

Regulation and Role of α E Integrin and Gut Homing Integrins in Migration and Retention of Intestinal Lymphocytes during Inflammatory Bowel Disease

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Targeting interactions between α 4 β 7 integrin and endothelial adhesion molecule MAdCAM-1 to inhibit lymphocyte migration to the gastrointestinal tract is an effective therapy in inflammatory bowel disease (IBD). Following lymphocyte entry into the mucosa, a subset of these cells expresses α E β 7 integrin, which is expressed on proinflammatory lymphocytes, to increase cell retention. The factors governing lymphocyte migration into the intestinal mucosa and α E integrin expression in healthy subjects and IBD patients remain incompletely understood. We evaluated changes in factors involved in lymphocyte migration and differentiation within tissues. Both ileal and colonic tissue from active IBD patients showed upregulation of ICAM-1, VCAM-1, and MAdCAM-1 at the gene and protein levels compared with healthy subjects and/or inactive IBD patients. β 1 and β 7 integrin expression on circulating lymphocytes was similar across groups. TGF- β 1 treatment induced expression of α E on both β 7⁺ and β 7⁻ T cells, suggesting that cells entering the mucosa independently of MAdCAM-1/ α 4 β 7 can become α E β 7⁺. ITGAE gene polymorphisms did not alter protein induction following TGF- β 1 stimulation. Increased phospho-SMAD3, which is directly downstream of TGF- β , and increased TGF- β -responsive gene expression were observed in the colonic mucosa of IBD patients. Finally, in vitro stimulation experiments showed that baseline β 7 expression had little effect on cytokine, chemokine, transcription factor, and effector molecule gene expression in α E⁺ and α E⁻ T cells. These findings suggest cell migration to the gut mucosa may be altered in IBD and α 4 β 7⁻, and α 4 β 7⁺ T cells may upregulate α E β 7 in response to TGF- β once within the gut mucosa. *The Journal of Immunology*, 2021, 207: 2245–2254.

The immune system relies on a complex network of mechanisms to enable immune surveillance of tissues. Migration of leukocytes into intestinal tissues involves chemokines, adhesion molecules, and integrins to mediate rolling along the endothelium, arrest following integrin activation, and binding to adhesion molecules and transmigration (1). Circulating T cells in the peripheral blood can enter the gut through an endothelial barrier in one of two ways: 1) naive T cells migrate to gut-associated lymphoid tissue or 2) memory T cells previously activated in gut-associated lymphoid tissue enter the lamina propria (LP) via systemic circulation (2).

Under normal homeostatic conditions, migration into the gut occurs through interactions between α 4 β 7 integrin expressed on both naive and memory T cells and MAdCAM-1 on the endothelium (2, 3). To a lesser extent, cells are thought to use α 4 β 1 (very late Ag 4 [VLA-4]) interacting with vascular adhesion molecule-1 (VCAM-1) and fibronectin (4), or α L β 2 (lymphocyte function-associated Ag) interacting with ICAM-1 to migrate to the intestine (5, 6). Recent data suggest that α 4 β 1 may be particularly important for migration to the small intestine in comparison with the colon (7). Once lymphocytes have entered the intestinal mucosa, a subset of T cells upregulates

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Abbreviations used in this article: CD, Crohn's disease; EMBARK, Emerging Biomarkers; FDR, false discovery rate; IBD, inflammatory bowel disease; IHC, immunohistochemistry; LP, lamina propria; MFI, mean fluorescence intensity; REC, research and ethics committee; UC, ulcerative colitis; VCAM-1, vascular adhesion molecule-1.

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α E β 7 (CD103) (8). α E β 7 binds to E-cadherin and increases the retention of cells in the intestinal mucosa (9).

Aberrant innate and adaptive mucosal immune responses to microbes in the gut lumen characterize both Crohn's disease (CD) and ulcerative colitis (UC), the two types of inflammatory bowel disease (IBD) (10). During inflammation, intestinal vascular endothelium can be activated by inflammatory cytokines, including TNF- α and IL-1 β , and other stimuli, such as LPS, to upregulate expression of VCAM-1 and ICAM-1 (11), which have the potential to alter normal lymphocyte migration patterns into the intestine. Early studies in IBD showed that MAdCAM-1 is upregulated in IBD samples (12), whereas results for VCAM-1 and ICAM-1 are mixed (13, 14). Investigating inflammation-induced changes in lymphocyte migration is important for understanding current anti-integrin therapies for IBD that target lymphocyte migration to the intestine.

Differences between blockade of α 4 integrin (natalizumab) (15), α 4 β 7 integrin heterodimer (vedolizumab) (16, 17), and β 7 integrin (etrolizumab) (18, 19), which blocks both α 4 β 7 and α E β 7 heterodimers, may be key to understanding differences in therapeutic efficacy. Both α 4 β 7 and α 4 β 1 are expressed by a large proportion of circulating T cells (20). α E β 7, in contrast, is expressed by very few circulating T cells but is highly expressed by intestinal T cells both in the LP and intraepithelial space (21). TGF- β 1 is a multifunctional cytokine present in the intestinal mucosa that can signal to T cells, is associated with commitment to the Th17 cell lineage, and also induces α E expression. Disease-associated risk alleles in *ITGAE*, the gene that encodes α E integrin, have been associated with differential levels of surface expression of α E following TGF- β 1 stimulation *in vitro* (22). It is unclear if circulating T cells require pre-existing expression of α 4 β 7 integrin to express α E β 7 integrin in the gut, perhaps by switching the pairing of the α -subunit on β 7⁺ cells from α 4 to α E through increased α E surface expression. This is of particular importance, as data from our group and others suggest that a subset of potentially pathogenic T cells expresses α E β 7 integrin in UC (21, 23, 24).

The present study aims clearly to define inflammation-induced changes in lymphocyte migration that are relevant to interpreting clinical data for therapies that inhibit integrin-mediated lymphocyte migration to the intestine. Blockade of α 4 β 7/MAdCAM-1 interaction has been shown to reduce lymphocyte trafficking. As we show in this study, induction of α E β 7 integrin on α 4 β 7⁻ cells is possible, suggesting that cells trafficking to the gut via inflammation-induced alternative mechanisms (e.g., via α 4 β 1/VCAM-1 or α L β 2/ICAM-1) may become α E β 7⁺ effector T cells. Our findings support both altered adhesion molecule patterns in the intestine in active IBD as well as differentiation of α E⁺ T cells from cells that initially enter the intestinal mucosa by non- α 4 β 7/MAdCAM-1 means.

Materials and Methods

Human subjects

Healthy volunteers and IBD patients were enrolled in separate studies at Newcastle University, and in a previously described Mayo Clinic (25) and multicenter observational IBD study (Emerging Biomarkers [EMBARC]) (26). Patient characteristics for all cohorts are shown in Supplemental Table I. In addition, peripheral blood and PBMCs were collected from healthy donors participating in the Genentech blood donor program with written, informed consent. Saliva was collected from Genentech donors and genotyped on the Global Screening Array (Illumina, San Diego, CA) and confirmed by targeted PCR genotyping.

For the Newcastle cohort, none of the IBD patients were taking any medications for their disease, including biologics, immunomodulators, steroids, or 5-ASA. Active disease in the ileum or distal colon was defined as endoscopically visible mucosal inflammation and active inflammation was confirmed via histological examination by a pathologist. Inactive disease in the ileum or distal colon was defined as absence of visible inflammation at endoscopy and absence of histological inflammation by pathologist examination.

Ethics

Written informed consent was obtained in accordance with research and ethics committee (REC) (Newcastle and North Tyneside 1 REC 10/H0906/41 and Newcastle and North Tyneside 2 REC 22/02; Mayo Clinic Institutional Review Board 10-006628) approval. These studies were performed according to the principles of the Declaration of Helsinki. The Western Institutional Review Board provided research and ethics oversight for the Genentech blood donor program and the EMBARK cohort has been previously described (26).

Microarray

RNA was isolated as previously described (23). Microarray data (Gene Expression Omnibus <https://www.ncbi.nlm.nih.gov/geo/>; accession number GSE 179285) were preprocessed and normalized using packages from the Bioconductor project (<http://bioconductor.org>), supplemented with scripts written in the R programming language (<http://r-project.org>). Feature-extracted data were background subtracted using the normal + exponential method from the limma Bioconductor package (27). Log-ratios for the test and reference channel were normalized using loess normalization (28). Datasets were independently normalized across samples using quantiles normalization (29). We filtered out probes from the microarrays that did not map to known Entrez Genes, and further reduced the data by selecting a single probe per gene, selecting the gene with the highest interquartile range as the exemplar for a given gene (30). Individual genes were plotted for each patient sample in the EMBARK cohort (26).

Immunohistochemistry

Staining was performed using a Discovery Ultra Autostainer (Ventana). For each immunohistochemistry (IHC) Ab, positive control tissue was initially used for optimization and identification of staining morphology, typically with archival paraffin-embedded tonsil, thymus, bone marrow, or spleen. Optimal Ag retrieval, primary Ab dilution, and detection kits were then determined by staining 4- μ m sections of intestine. All Ag retrieval was optimal using a Standard Cell Conditioning 1 buffer (Ventana) retrieval protocol for 64 min at 100°C. The following anti-human Abs were optimized: anti-pSMAD3 (phosphor-S423 and S425, rabbit monoclonal clone EP823Y, dilution 1:100; Abcam), anti-MAdCAM-1 (mouse monoclonal, clone 355G8; Thermo Fisher Scientific, dilution 1:100), anti-ICAM-1 (rabbit monoclonal, clone EPR4776, dilution 1:400; Abcam), and anti-VCAM-1 (rabbit monoclonal, clone EPR5047, dilution 1:250; Abcam). The Ultraview DAB Detection Kit (Ventana) was used for all Abs. Digital images were acquired using a Vectra 3 Slide Scanner (PerkinElmer) for cell adhesion molecules and an BX43 microscope (Olympus) for pSMAD3 IHC. Unless otherwise noted, representative images were selected from 10 samples stained in each group.

Flow cytometry

Expression of integrin β 7, β 1, and α E on CD3⁺ peripheral homing (CD45RA⁻ β 7 low), mucosal homing (CD45RA⁻ β 7 high), and naive (CD45RA⁺ β 7 intermediate) T cell subsets were assessed by flow cytometry. In brief, whole blood samples from Mayo Clinic UC, CD, and non-IBD patients were collected into sodium heparin vacutainer tubes and shipped overnight at ambient temperature to Genentech. To lyse the RBCs, 1 \times BD Pharm Lyse buffer (BD Biosciences, San Jose, CA) was added to the whole blood sample and allowed to incubate at room temperature for 20 min. Samples were washed three times with ice cold staining buffer (PBS + 2% FBS) followed by addition of normal mouse serum to block potential non-specific staining. Samples were incubated on ice for 20 min prior to staining aliquots with Ab mixtures containing fluorescently conjugated anti-CD3 (SP34-2), CD45RA (HI100), β 7 (FIB504), β 1 (MAR4), α E (Ber-ACT8), and/or the respective isotype controls (BD Biosciences, San Jose, CA). Samples were incubated on ice for 30 min, washed twice with staining buffer, and resuspended in 1% paraformaldehyde prior to acquisition on a BD LSR (BD Biosciences, San Jose, CA). A total of 75,000–100,000 lymphocyte gated events were acquired per sample. Flow cytograms were generated to establish the fraction of cells positive for each surface marker. Geometric mean fluorescent intensity (MFI) and percentage of gated cells for each T cell subset were assessed and reported accordingly.

In vitro cell culture experiments

T cells were purified from PBMCs by immunomagnetic negative selection using the EasySep Human T Cell Enrichment Kit (STEMCELL Technologies) and cultured with CD3/CD28 Dynabeads (Invitrogen) in complete RPMI medium-1640 (RPMI) containing 10% FCS at a 1:1 cell/bead ratio with or without 10 ng/ml TGF- β 1 (R&D Systems). In some experiments, an ALK5 inhibitor (SB-505124; Sigma-Aldrich) was included. For CFSE dilution experiments, purified T cells were labeled with 10 μ M CFSE, stimulated at a 2:1 cell/bead ratio, and harvested at times indicated.

In vitro stimulation of peripheral blood T cells

Frozen PBMCs were thawed with RPMI 1640 with 20% FCS, 50 IU/ml penicillin, 50 μ g/ml streptomycin, 2 mM glutamax, and 25 U/ml benzoxone (Sigma-Aldrich, St. Louis, MO) and rested overnight in 48-well culture plates in the incubator at 37°C. The next day, anti-CD3/CD28-coated Dynabeads (Thermo Fisher Scientific, Waltham, MA) were prepared per manufacturer instructions, resuspended in RPMI, and added to cells along with 5 ng/ml of recombinant TGF- β 1 (Cell Signaling Technologies, Danvers, MA), 30 U/ml of recombinant IL-2 (PeproTech, Cranbury, NJ) and, where indicated, in the presence of 20 μ g/ml etrolizumab (anti- β 7), vedolizumab (anti- α 4 β 7) or human IgG1 isotype control. After 6 d of stimulation, cells were harvested and stained. Viability was assessed using the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, MA). Cells were blocked with human TruStain Fc block (BioLegend, San Diego, CA), incubated on ice for 10 min, stained with an Ab mixture of anti-CD8-V500 (SK1), anti-CD3-FITC (UCHT1), anti-CD103-PE (Ber-ACT8), anti-CD4-PerCP-Cy5.5 (SK3) (BD Bioscience, San Jose, CA), and were added for 20 min. In some experiments, β 7 surface expression was assessed by staining with anti- β 7 FIB504 allophycocyanin (BD Biosciences, San Jose, CA) or anti- β 7 9D8 Alexa647 (Genentech, South San Francisco, CA). After washing, cells were resuspended in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) prior to analysis on a Canto II Flow Cytometer (BD Bioscience, San Jose, CA).

Comparison of gene expression in T cells with or without baseline β 7 expression

PBMCs from seven donors were phenotyped and sorted using a BD FACS Aria Fusion cell sorter (BD Biosciences) into CD3⁺ β 7⁻ α E⁻ and CD3⁺ β 7⁺ α E⁻ populations (Abs: anti-CD3 clone OKT3, anti- β 7 clone FIB504, anti- α E clone Ber-ACT8, anti- α L clone HI111, anti- β 1 clone TS2/16). A total of 5×10^5 cells were cultured in 1 ml of complete RPMI (20% FCS) supplemented with 10 ng/ml TGF- β 1 in a 1:1 ratio with anti-CD3/CD28 Dynabeads. Media was changed every 2–3 d with FCS reduced to 10% at the first media change. Following 10 d of culture, four cell populations, CD4⁺ α E⁺, CD4⁺ α E⁻, CD8⁺ α E⁺, and CD8⁺ α E⁻ (Abs: anti-CD4 clone RPA-T4, anti-CD8 clone HIT8a), cultured from the β 7⁺ and β 7⁻ progenitor populations were sorted using a BD FACS Aria Fusion Cell Sorter into 1% 2-ME RLT RNeasy lysis buffer (Qiagen). RNA was extracted using RNeasy Kits (Qiagen). Fifty-six total tubes were sorted based on the original β 7 surface expression in combination with CD4, CD8, and α E expression at the end of culture. Eight of these tubes had <1000 cells per group and two failed to yield enough RNA for analysis. First-strand cDNA was generated from 34 ng of total RNA using iScript (Bio-Rad Diagnostics, Hercules, CA). cDNA (2.125 ng) was preamplified using the TaqMan PreAmp Master Mix (Life Technologies, Carlsbad, CA) along with diluted TaqMan assays (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Quantitative PCR was performed using the Biomark HD system (Fluidigm 96.96 format).

Comparison of selected genes in sorted in vitro differentiated cells

A linear model was used to evaluate the effect of baseline expression of β 7 integrin on expression of selected genes following 10 d of culture with or without TGF- β 1 stimulation as described. Both fold change and statistical significance was determined for all genes tested and a false discovery rate (FDR) was calculated using the method of Benjamini and Hochberg (31). The analysis was performed independently for CD4⁺ and CD8⁺ T cells, with day 10 α E integrin expression included as a covariate.

Results

Adhesion molecules ICAM-1, VCAM-1, and MAdCAM-1 are upregulated in IBD

Previous work has shown that ICAM-1 and VCAM-1 have low expression whereas MAdCAM-1 is highly expressed on vascular endothelium in normal intestine (12–14). Although changes in patterns of expression have been described in IBD, shifts in overall gene expression have not been extensively evaluated. We therefore measured ICAM-1, VCAM-1 and MAdCAM-1 gene expression levels in healthy subjects and IBD patients. Consistent with historical protein data, we observed low but detectable levels of adhesion molecules in colonic and ileal biopsies from healthy subjects (Fig. 1A, 1C, 1E). ICAM-1 was highly upregulated in inflamed tissue from both UC and CD patients in comparison with uninflamed and healthy control tissue in both ileum and colon (Fig. 1A). In contrast,

VCAM-1 gene expression was only moderately increased in inflamed CD, but not UC, samples (Fig. 1C). MAdCAM-1 upregulation was observed in both inflamed UC and CD tissue (Fig. 1E). We found no difference in gene expression between uninflamed UC or CD and healthy subject samples for any of the three adhesion molecules (Fig. 1A, 1C, 1E).

Because migration patterns change during inflammation, gene expression changes may reflect differences in expression between distinct cellular compartments. Inflamed biopsies from both ileum and colon exhibited consistent increases in ICAM-1 gene expression (Fig. 1A) and widespread upregulation of ICAM-1 protein (Fig. 1B) compared with uninflamed biopsies and controls (Supplemental Fig. 1A, 1B). These findings are consistent with previous reports demonstrating ICAM-1 expression on monocytes and plasma cells as well as endothelial cells in inflamed tissue (28). VCAM-1 protein levels were less altered (Fig. 1D, Supplemental Fig. 1C, 1D). MAdCAM-1 staining showed increased staining limited to endothelial cells (Fig. 1F, Supplemental Fig. 1E, 1F), consistent with previous reports (32).

Expression of β 1 and β 7 integrins is unchanged on peripheral blood T cell populations in IBD

To better understand changes in integrin expression on lymphocytes from patients undergoing intestinal resection because of UC, CD, and diverticulitis (non-IBD), we evaluated β 1 and β 7 integrin expression on the surface of peripheral blood T cells.

Peripheral blood T cells were separated into three groups based on high surface expression of CD45RA (CD45RA⁺; naive T cells), low surface expression of CD45RA and high β 7 integrin (CD45RA⁻ β 7^{high}; mucosal homing T cells) or low β 7 integrin expression (β 7^{low}; peripheral homing T cells) (example of gating strategy is shown in Fig. 2A). The frequency of these different subpopulations in peripheral blood was similar between UC and CD and between IBD and non-IBD groups (Fig. 2B).

Expression of β 1 and β 7 integrins was observed on all T cell subsets with the exception of β 7 integrin on peripheral homing T cells (Fig. 2C, 2D). On mucosal homing T cells, both β 1 and β 7 integrin levels were similar between all patient groups (Fig. 2C, 2D). β 1 expression was 3-fold higher on peripheral blood homing T cells in comparison with naive and mucosal homing T cells (Fig. 2C), whereas β 7 expression was 4-fold higher on average on mucosal homing T cells in comparison with naive T cells (Fig. 2D). Similar findings were observed for all subsets for both CD4⁺ and CD8⁺ T cells, with the exception of β 1 expression, which was only 2-fold higher on peripheral blood homing CD8⁺ T cells in comparison with naive and mucosal homing cells CD8⁺ T cells (Supplemental Fig. 2).

Induction of α E integrin on T cell populations through TGF- β 1 stimulation is not dependent on baseline β 7 expression

The α E β 7 integrin is expressed by a very low proportion of peripheral blood T lymphocytes. T cell expression of α E integrin is known to be TGF- β inducible (30–32), as was confirmed in our experiments (Fig. 3). Both CD4⁺ and CD8⁺ T cells responded to increasing concentrations of TGF- β 1 with increasing frequency of surface α E expression (Fig. 3A, 3B). A dose-response relationship was observed for α E induction, with a higher frequency of CD8⁺ α E⁺ T cells at all doses tested in comparison with CD4⁺ α E⁺ T cells. Treatment with an ALK5 (TGF- β receptor I kinase) inhibitor demonstrated that α E upregulation in both CD4⁺ and CD8⁺ T cells required TGF- β RI signaling (Fig. 3A, 3B). CFSE dye was used to follow α E upregulation in dividing CD4⁺ and CD8⁺ T cells following stimulation with anti-CD3/CD28 and 10 ng/ml TGF- β 1 (Fig. 3C). A lower overall percentage of CD4⁺ T cells underwent α E upregulation, yet achieved maximal frequency of α E positivity more rapidly than CD8⁺ T cells (Fig. 3D). α E expression levels were also higher on CD8⁺ T cells

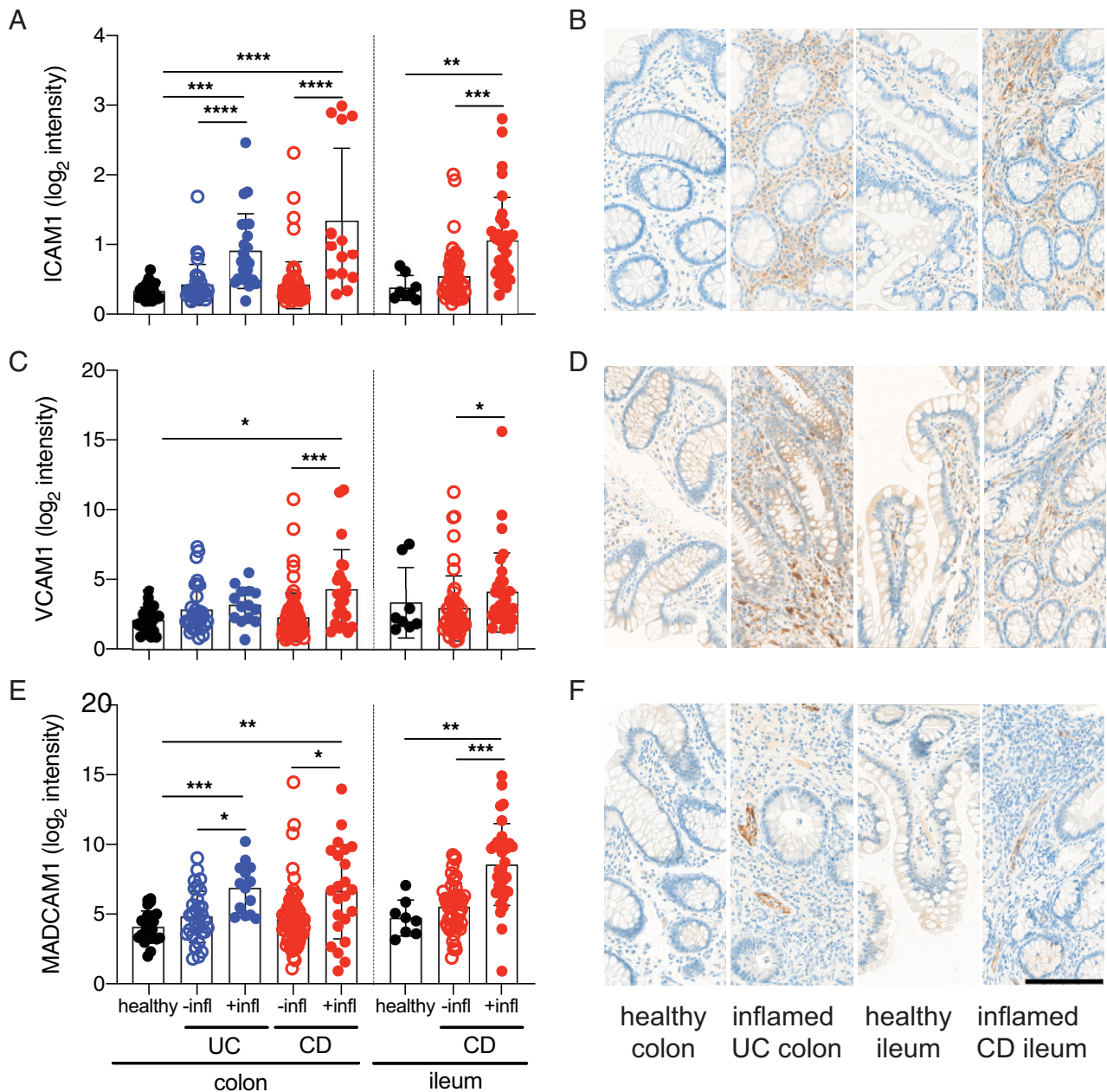


FIGURE 1. Upregulation of adhesion molecules ICAM-1, VCAM-1, and MAdCAM-1 in IBD. Gene expression and IHC of adhesion molecules in intestinal biopsy samples from healthy subjects and IBD patients from the EMBARK and Newcastle cohorts. **(A)** ICAM-1 gene expression levels in normal mucosal biopsies from healthy subjects (black), uninflamed (open circles), and inflamed (closed circles) biopsies from the colon and ileum. **(B)** Examples of ICAM-1 protein localization by IHC in (left to right) healthy colon, inflamed UC colon, healthy ileum, and inflamed CD ileum. **(C)** VCAM-1 gene expression and **(D)** protein localization by IHC in inflamed and uninflamed biopsies from the colon and ileum of healthy subjects and IBD patients. **(E)** MAdCAM-1 gene expression and **(F)** protein localization by IHC in inflamed and uninflamed biopsies from the colon and ileum of healthy subjects and IBD patients. Statistical significance of indicated comparisons using the Kruskal–Wallace test. For IHC, 10 samples were stained per group and a single representative image is shown at 20 \times magnification. Scale bar, 100 μ M. * p < 0.05, ** p < 0.01, *** p < 0.001. –infl, uninflamed; +infl, inflamed.

and continued to increase over later cell divisions, whereas CD4⁺ T cells had lower MFIs, yet achieved these expression levels at earlier cell divisions (Fig. 3E). α E MFI on CD4⁺ α E⁺ lymphocytes were not dependent on the number of cell divisions, whereas α E MFI of CD8⁺ α E⁺ T cells increased with successive cell division (Fig. 3E). A similar level of upregulation of α E in response to anti-CD3/CD28 and 10 ng/ml TGF- β 1 was observed on both CD4⁺ and CD8⁺ T cells from peripheral blood of IBD patients (Supplemental Fig. 3A). No effect on α E induction was observed when therapeutically relevant anti-integrin Abs (anti- α 4 β 7 or anti- β 7) were included in the culture for the duration of the stimulation (Supplemental Fig. 3B).

TGF- β 1-driven induction of α E expression on CD4⁺ T cells has been reported to be reduced in sarcoidosis patients carrying a genetic polymorphism in the promoter of the *ITGAE* gene tagged by the rs2981 single nucleotide polymorphism (22). We evaluated reported genetic risk alleles in the *ITGAE* gene in genotyped healthy donors to assess effects on surface α E expression. In contrast to previous studies, we found no association between rs2981 and α E expression by frequency or surface levels in either CD4⁺ (Fig. 3F) or CD8⁺ T cells (Fig. 3G). We also observed no significant differences in α E expression by frequency or surface levels for described intronic and exonic alleles (data not shown).

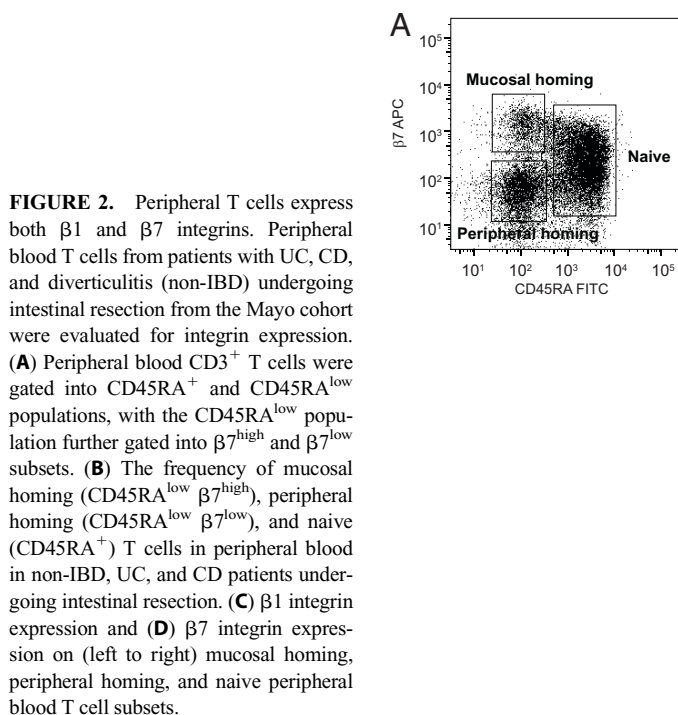
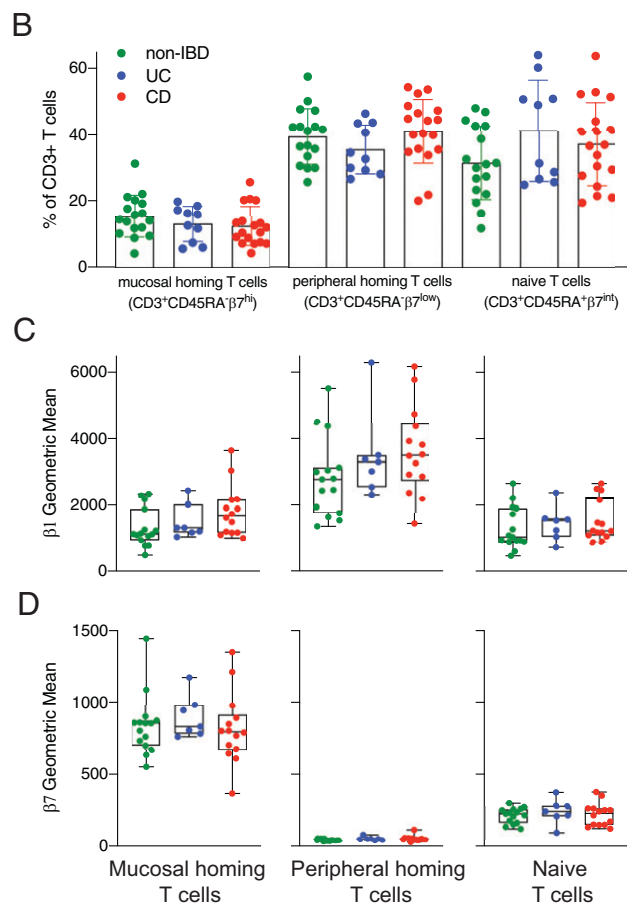


FIGURE 2. Peripheral T cells express both $\beta 1$ and $\beta 7$ integrins. Peripheral blood T cells from patients with UC, CD, and diverticulitis (non-IBD) undergoing intestinal resection from the Mayo cohort were evaluated for integrin expression. **(A)** Peripheral blood $CD3^+$ T cells were gated into $CD45RA^+$ and $CD45RA^{low}$ populations, with the $CD45RA^{low}$ population further gated into $\beta 7^{high}$ and $\beta 7^{low}$ subsets. **(B)** The frequency of mucosal homing ($CD45RA^{low} \beta 7^{high}$), peripheral homing ($CD45RA^{low} \beta 7^{low}$), and naive ($CD45RA^+$) T cells in peripheral blood in non-IBD, UC, and CD patients undergoing intestinal resection. **(C)** $\beta 1$ integrin expression and **(D)** $\beta 7$ integrin expression on (left to right) mucosal homing, peripheral homing, and naive peripheral blood T cell subsets.



Although TGF- $\beta 1$ can induce αE expression on both $CD4^+$ and $CD8^+$ T cells, the baseline requirement for $\beta 7$ expression prior to upregulation of αE has not been extensively evaluated (33). We sorted T cells by $\beta 7$ expression and CD4 or CD8 lineage prior to stimulation with anti-CD3/CD28 and TGF- $\beta 1$. Following in vitro stimulation, both $\beta 7^+$ and $\beta 7^-$ T cells upregulated αE expression (Fig. 3H, 3I). Both $\beta 7^+$ and $\beta 7^-$ $CD4^+$ and $CD8^+$ T cells upregulated αE expression in response to TGF- $\beta 1$ by day 6–8, with $\sim 20\%$ relatively fewer αE^+ cells observed in baseline $\beta 7^-$ cells (Fig. 3H, 3I). These data confirm that both $\beta 7^-$ $CD4^+$ and $CD8^+$ T cells can be induced to upregulate αE following TGF- $\beta 1$ stimulation in vitro, although the frequency of cells with inducible αE expression is consistently lower than for cells that already have surface $\beta 7$ expression.

pSMAD3 and increased TGF- β -inducible gene expression in IBD intestinal mucosa

TGF- β regulates transcription of the αE and $\beta 7$ integrin genes (*ITGAE* and *ITGB7*, respectively) (33–35). Serine/threonine phosphorylation of SMAD2 and SMAD3 following TGF- β activation of ALK5 (TGF- $\beta R1$) as well as NFAT-1 activity have been shown to drive expression of *ITGAE* (36, 37). Indeed, both NFAT-1 and SMAD binding sites have been identified in the *ITGAE* promoter and enhancer (36). The *ITGB7* promoter contains two functional TGF- β response elements (35). Conversely, TGF- β leads to reduced transcription of the $\alpha 4$ integrin subunit (35).

We used IHC to evaluate changes in SMAD3 phosphorylation (pSMAD3) in biopsies from IBD patients in comparison with healthy subjects. In healthy subjects, pSMAD3 was largely limited to the epithelium in both the colon (Fig. 4A) and ileum (Fig. 4B), with a few scattered mononuclear cells also staining positive. The majority of epithelial cells displayed positive staining for pSMAD3

in the ileum, with a lower frequency of positive cells in the colon. pSMAD3 $^+$ mononuclear cells were also more frequent in the ileum than in the colon of healthy subjects. Colonic biopsies from UC patients with active disease showed increased infiltration of mononuclear cells, many of which were pSMAD3 $^+$ (Fig. 4C). Epithelial staining for pSMAD3 in UC biopsies was more intense than in healthy colonic tissue. Similarly, colonic biopsies from CD patients showed increased epithelial staining intensity (Fig. 4D). Ileal biopsies from CD patients had localized increases in pSMAD3 $^+$ mononuclear cells in inflamed areas (Fig. 4E) with overall similar intensity of pSMAD3 in the epithelium compared with healthy ileum (Fig. 4B).

Analysis of TGF- β family members and receptor expression in isolated cells using single-cell RNA-sequencing data (38) showed that TGF- $\beta 1$ is the predominant family member expressed in the colon and is expressed primarily by immune cells, whereas TGF- β receptor expression was found on both immune and epithelial cells (Fig. 4F). TGF- $\beta 3$ was detected at low levels whereas TGF- $\beta 2$ was not detectable. Expression of TGF- $\beta 1$ and both TGF- β receptors was similar between healthy, inflamed, and uninfamed biopsies. TGF- β induces a much broader gene expression program beyond *ITGAE* and *ITGB7*, including common and distinct elements in different cell types (39). A common gene induced across cell types is *EGR1*, a transcription factor (37) with binding sites near TGF- $\beta 1$ target genes, including *SMAD7*, *IDI1*, and *SKIL* (40). Expression of the TGF- β -inducible genes *EGR1* and *EGR2* in intestinal biopsies from healthy subjects and IBD patients (Supplemental Fig. 3E, 3F). Increased gene expression of both *EGR1* and *EGR2* was observed in inflamed colonic biopsies versus healthy colon or uninfamed biopsies from IBD patients. There was no significant difference in *EGR1* or *EGR2* gene expression in ileal biopsies, consistent with the stronger pSMAD3 staining observed in the colon in comparison with the ileum.

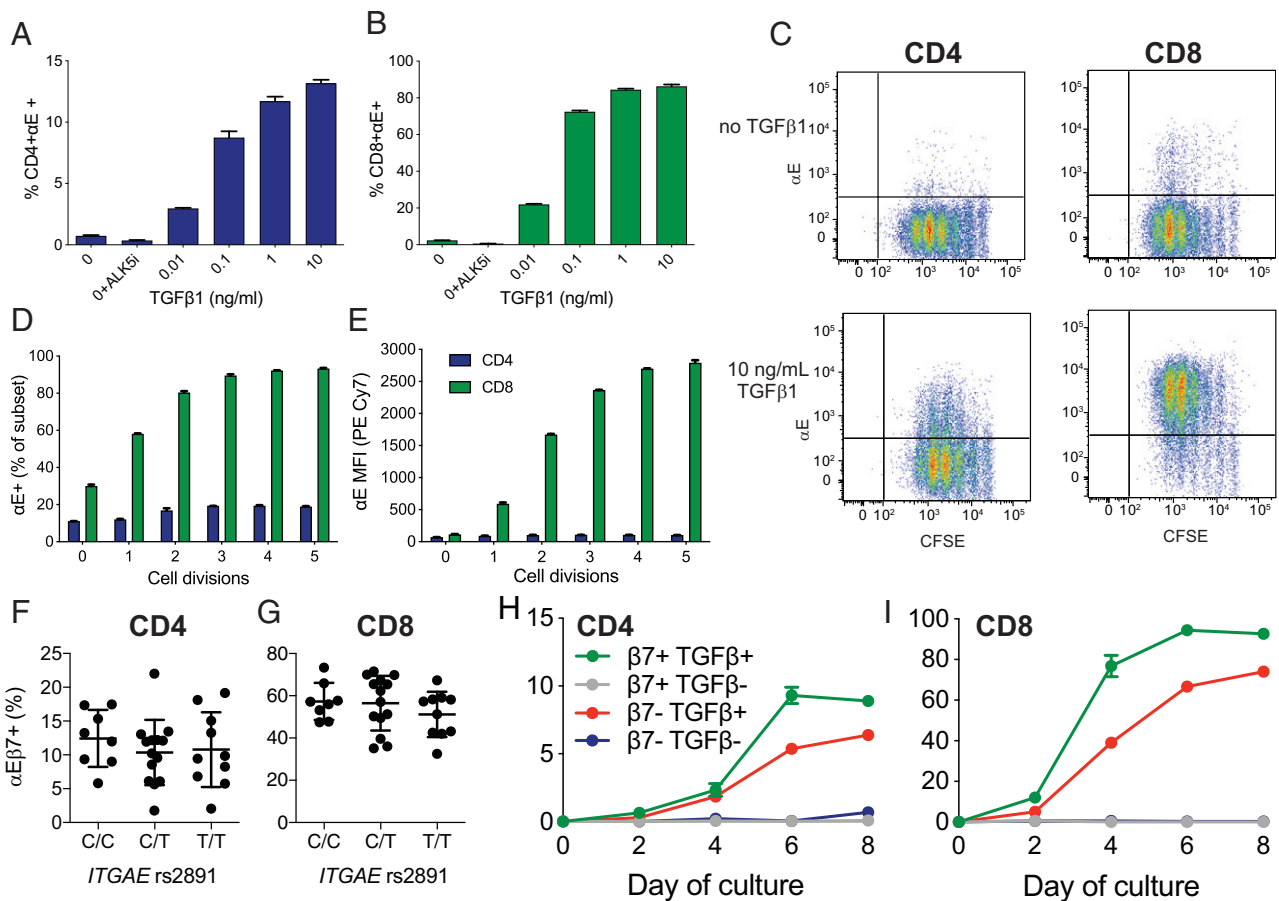


FIGURE 3. α E integrin can be induced by TGF- β 1 and anti-CD3/CD28 stimulation of peripheral blood T cells independent of baseline β 7 integrin expression. Peripheral blood T cells from healthy donors were stimulated for 4–9 d with anti-CD3/CD28 \pm TGF- β 1. Increasing cell surface expression of α E integrin was observed on (A) CD4 $^{+}$ and (B) CD8 $^{+}$ peripheral blood T cells in response to increasing doses of TGF- β 1 after 9 d of culture. Data are representative of two independent experiments. No α E cell surface expression was found on either T cell population when ALK5 inhibitor was added to the culture. (C) CFSE dilution at 4 d shows increased α E expression on CD4 $^{+}$ and CD8 $^{+}$ T cells with increasing cell division in the presence of TGF- β 1. Increases in both the (D) overall percentage of α E $^{+}$ T cells and the (E) MFI of α E on both CD4 $^{+}$ and CD8 $^{+}$ T cells. Data in (C)–(E) are representative of three independent experiments. (F and G) PBMCs from *ITGAE* rs2891 genotyped healthy subjects with either C and/or T alleles were stimulated with anti-CD3/CD28/TGF- β 1 for 6 d, then CD4 $^{+}$ and CD8 $^{+}$ T cells were evaluated for α E expression. (H and I) Peripheral blood T cells were sorted by baseline expression of β 7 integrin and stimulated with anti-CD3/CD28 \pm TGF- β 1 (10 ng/ml) for 8 d. α E integrin expression was observed following TGF- β 1 stimulation on both β 7 $^{-}$ and β 7 $^{+}$ CD4 $^{+}$ and CD8 $^{+}$ T cells. Data are representative of three independent experiments.

Gene expression in induced α E $^{+}$ T cells is independent of baseline β 7 expression status

pSMAD3 levels in the inflamed colon suggest that TGF- β signaling is increased in active colonic disease. We hypothesized that upregulation of α E integrin by local active TGF- β would not require pre-existing expression of the α 4 β 7 dimer and that both β 7 $^{-}$ and β 7 $^{+}$ peripheral blood T cells can induce α E following migration into the intestinal mucosa. To evaluate potential differences in the α E $^{+}$ T cell phenotype due to initial β 7 integrin expression, we developed a two-step in vitro culture and cell sorting approach to enable comparison of gene expression between these populations. Data from seven donors confirmed expression of α E integrin on <1% of peripheral blood CD4 $^{+}$ T cells and \sim 3% of peripheral blood CD8 $^{+}$ T cells, with 80% of CD4 $^{+}$ T cells and 60% of CD8 $^{+}$ T cells showing high expression of β 7 integrin (Fig. 5A). Ubiquitous expression of α L integrin was observed on peripheral CD4 $^{+}$ and CD8 $^{+}$ T cells (Fig. 5B). β 1 integrin was observed on \sim 90% of CD4 $^{+}$ T cells and nearly all CD8 $^{+}$ T cells (Fig. 5C). Initial cell sorting was based on β 7 expression, with low/negative β 7 (peripheral homing) and high β 7 (mucosal homing) T cells sorted from seven healthy donors (Fig. 5D, 5E). Following 10 d of culture with anti-

CD3/CD28 beads and 10 ng/ml TGF- β 1, a subset of T cells had upregulated α E surface expression. Higher induction of α E was again observed on CD8 $^{+}$ in comparison with CD4 $^{+}$ cells, and cells were sorted into α E $^{+}$ CD4 $^{+}$, α E $^{-}$ CD4 $^{+}$, α E $^{+}$ CD8 $^{+}$, and α E $^{-}$ CD8 $^{+}$ fractions (Fig. 5F). Consistent with upregulation of α E surface expression in response to TGF- β 1, there was a clear increase in β 7 MFI in all cell groups over the 10 d of culture (Fig. 5G).

Analysis using a linear model approach to evaluate the effect of baseline β 7 expression was performed independently for CD4 $^{+}$ and CD8 $^{+}$ T cells using α E surface expression at day 10 as a covariate (Fig. 5H). T cell lineage genes, TGF- β 1 response genes, and genes associated with α E β 7 integrin (18, 20) were selected for evaluation based on expression in cell types of interest. For CD4 $^{+}$ T cells, baseline β 7 integrin expression had a nominally significant (unadjusted $p < 0.05$) effect on the following genes: *ITGA4*, *GATA3*, *GZMA*, *BATF*, *HIFA*, *ICOS* and *IRF4*, although none of these genes had an FDR below 0.1. For CD8 $^{+}$ T cells, *STAT1*, *ITGB1*, *ITGA4*, *CD127*, and *CD4* demonstrated a significant ($p < 0.05$) effect of baseline β 7 integrin expression. Both *STAT1* and *ITGB1* had an FDR below 0.05. The majority of genes showed no difference in expression related to initial β 7 expression levels.

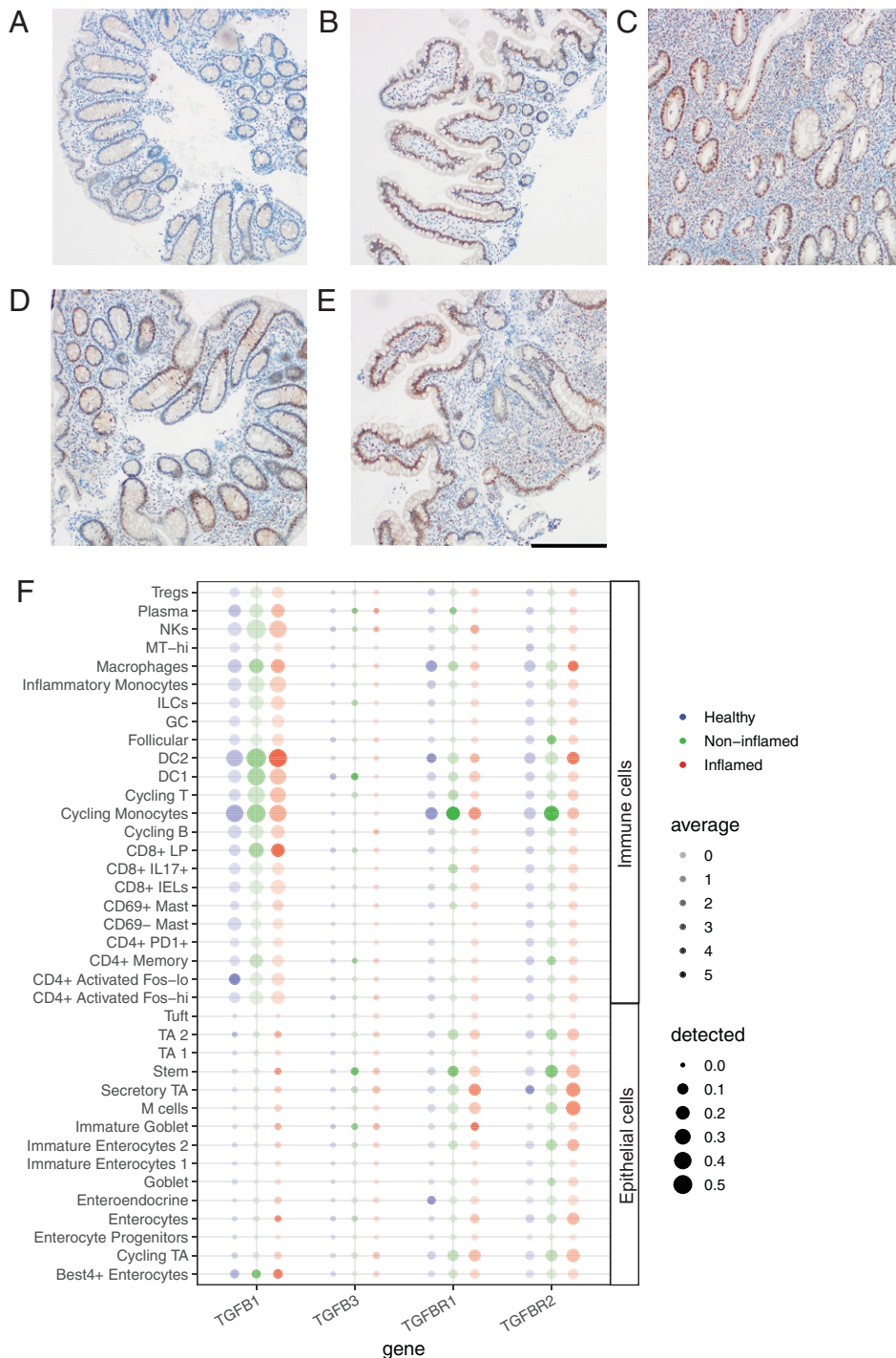


FIGURE 4. Inflamed colonic tissue exhibits increased pSMAD3 with TGF-βR expression on immune and epithelial cells. Intestinal biopsies from healthy subjects or IBD patients from the Newcastle cohort were evaluated for evidence of TGF-β-induced signaling or gene expression. pSMAD3 expression in the colon and ileum during active IBD. pSMAD3 (DAB; brown) staining is shown for (A) colon and (B) ileum for matched tissue biopsies from a healthy subject. Actively inflamed colonic biopsies from a (C) UC and (D) CD patient. (E) Representative image of an inflamed ileal biopsy from a CD patient. Images are representative of staining from five subjects in each group. Images are shown at 10× magnification. Scale bar, 200 μM. (F) Expression of TGF-β1, TGF-β3 and their receptors was evaluated in single cell RNA-sequencing data from healthy volunteers (*n* = 12) and IBD patients (*n* = 18). Upregulation of TGF-βR1 and TGF-βR2 was observed in inflamed colonic tissue.

Discussion

Inflammatory events have the potential to change established patterns of cellular migration and lymphocyte differentiation in patients with IBD. Under homeostatic conditions, lymphocytes activated within mucosal lymph nodes to express high levels of α4β7 integrin as well as naive cells with intermediate α4β7 integrin expression circulate through the intestinal mucosa. In IBD, activation of inflammatory cascades can alter expression patterns of adhesion molecules that serve as entry points into mucosal tissues. Increased levels of adhesion molecules on the intestinal endothelium may alter lymphocyte migration into the gut. We evaluated VCAM-1, ICAM-1, and

MAdCAM-1 gene and protein expression in healthy mucosa from normal subjects and uninflamed and inflamed mucosa from patients with IBD. Although inflamed mucosa had generally increased expression of adhesion molecules, ICAM-1 was broadly upregulated, consistent with its known expression by infiltrating monocytes and plasma cells, whereas VCAM-1, and in particular MAdCAM-1, were more restricted in expression to endothelial cells. Subsets of peripheral blood T cells can express both α4β7 and/or α4β1, consistent with previous data showing that T cells express multiple integrins for entry into mucosal tissues (20). Once cells have migrated into the mucosa, induction of other integrins can occur.

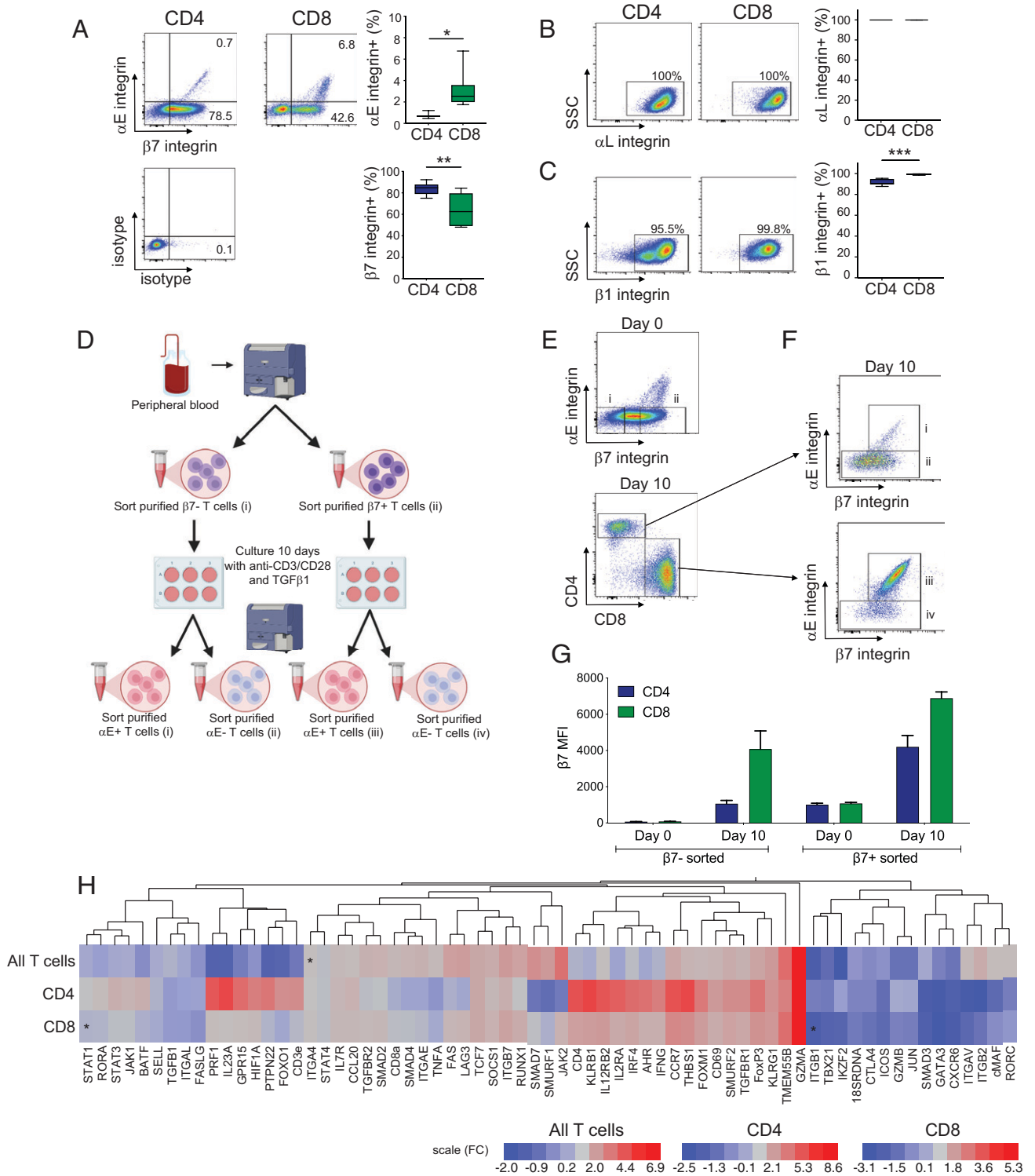


FIGURE 5. Baseline β7 expression does not drive differences in gene expression in αE⁺ and αE⁻ T cells. **(A)** PBMCs were obtained from seven healthy donors and evaluated for surface expression of (A) αE and β7 **(B)** αL and **(C)** β1 integrin subunits. Representative FACS plots of CD4⁺CD8⁺ and CD4⁺CD8⁻ T cell expression are shown with accompanying isotype control staining and Tukey plots of the percentage of CD4⁺ and CD8⁺ cells that express each integrin subunit. **(D)** Culture strategy for evaluation of the effect of baseline β7 integrin expression on αE induction and gene expression, showing final groups in analysis. **(E)** PBMCs were sort purified into 1) β7⁻ and 2) β7⁺ fractions, then stimulated for 10 d with anti-CD3/CD28 plus TGF-β1 for 10 d. **(F)** After 10 d of culture, four cell populations 1) CD4⁺αE⁺, 2) CD4⁺αE⁻, 3) CD8⁺αE⁺, and 4) CD8⁺αE⁻ were sorted from baseline β7⁻ and from baseline β7⁺ cultures, yielding eight total groups for gene expression analysis from each of the seven donors. **(G)** Baseline and day 10 levels of β7 surface expression were measured and increased β7 was observed in all groups. **(H)** Fold change of candidate genes (cytokines, chemokines, cytokine, and chemokine receptors, signaling molecules, transcription factors, T cell surface markers, and effector molecules) is shown. Significant differences are indicated by an asterisk.

We demonstrate that T cells without α4β7 expression can be induced to express αE integrin following exposure to TGF-β1 under activating conditions. Downstream TGF-β gene expression and

pSMAD3, a key part of the TGF-β signaling pathway, are both increased in the colon of IBD patients in comparison with healthy subjects, suggesting a TGF-β rich environment. TGF-β1 was the

predominant TGF- β family member by gene expression in colonic macrophages, T cells and DCs, whereas TGF- β R1 and TGF- β R2 are expressed on monocytes, T cells and epithelial cells. Finally, following the induction of α E expression on T cells that were either α 4 β 7⁺ or α 4 β 7⁻ by TGF- β 1, gene expression was largely not dependent on initial β 7 expression. This suggests that T cells that undergo α 4 β 7-independent migration into mucosal tissue sites can give rise to inflammatory α E⁺ T cells and therefore that α 4 β 7-directed therapy alone may be insufficient to modulate α E β 7⁺ lymphocyte retention in vivo. How frequently this occurs in the human gut cannot be measured using current clinical techniques.

IBD may increase the likelihood that both α 4 β 7⁺ and α 4 β 7⁻ T cells enter the intestinal mucosa through compensatory homing mechanisms. Increased expression of ICAM-1 and VCAM-1 (Fig. 1), which has been shown to resolve at the gene expression level following anti-TNF treatment (41), may enable entry of these cells into the intestinal mucosa in IBD. Our data in healthy subjects were from two age-matched cohorts and showed increased expression of adhesion molecules at the gene expression and protein level. We found that peripheral blood T cells coexpress β 1 and α 4 β 7 on a significant fraction of mucosal homing T cells and naive T cells (Figs. 2 and 5). Previous reports show an increase in peripheral blood α 4⁺ β 1^{high} cells and a corresponding increase in α 4⁺ β 1^{high} T cells in intestinal mucosa from CD patients (7). In the absence of a human Ab specific for the α 4 β 1 dimer, it remains to be determined whether α 4 β 1 is expressed on the cell surface of circulating cells, or whether β 1 is paired with another of the 10 α -integrin chains that are known to form β 1 heterodimers. Experiments using humanized mice and mouse models show that lymphocytes use compensatory homing mechanisms to enter the mucosa; this process can be blocked using a small molecule inhibitor specific for α 4 β 1 (7).

Once T cells have entered the LP, TGF- β 1 can induce upregulation of α E through SMAD phosphorylation. Our data suggest that TGF- β -induced gene expression may occur more readily upon entry into the colon than the ileum during active IBD where increases in pSMAD3 and TGF- β -inducible genes were observed. Previous work has shown that colonic α E⁺CD4⁺ T cells express increased inflammatory cytokines such as IL-17 and IFN- γ (21). The frequency of α E⁺CD4⁺ T cells is increased in UC, which is consistent with our observations of increased TGF- β signaling. When we induced α E expression by treating activated T cells in vitro, we detected few gene expression differences for inflammatory cytokines or effector molecules between cells that were originally α 4 β 7⁺ and α 4 β 7⁻. These data suggest that cells that migrate into the mucosa using compensatory homing mechanisms can develop an α E-associated inflammatory phenotype. Abs that block α E integrin suppress accumulation of inflammatory lymphocytes in the intestinal mucosa (9, 24), indicating that once α E β 7 is induced, it increases cellular retention in the mucosa. The decrease in cellular accumulation was more pronounced at later time points in a humanized mouse model, consistent with effects on retention rather than initial homing (24).

Recent studies have specifically addressed migration of cells into tissue, which is challenging to directly evaluate in clinical studies. The effect of vedolizumab on lymphocyte trafficking was recently described in a study that followed labeled cells using imaging, but observed no change in T cell migration to the intestinal mucosa (42). Flow cytometry within the same study protocol also failed to demonstrate a change in T cell frequency following 14 wk of vedolizumab treatment, supporting the idea that alternative migratory pathways following treatment with vedolizumab may be active. A separate study observed decreased α 4 β 7⁺ within intestinal T cell subsets but no change in α E β 7⁺ T cells in the colon, whereas also noting incomplete occupancy on intestinal CD8⁺ T cells (43). Our

in vitro differentiation studies show that initial β 7 expression is not required for upregulation of α E. Indeed, patients treated with vedolizumab have been found to have an increase in α E⁺ CD8⁺ T cells in peripheral blood during maintenance time points, suggesting that blockade of α 4 β 7 does not impair α E upregulation (43). Taken together, these findings suggest that blockade of α 4 β 7 alone may not be sufficient to inhibit T cell trafficking to the intestinal mucosa, and β 7⁻ negative cells may undergo induction of α E β 7 once in the mucosa, increasing retention of inflammatory T cells.

Inhibition of lymphocyte trafficking has proven to be an effective strategy for treating both UC and CD, yet in a significant fraction of patients, medication fails to induce and maintain remission. Although therapies designed to inhibit migration through blockade of α 4 β 7 or MAdCAM-1 have shown efficacy, studies showing that blockade of α 4 β 7/MAdCAM-1 interactions alone may leave additional homing and retention pathways intact. Consistent with this, studies evaluating changes in inflammatory gene expression following treatment with anti-integrins show that few genes are significantly changed by vedolizumab (anti- α 4 β 7) treatment in comparison with inhibition of TNF- α at early time points in UC patients (44). Our more recent phase 3 study showed that etrolizumab significantly decreases lymphocyte gene expression at week 14 in CD patients (9), consistent with data from a phase 2 study of etrolizumab in UC (18). Continued migration of lymphocytes through α 4 β 7-independent compensatory mechanisms and/or increased retention of lymphocytes following α E β 7 upregulation might yield insights into patients who do not respond to vedolizumab treatment.

In conclusion, we demonstrate that α E β 7 can be induced on both α 4 β 7⁺ and α 4 β 7⁻ T cells following exposure to TGF- β 1, and we show evidence for increased TGF- β activity in the colon of patients with active IBD. These data, in combination with an increase in adhesion molecules in active IBD, highlight the potential for increased compensatory migration mechanisms to the gut mucosa beyond α 4 β 7/MAdCAM-1 in active inflammation that can result in α E⁺ T cell accumulation. Inflammatory gene expression in α E⁺ T cells was largely unchanged between T cell populations that were originally α 4 β 7⁺ and α 4 β 7⁻ T cells. These data suggest that IBD patients treated with Abs that block α 4 β 7 or MAdCAM-1 may benefit from additional integrin and/or inflammatory cytokine blockades to further inhibit inflammatory cell migration to the gut mucosa.

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Disclosures

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Key Points

- Adhesion molecules are upregulated in inflamed intestinal mucosa in IBD patients.
- Baseline $\beta 7$ expression does not impact αE induction or gene expression in T cells.
- Phospho-SMAD3 is increased in inflamed mucosa in IBD.