin, we next sought to determine whether CD4+ $\alpha E$ + T cells also had a Th1 phenotype using qPCR examination of T cells and flow cytometry of PMA/ionomycin-stimulated colonic T cells. Sort-purified CD4+ $\alpha E$ + T cells from control subjects demonstrated higher gene expression of TNF $\alpha$  compared with CD4+ $\alpha E$ - T cells. A trend to higher IFN $\gamma$  expression by CD4+ $\alpha E$ + T cells in control subjects was also observed [ $p = 0.09$ , Figure 4A].

The relationship of higher effector gene expression of sort-purified CD4+ $\alpha E$ + T cells in comparison to CD4+ $\alpha E$ - T cells was more striking in active UC patients [Figure 4B]. The frequency of IFN $\gamma$  protein-expressing cells and the level of IFN $\gamma$  transcripts were significantly increased in CD4+ $\alpha E$ + compared to CD4+ $\alpha E$ - cells in UC patients, and the frequency of IFN $\gamma$ -producing cells was overall decreased in the CD4+ $\alpha E$ - population in patients with UC [Figure 4C]. CD8+ $\alpha E$ + T cells also had increased IFN $\gamma$ -expressing potential compared to CD8+ $\alpha E$ - cells by protein and gene expression [Supplementary Figures 3B & 4B].

Similar relationships of protein and transcript level increases were observed for TNF $\alpha$  expression in the CD4+ $\alpha E$ + compared to CD4+ $\alpha E$ - population [Figure 4A, B&D]. In contrast to these findings in CD4+ T cells, no difference in TNF $\alpha$  protein or gene expression was seen between CD8+ $\alpha E$ + and CD8+ $\alpha E$ - cells [Supplementary Figures 3B & 4C].

Although gene expression of the Th2 cytokines IL-4 and IL-13 by CD4+ T cells in the colon was very low in both controls and UC, a small but significant increase in IL-4 gene expression in CD4+ $\alpha E$ - cells relative to CD4+ $\alpha E$ + cells was observed [Figure 4A&B] in control subjects and UC patients. An increase in IL-13 gene expression was also seen in CD4+ $\alpha E$ - cells relative to CD4+ $\alpha E$ + cells in active UC.

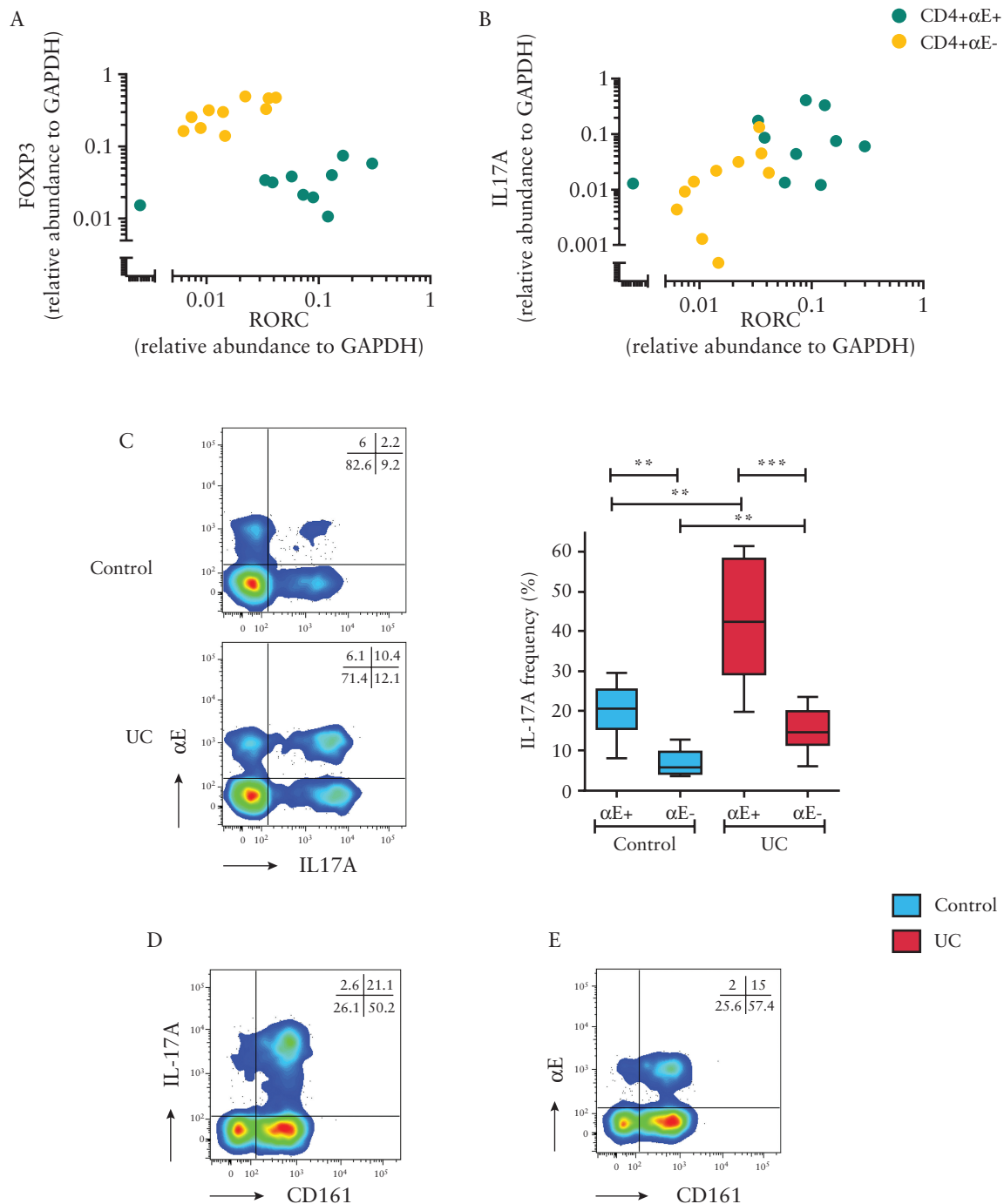


**Figure 2.** Lymphocytes with a regulatory phenotype are expanded in the colonic lamina propria [LP] during ulcerative colitis and lack  $\alpha E$  integrin. Dual stain IHC was performed on formalin-fixed paraffin-embedded colonic biopsies to evaluate co-expression of the  $\alpha E$  integrin [red] with FOXP3 [green]. Single and dual expressing cells [yellow, indicated by arrowheads] were enumerated using the Nuanco Multispectral Tissue Imaging System combined with automated morphometric analysis. [A] A representative Nuanco spectrally unmixed image is shown for a patient with active UC. Scale bar represents 50  $\mu m$ . Automatic cell counting was used to enumerate [B] FOXP3+ $\alpha E$ +, [C] FOXP3+ $\alpha E$ - cells and [D] FOXP3- $\alpha E$ + cells per mm<sup>2</sup> of cell area within the epithelium or LP in control subjects [n = 16] and patients with active UC [n = 14]. In a separate study cohort, FACS-sorted unstimulated CD4+ $\alpha E$ + and CD4+ $\alpha E$ - colonic lymphocytes were analysed for TReg gene expression in [E] control subjects [n = 8] and [F] patients with active UC [n = 10].

### 3.5. CD4+ $\alpha E$ + T cells display a Th17/Th1 phenotype that is exaggerated in UC

Recent studies have identified that in contrast to Th1 cells, which demonstrate stable lineage commitment after differentiation from naive cells, Th17 cells retain some degree of plasticity which can give rise to so-called Th17/Th1 dual IL-17A-positive and IFN $\gamma$ -positive cells.<sup>37</sup> These potentially pathogenic cells have previously

been identified in Crohn's disease.<sup>38</sup> We therefore used flow cytometry to explore if Th17/Th1 cells were present in UC and if there was any differential phenotype relative to  $\alpha E$  expression. Dual IL-17A+IFN $\gamma$ + T cells were identified in control subjects and active UC [Figure 5A&B]. Notably, the frequency of dual cytokine-producing CD4+ $\alpha E$ + cells was increased 2-fold in UC compared to control tissue samples. CD8 lymphocytes had a very low potential to concurrently produce both IL-17A and IFN $\gamma$  [Supplementary Figure 4D].

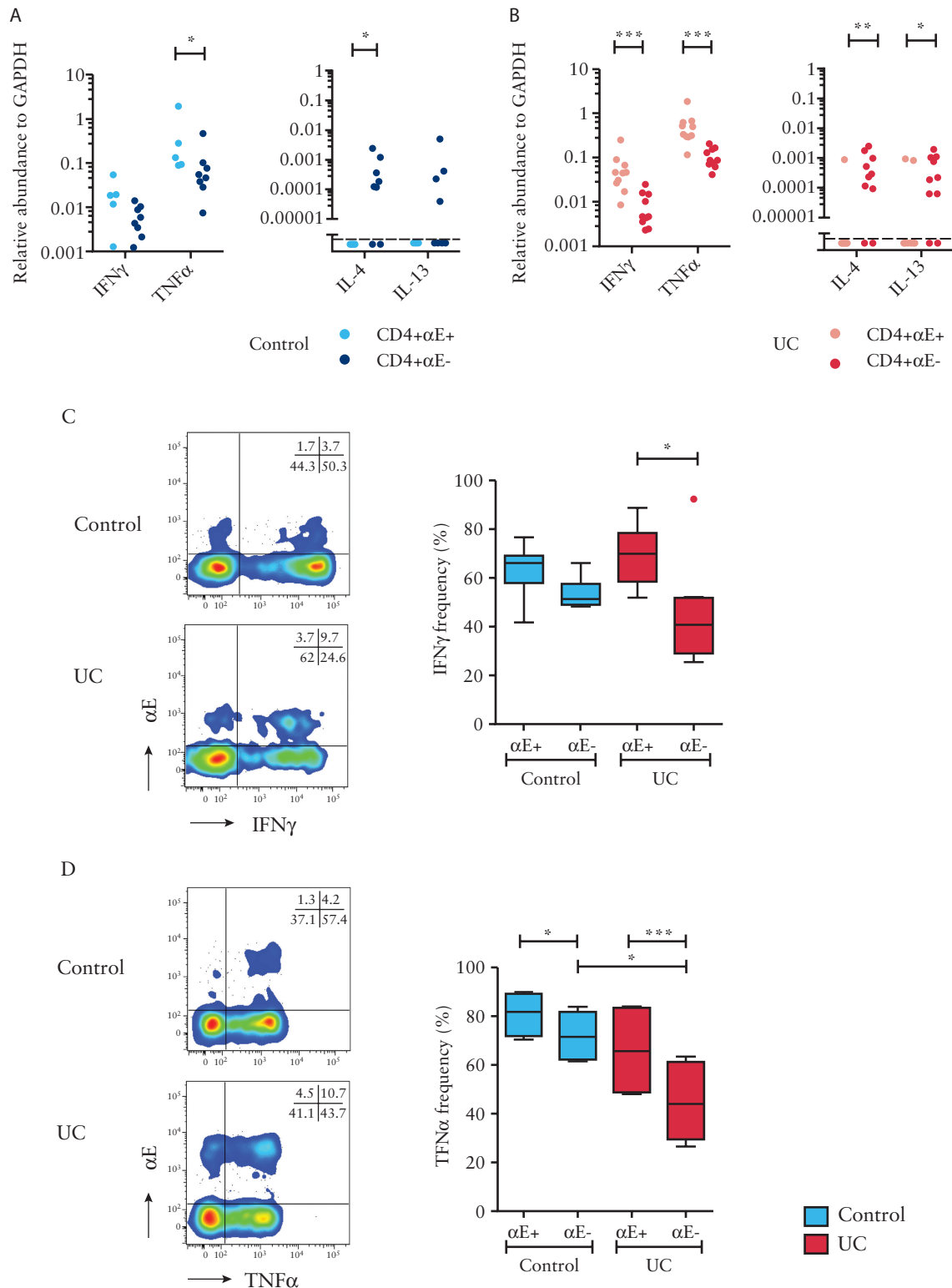


**Figure 3.**  $\alpha$ E integrin expression by colonic CD4<sup>+</sup> T lymphocytes is associated with Th17 differentiation. FACS-sorted, unstimulated CD4<sup>+</sup> $\alpha$ E<sup>+</sup> and CD4<sup>+</sup> $\alpha$ E<sup>-</sup> T lymphocytes isolated from active UC colonic biopsies were analysed for gene expression of [A] FOXP3 vs RORC and [B] IL17A vs RORC [ $n = 10$  patients]. [C] In a separate study cohort, IL-17A protein expression in CD4<sup>+</sup> $\alpha$ E<sup>+</sup> and CD4<sup>+</sup> $\alpha$ E<sup>-</sup> T cells was evaluated by FACS in colonic *ex-vivo* stimulated T lymphocytes from control subjects [ $n = 6$ ] and patients with active UC [ $n = 8$ ]. Representative two-colour FACS plots from control and UC patients are shown alongside summary Tukey box plots. Representative flow cytometry showing CD4<sup>+</sup> T cell CD161 co-expression with [D] intracellular IL-17A, and [E] surface  $\alpha$ E staining on a patient with active UC.

#### 4. Discussion

A substantial fraction of gut-associated T lymphocytes express the  $\alpha$ E integrin, yet the phenotype of these cells in humans and their potential contribution to diseases such as UC remain largely unknown. TGF $\beta$ , which plays a key role in the differentiation of both Th17 cells and induced TRegs depending on the cytokine milieu,<sup>37,39</sup> also induces  $\alpha$ E expression on T cells. In this study, utilizing two independent

cohorts, one examined for protein expression by IHC and FACS and one examined for gene expression by qPCR, we consistently observed CD4<sup>+</sup> $\alpha$ E<sup>+</sup> lymphocytes to be enriched in mucosal effector T cell populations expressing several inflammatory cytokines, including Th17 and Th1, but not Th2. The recently described Th17/Th1 lineage cells, which express T-bet and RORC and are potentially potent effector T cells, were also increased within CD4<sup>+</sup> $\alpha$ E<sup>+</sup> cells.<sup>37</sup> Consistent with their increased expression of IL-17A, we found that the majority of

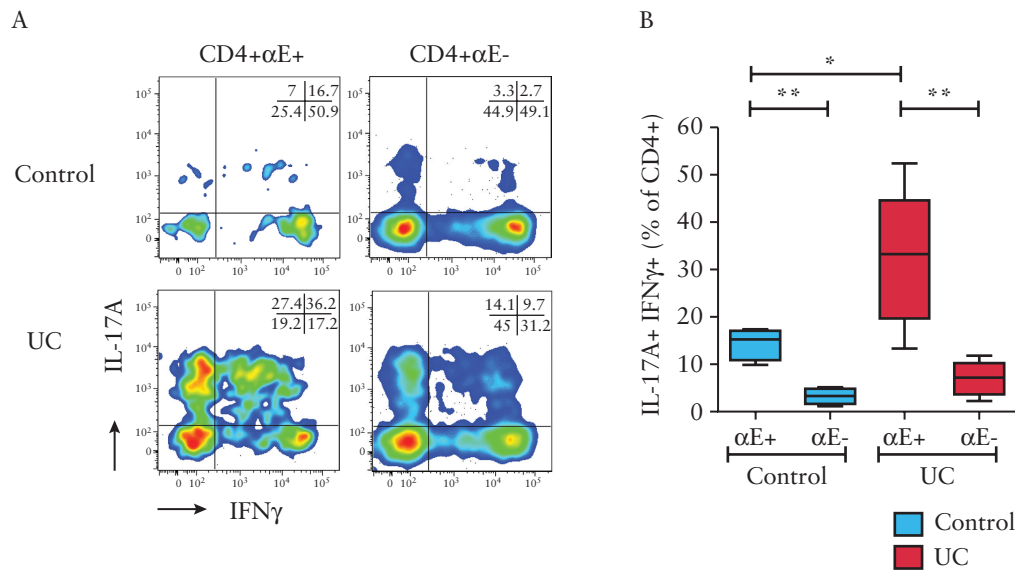


**Figure 4.** Colonic CD4+ $\alpha$ E+ lymphocytes express higher levels of Th1 cytokines relative to CD4+ $\alpha$ E- lymphocytes. FACS-sorted CD4+ $\alpha$ E+ and CD4+ $\alpha$ E- T lymphocytes from colonic biopsies harvested from [A] control subjects [ $n = 8$ ] and [B] patients with active UC [ $n = 10$ ] were evaluated for gene expression of IFN $\gamma$ , TNF $\alpha$ , IL-4 and IL-13. In a separate study cohort, *ex-vivo* stimulated T cells were evaluated by flow cytometry for intracellular [C] IFN $\gamma$  [ $n = 6$  control subjects and  $n = 8$  patients with active UC], and [D] TNF $\alpha$  protein expression [ $n = 4$  control subjects and  $n = 6$  patients with active UC]. Representative two-colour FACS plots from control and UC patients are shown alongside summary Tukey box plots.

CD4+ $\alpha$ E+ T cells also express CD161, a surface marker previously found to be highly enriched in the Th17 subset.<sup>36</sup> These data suggest that TGF $\beta$ -driven differentiation into the Th17 lineage, rather than the

Treg lineage, is favoured in CD4+ $\alpha$ E+ T cells in the gut. Previous studies have identified Th17/Th1 lineage cells that are enriched in Crohn's disease patients.<sup>37</sup> Our data extend these findings into UC, indicating





**Figure 5.**  $\alpha$ E integrin expression by CD4+ T lymphocytes is associated with a Th17/Th1 phenotype, which is enhanced in ulcerative colitis. *Ex-vivo* colonic CD4+ T cells were stimulated and analysed by flow cytometry for dual cytokine production of IL-17A and IFN $\gamma$ . [A] Representative two-colour FACS plots of IL-17A vs IFN $\gamma$  for CD4+ $\alpha$ E+, CD4+ $\alpha$ E- cells. [B] Summary Tukey box plots of double positive IL-17A+IFN $\gamma$ + lymphocyte frequency and double negative IL-17A-IFN $\gamma$ - lymphocyte frequency [ $n = 4$  control subjects and  $n = 6$  UC patients].

that Th17/Th1 cells are present in UC and are most frequent in the CD4+ $\alpha$ E+ population. In addition, CD4+ $\alpha$ E+ cells in UC had higher transcription and expression of the pro-inflammatory cytokine TNF $\alpha$ , which although classically associated with Th1 cells, can also be produced by Th17 cells,<sup>40</sup> suggesting that the pro-inflammatory functions of these cells may extend beyond production of only IL-17A and IFN $\gamma$ .

As we observe substantial enrichment of regulatory markers in  $\alpha$ E- human intestinal T cells,  $\alpha$ E integrin is probably not an exclusive marker for regulatory T cells in humans. TRegs can suppress effector functions of other T lymphocytes, in part through the production of IL-10 and TGF $\beta$ 1. In mice, TReg populations expressing  $\alpha$ E have been identified and shown to suppress inflammation in a number of disease models, including colitis,<sup>17</sup> chronic graft versus host disease<sup>18</sup> and antigen-induced arthritis.<sup>15</sup> In human UC and control subjects we found CD4+ $\alpha$ E+ lymphocytes have reduced transcription of the regulatory T cell-associated gene FOXP3 relative to their CD4+ $\alpha$ E- counterparts. Competitive antagonism has previously been demonstrated in transcription of FOXP3 and RORC, stabilizing, respectively, the TReg and Th17 phenotype.<sup>41</sup> Consistent with these findings, we demonstrated that CD4+ T cells sorted on the basis of  $\alpha$ E surface expression and analysed for RORC and FOXP3 gene expression, cluster into RORC<sup>hi</sup> FOXP3<sup>lo</sup> [CD4+ $\alpha$ E+] and RORC<sup>lo</sup> FOXP3<sup>hi</sup> [CD4+ $\alpha$ E-] subsets, suggestive of distinct functions. Although FOXP3 may be expressed by activated<sup>30</sup> or a minor non-regulatory T cell subset,<sup>42</sup> other regulatory T cell-associated genes [CTLA-4, GARP, ICOS, IL-10] were also upregulated in CD4+ $\alpha$ E- T cells. While none of these genes are specific to TRegs, for instance IL-10 can also be produced by activated Th1 cells,<sup>43</sup> the aggregate pattern of gene expression observed in this study suggests strongly that the majority of human CD4+ $\alpha$ E+ T cells do not have an enhanced regulatory function, and that the majority of T cell-mediated immunoregulatory activity is contained within the CD4+ $\alpha$ E- population.

Taken together, these data support a model in which the mucosal CD4+ $\alpha$ E+ population, already skewed towards an effector T cell phenotype in the steady state, becomes highly enriched in cells capable of producing pro-inflammatory cytokines. These cytokines have been shown to contribute to the epithelial damage and dysfunction characteristic of

UC.<sup>44-46</sup> The mechanisms responsible for promoting the differentiation and activation of CD4+ $\alpha$ E+ T cells *in vivo* remain largely unknown, but could involve factors directly produced by intestinal epithelial cells such as TGF $\beta$ 1, which can induce  $\alpha$ E expression and a Th17 phenotype.<sup>10,47</sup> Thus, cross-talk between CD4+ mucosal lymphocytes and dysfunctional epithelial cells may contribute to the pathobiology of UC.<sup>9</sup>

Etolizumab has recently demonstrated efficacy during a Phase II clinical trial in moderate to severe UC.<sup>24</sup> This monoclonal antibody is selective for the  $\beta$ 7 integrin subunit and thus targets both  $\alpha$ E $\beta$ 7 and  $\alpha$ 4 $\beta$ 7 integrin-expressing cells. Notably, etrolizumab treatment was associated with a significant reduction in the number of  $\alpha$ E+ cells within the colonic epithelial compartment of patients responding to therapy. In addition, post-hoc analysis demonstrated higher remission rates in patients with higher baseline colonic  $\alpha$ E expression as measured by IHC or whole-biopsy qPCR.<sup>24,25</sup> Together with data presented in the current study, these findings suggest that blockade of  $\alpha$ E $\beta$ 7:E-cadherin interactions by etrolizumab may interfere with epithelial cell-lymphocyte localization<sup>48</sup>, and could function in combination with inhibition of  $\alpha$ 4 $\beta$ 7:MAAdCAM-mediated lymphocyte trafficking, or independently of  $\alpha$ 4 $\beta$ 7 where  $\alpha$ E $\beta$ 7 is expressed in isolation, to reduce colonic inflammation in UC.<sup>24</sup> It is not clear whether  $\alpha$ E:E-cadherin interactions are required for effector T cell function, or whether  $\alpha$ E is simply a biomarker of a cytokine-responsive mucosa-associated cell. Specifically, our study identified very limited phenotypic differences between  $\alpha$ E+ and  $\alpha$ E- colonic CD8+ T cells. However,  $\alpha$ E is likely to influence the spatial localization of CD8+ T cells relative to the epithelium and the immunological synapse may influence release of cytotoxic granules. Thus, further study will be required in larger patient groups as well as additional studies focused on identifying the pathways responsible for CD4+ $\alpha$ E+ and CD8+ $\alpha$ E+ T cell recruitment, differentiation and activation in the absence and presence of factors blocking interaction of the integrin with E-cadherin.

The goal of personalized medicine in heterogeneous diseases such as UC, in which treatment paradigms are based on knowledge of the underlying biology of disease using testable biomarkers or genetics, will require careful and iterative investigation of pathobiology in the context of clinical trials. Our findings highlight

the importance of establishing the function of cell populations in directly relevant human tissues and patient groups. Mixed results have been reported for the efficacy of targeting  $\beta 7$  integrins or corresponding cell adhesion molecules in murine models of IBD,<sup>49–54</sup> and one possible explanation is key species differences in function of leukocyte populations that are blocked from entry or retention within the gut. Differences in human cell biology demonstrated in this study from previous reported murine research highlight the importance of directly evaluating target cell populations in patient samples to ultimately enable appropriate patient selection strategies. The specific contribution of  $\alpha\text{E}^+$  T cells to maintenance and loss of intestinal homeostasis will need to be evaluated in the context of integrin blockade in patients, ideally in ongoing Phase III studies, to further clarify the mechanisms by which integrin-targeted therapies function in UC, and whether these cells may be of interest for patient-selection strategies.

## Funding

This work was supported by the Wellcome Trust [grant number 093885/Z/10/Z to CAL]; by the Biomedical Research Centre of Guy's and St Thomas' Hospitals and King's College London, by the NIHR Newcastle Biomedical Research Centre, and by Genentech Inc. CAL is a Clinical Lecturer supported by the National Institute for Health Research [NIHR].

## Conflict of Interest

During this study GWT, JEA, LD, SO'B, MEK and JGE were full-time employees of Genentech, Inc., a member of the Roche Group. Financial or non-financial research support, educational funds or compensation for consulting, speaking or teaching has been received from: CAL: Genentech, Techlab, Immundiagnostik, Roche Tissue diagnostics, Takeda; JCM: AbbVie, Ferring, Genentech, Takeda; DG: Genentech, Cerimon Pharmaceuticals; PI: AbbVie, MSD, Ferring, Vifor, Genentech, Takeda, Warner Chilcott, Shire, Johnson & Johnson, Falk, Pharmacosmos; GO'B: Genentech; DEJ: Genentech; AH: HS-Lifesciences, ImmunoQure, Genentech, MedImmune; JAK: Genentech, GlaxoSmithKline, Intercept Pharmaceuticals. AL and MBP have nothing to disclose.

## Acknowledgments

We are grateful to the patients who contributed tissue specimens. We thank Stacey Newton, Thomas Ness [Newcastle upon Tyne Hospitals] and Melissa Gonzalez-Edick [Genentech] for their contributions to this study. We would also like to thank the flow cytometry facilities at Newcastle University and Kings College London.

## Author Contributions

CAL, JCM, DG, SOB, AH, MEK, JGE and JAK participated in study design. CAL, JCM, AKL, PI and MBP participated in patient recruitment and tissue acquisition. All authors were responsible for data analysis and interpretation. CAL, MEK, JGE and JAK drafted the initial manuscript. All authors participated in subsequent manuscript redrafting. The authors confirm this manuscript has not been previously published and is not under consideration for publication elsewhere.

## Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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