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Dual targeting of lymphocyte homing and retention through $\alpha 4\beta 7$ and $\alpha E\beta 7$ inhibition in inflammatory bowel disease

Highlights
- Blockade of $\alpha 4\beta 7$ and $\alpha E\beta 7$ reduces CD8$^+$ T cells in the gut mucosa more than $\alpha 4\beta 7$ alone
- Anti-$\alpha E\beta 7$ or -E-cadherin reduces retention and increases egress of T cells in the gut
- $\alpha E\beta 7^+$ intestinal T cells are proinflammatory and have little to no regulatory markers
- Etrolizumab reduces mucosal inflammatory T cell genes in patients with Crohn disease

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In brief
Dai et al. demonstrate the cooperative roles $\alpha 4\beta 7$ and $\alpha E\beta 7$ integrins have in CD8$^+$ T cell accumulation in the gut mucosa in a mouse model of trafficking. Intestinal biopsies from patients with Crohn disease that has been treated with etrolizumab (anti-$\beta 7$) show treatment-specific reductions in gene expression associated with CD8$^+$ cytotoxic T cells.
Dual targeting of lymphocyte homing and retention through $\alpha 4\beta 7$ and $\alpha E\beta 7$ inhibition in inflammatory bowel disease

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SUMMARY

Anti-integrins are therapeutically effective for inflammatory bowel disease, yet the relative contribution of $\alpha 4\beta 7$ and $\alpha E\beta 7$ to gut lymphocyte trafficking is not fully elucidated. Here, we evaluate the effect of $\alpha 4\beta 7$ and $\alpha E\beta 7$ blockade using a combination of murine models of gut trafficking and longitudinal gene expression analysis in etrolizumab-treated patients with Crohn’s disease (CD). Dual blockade of $\alpha 4\beta 7$ and $\alpha E\beta 7$ reduces CD8+ T cell accumulation in the gut to a greater extent than blockade of either integrin alone. Anti-$\alpha E\beta 7$ reduces epithelial:T cell interactions and promotes egress of activated T cells from the mucosa into lymphatics. Inflammatory gene expression is greater in human intestinal $\alpha E\beta 7^+$ T cells. Etrolizumab-treated patients with CD display a treatment-specific reduction in inflammatory and cytotoxic intraepithelial lymphocytes (IEL) genes. Concurrent blockade of $\alpha 4\beta 7$ and $\alpha E\beta 7$ promotes reduction of cytotoxic IELs and inflammatory T cells in the gut mucosa through a stepwise inhibition of intestinal tissue entry and retention.

INTRODUCTION

To mount an efficient immune response, lymphocytes travel between secondary lymphoid organs and mucosal tissues to enable antigen recognition, leading to activation and expansion. The gastrointestinal tract harbors a high antigenic load derived from food and microbiota and is a highly dynamic compartment in terms of immune cell movement.1 In the mesenteric lymph node (mLN), activated T lymphocytes are imprinted in a specialized microenvironment that results in increased expression of integrin $\alpha 4\beta 7$ and CCR9.2,3 Elevated $\alpha 4\beta 7$ enables T cells to have an increased capacity to adhere to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) on endothelial venules in the lamina propria (LP).4 Within the LP, T cells further upregulate $\alpha E$ integrin expression in response to the transforming growth factor $\beta$ (TGF-$\beta$)-rich environment.5,6 E-cadherin expressed by intestinal epithelial cells serves as a high-affinity ligand for $\alpha E\beta 7$.7–9 $\alpha E\beta 7$ is also expressed on a subset of dendritic cells in both mouse and human and are involved in T cell differentiation and imprinting of homing receptors.10 Gut-resident T cells also express CD69 to repress S1PR1-dependent tissue egress into lymphatics through CD69:S1P1 complex formation, another mechanism of lymphocyte retention in tissue.11,12

Inflammatory bowel disease (IBD) encompasses both ulcerative colitis (UC) and Crohn’s disease (CD) and is characterized by aberrant inflammatory responses in the gastrointestinal (GI) tract.13–15 Patients with IBD have increased activated lymphocytes in gut tissues, and anti-integrin therapies are efficacious.16,17 Natalizumab, which blocks the $\alpha 4$ subunit of the $\alpha 4\beta 7$ and $\alpha 4\beta 1$ integrin heterodimers, was the first anti-integrin antibody approved for use in CD in 2004.17 Vedolizumab is an anti-integrin monoclonal antibody that blocks the $\alpha 4\beta 7$...
heterodimer while sparing α4β7 integrin and is approved for both UC and CD.\textsuperscript{18,19} Etrolizumab,\textsuperscript{20–23} an investigational monoclonal antibody that blocks the β7 subunit of both α4β7 and αEβ7, is currently under development for CD.\textsuperscript{24}

Despite the potential for gut-homing cells to be differentially affected by the integrin heterodimers targeted by these therapeutics, the relative contribution of α4β7 and αEβ7 in lymphocyte homing and retention is not yet fully understood.\textsuperscript{25} Here, we established a surgical photoconversion system to investigate the integrin dependencies of T cell homing and retention in the gut and tissue egress to the mLN in a mouse model. Single-cell RNA sequencing (RNA-seq) and flow cytometry show that human inflammatory intestinal CD8\textsuperscript{+} T cell subsets, and not regulatory T cells, have high expression of αEβ7. Consistent with that, we demonstrate a significant reduction of genes associated with cytotoxicIELs in patients with CD enrolled in a placebo-controlled study of etrolizumab (ClinicalTrials.gov: NCT02394028), which showed efficacy in an exploratory subcohort analysis.\textsuperscript{26} Taken together, these mechanistic mouse model studies and human clinical studies suggest that targeting β7 integrin will effectively reduce inflammatory CD8\textsuperscript{+} T cells and intestinal inflammation through dual effects on lymphocyte homing and retention.

RESULTS

Migration of CD8\textsuperscript{+} T cells from mLN to gut mucosa is additively inhibited by combined blockade of α4β7 and αEβ7

To evaluate the role of integrins in T cell trafficking from mLN into the gut mucosa, we established a surgical photoconversion procedure in the KikGR transgenic mouse model.\textsuperscript{27} KikGR protein changes color from green to red fluorescence upon exposure to violet light, which in these experiments was focused specifically on the mLN, thus “stamping” cells with red fluorescence to enable tracking their migration out of the mLN. As a proof-of-concept of this model, we evaluated steady-state mucosal immune cell trafficking from the mLN of control or FTY720 (a S1PR1 agonist)-dosed animals after photoconversion. Lymphocyte egress and re-distribution to various tissues was analyzed 16 h after photoconversion. Because of the critical role of S1PR1 in T cell egress from lymph nodes, most KikR\textsuperscript{+} photo-stamped T cells are confined to the mLN of FTY720-treated animals (Figure 1A). In contrast, dynamic egress of mLN T cells was observed in vehicle-treated animals, with re-distribution of photo-stamped KikR\textsuperscript{+} T cells to secondary lymphoid organs, including the spleen, inguinal LN, and Peyer’s patches, as well as to the small intestine and colon (Figure 1A). Photo-stamped cells that migrated to the secondary lymphoid organs were mostly CD62L\textsuperscript{hi}CD44\textsuperscript{low} naive T cells, whereas T cells that had migrated into the colon LP were predominantly CD44\textsuperscript{hi} effector/memory T cells (Figure S1A).

After the successful proof-of-concept study, this model was used to evaluate anti-α4β7 and anti-β7 effects on T cell trafficking between the mLN and intestine. Anti-α4β7 blockade reduced the frequency of migrated KikR\textsuperscript{+} CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells by 50% in the LP compartment of the colon and small intestine (Figures 1B–1D and S1B). Anti-β7 antibody blockade, which blocks both α4β7 and αEβ7, showed greater inhibition of CD8\textsuperscript{+} T cells in comparison with that of CD4\textsuperscript{+} T cells (Figures 1B–1D and S1B). Although most of the mLN CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells express α4β7, αE expression is found on more than 60% of CD8\textsuperscript{+} T cells but less than 10% of CD4\textsuperscript{+} T cells (Figure S1C). Therefore, increased inhibition of CD8\textsuperscript{+} T cells by anti-β7 is likely due to the differential expression of αE integrin between CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells.

An anti-αE blocking antibody, which only binds to the αEβ7 heterodimer, was next used to further ascertain the individual contribution of α4β7 and αEβ7 in CD8\textsuperscript{+} T cell migration from mLN to the gut.\textsuperscript{28} Treatment with anti-αE blocking antibody significantly reduced photo-stamped KikR\textsuperscript{+} CD8\textsuperscript{+} T cells in the colon and small intestine as compared with control antibody (Figures 1E and 1F). As was previously observed with the β7 blockade, combined blockade of α4β7 and αEβ7 further reduced KikR\textsuperscript{+} CD8\textsuperscript{+} T cells ~50% more than blockade of either α4β7 or αEβ7 alone (Figures 1E and 1F). Taken together, our data suggest that blockade of α4β7 and αEβ7 has an additive effect in reducing CD8\textsuperscript{+} T cell trafficking to the small and large intestine.

Combined blockade of α4β7 and αEβ7 reduced trafficking of antigen-specific T cells in comparison with blockade of α4β7 or αEβ7 alone

To test the role of α4β7 and αEβ7 in antigen-dependent T cell migration to the intestinal LP and epithelium, we used an oral antigen-challenge model to elicit an immune response in the small intestine.\textsuperscript{29} Ovalbumin (OVA)-specific T cell receptor (TCR) transgenic MHC-I-restricted (OT-1) T cells were adoptively transferred into wild-type (WT) mice before oral immunization with OVA and cholera toxin (CT:OVA). Anti-integrin antibodies were administered to the recipient mice 3 days after CT:OVA immunization when activated T cells began to migrate from the mLN to the small intestine. Treatment with anti-α4β7 or anti-αE blockade led to a reduction in OT-1 T cells in both the LP and intraepithelial compartments, whereas the combination of these blocking antibodies resulted in further reduction of OT-1 T cells in both compartments (Figures 2A and 2B). In this model, blockade of both α4β7 and αEβ7 leads to a greater reduction of antigen-specific CD8\textsuperscript{+} T cells in comparison with blockade of either α4β7 or αEβ7 alone.

Blockade of αEβ7 or E-cadherin diminishes CD8\textsuperscript{+} T cell interaction with intestinal epithelium

We next asked whether blockade of E-cadherin, the only known ligand of αEβ7, also regulates CD8\textsuperscript{+} T cell accumulation similarly to anti-αE blockade. A blocking antibody against E-cadherin was used to disrupt E-cadherin:αEβ7 interactions after CT:OVA oral immunization.\textsuperscript{28} OT-1 CD8\textsuperscript{+} T cell accumulation in the intestinal mucosa was similarly reduced by anti-E-cadherin or anti-αE (Figure 3A), confirming that E-cadherin:αEβ7 interaction is critical for T cell accumulation in the gut epithelium.

T cells travel along and through the mesh-like structure of the basement membrane, an extracellular matrix composed of collagen and laminin, and interact with intestinal epithelial cells.\textsuperscript{30,31} E-cadherin protein is found on the basolateral side of the intestinal epithelium (Figure 3B; Video S1). Activated OT-1 T cells expressing red fluorescent protein (OT-1-tdTomato)
were used to enable live cell imaging. OT-1-tdTomato T cells localized to the basement membrane in close proximity to basolateral surfaces of epithelial cells, with extended cell bodies suggesting activation (Figure 3B; Video S1). Upon blockade of αEβ7 or E-cadherin, OT-1-tdTomato T cells preferentially localize to the LP with a few T cells observed in the epithelium (Figure 3B).

To follow T cell-epithelial cell interactions over time, OT-1-tdTomato CD8+ T cells were transferred into E-cadherin/cyan fluorescent protein (CFP) reporter mice, and activated T cells were tracked from the luminal side of the small intestine using two-photon intravital imaging (Figure 3C). As previously reported,30,32 activated T cells migrated toward subepithelial regions and exhibited dynamic interactions with epithelial cells during movement across the basement membrane (Figure 3C; Video S2). Upon αEβ7 blockade, activated T cells were observed to migrate across the basement membrane to reach the subepithelial regions less frequently (Figure 3C; Video S3). Quantification of individual T cells showed a statistically significant increase in maximum track speed (Figure 3D) and track length (Figure 3E) after αEβ7 blockade in comparison with the control. Because the blockade of αEβ7 led to increased T cell motility and reduced T cell-epithelial cell contact, our findings are consistent with a critical role for αEβ7 and E-cadherin in...
mediating tissue retention through sustained interactions between activated T cells and the epithelium.

**Blockade of αEβ7 increases T cell migration from the gut to the mLN**

Lymphatic vessels serve as conduits for lymphocyte movement out of the gut and into the mLN. The decrease in tissue retention after αEβ7 blockade may, therefore, directly affect T cell egress through lymphatics. To test that hypothesis, activated KikGR+OT-1+ cells were photo-stamped within the small intestine to enable lymphocyte tracking, and both KikR+OT-1+ cells remaining in the small intestine and KikR+OT-1+ cells in the mLN were evaluated 16 h later (Figure S2A). αEβ7 or E-cadherin blockade led to an ∼50% reduction in KikR+OT-1+ T cells in the small intestine (Figures 4A and S2B) and increased KikR+OT-1+ cells in the MLN by 3-fold (Figures 4B and S2C). Treatment with anti-αEβ7 antibody did not alter KikR+OT-1+ cells in the small intestine or mLN in comparison with the control, but the total number of OT-1+ cells was reduced, making the percentage similar to the FTY720 treatment (Figures 4A, 4B, S2B, and S2C).

T cell migration through the lymphatic vessels to the draining LNs is dependent on S1PR1. Treatment with FTY720, a S1PR1 agonist, increased T cells in the small intestine and reduced T cells in the mLN in comparison with the control (Figures 4A, 4B, S2B, and S2C). Consistent with an effect on lymphocyte egress from the mucosa, photo-stamped T cells were rare in intestinal lymphatics of FTY720-treated mice. In contrast, αEβ7 blockade increased T cell frequency within the lymphatic vessels (Figures 4C and 4D). Taken together, these findings support a critical role for αEβ7, but not αEβ7, in T cell retention within gut tissues, and the blockade increases subsequent egress of activated effector T cells from the mucosa to the draining LNs.

αEβ7 expression is restricted to cytotoxic CD8+ IELs and proinflammatory CD4+ T cell subsets in human colonic tissue

Studies in a photoconvertible mouse model demonstrated additive roles for αEβ7 and αEβ7 integrins in T cell migration and retention, suggesting targeting those complementary mechanisms with anti-integrin therapies may be important in IBD. Analysis of αE-expressing pooled IEL and LP immune cells in healthy and IBD human colonic biopsies showed that, similar to the mouse, most CD8+ T cells express αEβ7 (~80%), whereas only ~10% of the CD4+ T population are αEβ7+ (Figures 5A and S3A). αEβ7 CD4+ T cells produced higher levels of inflammatory cytokines, including interleukin 17 (IL-17) and interferon γ (IFN-γ) as compared with αE CD4+ T cells (Figures 5B and S3B). Human colonic regulatory T cells (CD4+CD25hiCD127lowFoxp3+) have low to absent αEβ7 expression as compared with non-regulatory T cells, in contrast to the proinflammatory phenotype of αE+CD4+ T cells (Figures 5C and S3C). Comparison of human and mouse T cells, using the *Helicobacter hepaticus* anti-IL10R model of colitis, suggested similarities in αE+CD8+ T cells (Figures 5H, S3D, and S3G), whereas the proinflammatory state of αE+CD4+ human T cells is directly opposite that of murine αE+CD4+ T cells, which have a regulatory T cell phenotype and produce significantly less inflammatory cytokines (Figures S3E and S3F).

Single-cell sequencing of biopsies containing both IELs and lamina propria lymphocytes (LPLs) revealed cellular heterogeneity of human colonic T cells with five main lymphocyte sub-clusters: CD8+ IELs, LP CD8+ TCRγδ+CD4+, effector/memory T cells, CD4+ regulatory T cells, and ILC3 cells (Figures 5D, S4A, and S4B; Data S1). The αE transcript is most abundant in CD8+ IELs (Figure 5E), consistent with surface protein expression (Figure 5A), and cytokine genes, including granzyme A and GZMB, perforin (PRF1), and NK receptors (KLRD1 and NKG7), are expressed in >80% of CD8+ IELs (Figures 5A and S4D). Further sub-clustering of CD8+ IELs identified four major sub-clusters: CD8+TCRγδ+, CD8+TCRβ+, CD8+TCRβ+IFN-γ+, and CD8+TCRβ+TIGIT+CD96+ IELs (Figure S4C). Despite their distinct TCR usage, cytokine profiles, and inhibitory receptor expression, all four CD8+ IEL subpopulations have similarly high expression of αE and cytokine genes, including GZMA and GZMB, perforin (PRF1), and NK receptors (KLRD1 and NKG7) (Figure S4D). Deep sequencing of sorted αE+ or αE-CD8+ T cells (Figure 5F) demonstrated αE+CD8+ IELs expressed even higher levels of cytokine genes compared with αE-CD8+ IELs (Figure 5G). Flow cytometry confirmed these findings, with αE+CD8+ T cells exhibiting higher level of GZMA and...
GZMB, PRF1, NK receptors, and other tissue-resident T cell markers, such as CD49a (Figure 5H). Taken together, our data indicate that αE expression by CD8+ cytotoxic IELs is associated with expression of cytotoxic genes, IFN-γ, and chemokines CCL3–5.

Blockade of β7 integrin by etrolizumab reduces cytotoxic CD8+ IEL gene expression in intestinal biopsies

Our mechanistic studies suggest β7 blockade may alter CD8+ T cell subset accumulation in intestinal tissue in patients with IBD. We tested the effect of β7 blockade in patients with CD...
who were enrolled in a phase III study of etrolizumab. Colonic or ileal biopsies were taken for RNA-seq before randomization to etrolizumab or placebo treatment arms and at 14 weeks after treatment. Etrolizumab-treated patients showed significant decreases in integrin gene expression with reductions in β7 integrin (ITGB7) and α4 integrin (ITGA4) (Figure 6A) in both the ileum and the colon. Although decreased expression of αE integrin (ITGAE) was observed after etrolizumab treatment, the difference was not statistically significant relative to placebo (Figures 6A and S5A).

Significant post-treatment changes in gene expression were observed in etrolizumab-treated patients but not in placebo-treated controls (Figures 6B and 6C). Comparison of baseline gene expression between areas with active disease and areas without active disease showed an inverse correlation between genes altered by etrolizumab treatment and genes associated with endoscopic activity (Figures 6D and 6E), suggesting a treatment-specific reduction in active inflammation by etrolizumab. The genes most downregulated by etrolizumab include genes associated with B cells (IGHG1, PAX5, and CD79B), T cells (CD8A, CD5, and CD28), and inflammatory cytokines (IFN-γ, IL-23, and TNF) (Figure 6B).

Because CD can affect the entire gastrointestinal tract, sub-analyses by anatomic region were performed. Gene expression changes between active and inactive disease were similar in both the ileum and colon (Figures S5A and S5B), and inflammatory gene expression was also similarly reduced in both locations after etrolizumab treatment (Figures S5C). These data demonstrate that inflammation-associated genes are downregulated by etrolizumab treatment in the colon and ileum. No correlation was observed in placebo-treated patients (Figure 6E).

To identify the T cell subsets affected by etrolizumab treatment, gene modules specific for each type of T cell were generated using our single-cell RNA-seq data (Figure 5D; Data S1). Signature scores were calculated for each of the gene modules in the bulk RNA-seq data from the CD cohort. CD8+ cytotoxic IEL and CD8+ LP T cell signature genes decreased in etrolizumab-treated patients in comparison with those treated with placebo (Figures 6F, S5B, and S7). Other T cell subsets also decreased from baseline in etrolizumab-treated patients, but there was no statistically significant difference between the etrolizumab and the placebo arms (Figure 6F). CD8+ cytotoxic IEL-specific genes, such as CD8A, NKG7, and CCL5, were strongly downregulated after etrolizumab treatment (Figures 6G and S5C).

Vedolizumab, which blocks α4β7 integrin, has been suggested to inhibit intestinal T cell trafficking, yet a recent study analyzing pre- and post-treatment colonic biopsies did not find any reduction in CD4+ and CD8+ T cells at 14 weeks. A study in a small cohort of HIV-infected patients with IBD showed an increase in total T cells in the terminal ileum, with no change in CD4/CD8 ratio, after vedolizumab treatment. To assess the effect of vedolizumab on cytotoxic IELs, we analyzed publicly available biopsy microarray data from patients with UC before and 12 weeks after treatment with vedolizumab. Similar to Zeissig et al., we did not observe a significant reduction of CD8+ cytotoxic IELs or CD8+ LP T cell-associated genes (Figure S7). Taken together with the reduction in CD8+ cytotoxic IELs and CD8+ LP T cell-associated genes after etrolizumab treatment, these data suggest that blockade of both α4β7 and α4β7 integrin gene expression with reductions in β7 integrin (ITGB7) and α4 integrin (ITGA4) (Figure 6A) in both the ileum and the colon. Although decreased expression of αE integrin (ITGAE) was observed after etrolizumab treatment, the difference was not statistically significant relative to placebo (Figures 6A and S5A). Significant post-treatment changes in gene expression were observed in etrolizumab-treated patients but not in placebo-treated controls (Figures 6B and 6C). Comparison of baseline gene expression between areas with active disease and areas without active disease showed an inverse correlation between genes altered by etrolizumab treatment and genes associated with endoscopic activity (Figures 6D and 6E), suggesting a treatment-specific reduction in active inflammation by etrolizumab. The genes most downregulated by etrolizumab include genes associated with B cells (IGHG1, PAX5, and CD79B), T cells (CD8A, CD5, and CD28), and inflammatory cytokines (IFN-γ, IL-23, and TNF) (Figure 6B). Because CD can affect the entire gastrointestinal tract, sub-analyses by anatomic region were performed. Gene expression changes between active and inactive disease were similar in both the ileum and colon (Figures S5A and S5B), and inflammatory gene expression was also similarly reduced in both locations after etrolizumab treatment (Figures S5C). These data demonstrate that inflammation-associated genes are downregulated by etrolizumab treatment in the colon and ileum. No correlation was observed in placebo-treated patients (Figure 6E).

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**DISCUSSION**

IBD is a chronic relapsing-remitting intestinal inflammatory disorder associated with an increase in activated lymphocytes in the intestinal mucosa. Etrolizumab, an anti-β7 integrin monoclonal antibody, is being tested for efficacy in CD in phase III (ClinicalTrials.gov: NCT02394028). Previous reports using humanized mouse models have shown that α4β7 is critical for homing of T cells to the colon and that blockade of αEβ7 can further decrease accumulation of CD8+ T cells. Here, we applied a surgical photoconversion system to quantify T cell homing and...
Figure 6. Etrolizumab treatment significantly reduces expression of genes associated with CD8⁺ cytotoxic IELs

Ileal or colonic biopsies were taken before treatment and at 14 weeks after treatment in a randomized placebo-controlled trial of etrolizumab (anti-β7 integrin) in patients with moderately to severely active CD.

(A) Fold change of integrin genes at 14 weeks after etrolizumab or placebo treatment. Each dot is an individual patient sample pair. Points are colored by sampling location (blue, colon; green, ileum). Boxes show the standard error, with the middle bar showing the group mean value.

(B and C) Volcano plots showing the log_{2}-fold change and p-value of each gene, comparing baseline to week-14 samples from etrolizumab-treated patients (B) or placebo-treated patients (C). Each gene is represented by a point, with genes shown in red undergoing a >1.5-fold change at a false-discovery rate (FDR) < 0.05.

(D and E) Scatterplots showing the correlation of the log_{2}-fold changes of individual genes in endoscopically active, versus inactive, disease and the log_{2}-fold changes observed between screening and week-14 samples from (D) etrolizumab-treated patients or (E) placebo-treated patients. Each point represents an individual gene.

(legend continued on next page)
retention and found that a combined blockade of α4β7 and αEβ7 is more effective in reducing CD8+ T cell accumulation in gut tissue than is blocking either integrin alone in an antigen-specific mouse model. Further, αEβ7 and E-cadherin mediate interactions of activated CD8+ T cells with the basolateral epithelium and prolong tissue retention time through inhibiting intestinal tissue egress. Our findings confirm that blockade of α4β7 and αEβ7 integrins inhibit T cell intestinal accumulation and extend their role to tissue entry and retention in a respective stepwise manner to inhibit T cell accumulation in the gut mucosa in a mouse model. Single-cell RNA-seq and flow cytometry of human colonic T cells show that most αE+ T cells are pro-inflammatory. Intestinal biopsy data from a placebo-controlled study of patients with CD demonstrate etrolizumab-specific reduction in genes associated with gut CD8+ cytotoxic IELs, along with a broad decrease of genes associated with active inflammation, consistent with a role for α4β7 and αEβ7 integrins in CD8 T cell accumulation in the gut mucosa.

Gastrointestinal immune priming and tissue trafficking is achieved through the induction of integrin and chemokine receptors that mediate gut homing of T cells activated within intestinal-draining lymphoid organs. Integrin-dependent gut trafficking is not all encompassing. T cells can still migrate to the gut during inflammation in the absence of α4β7 expression, and anti-α4β7 blockade only partially reduces T cell trafficking to the gut (Figure 1). Integrins α4β1 and αLβ2 may also mediate intestinal homing, a mechanism of particular importance in the ileum. Expression of addressins VCAM-1 and ICAM-1 on intestinal vascular endothelium can mediate adhesion and may be upregulated under inflammatory conditions, although reports in IBD are mixed.

CD8+ T cells express high levels of αEβ7; however, human CD8+ IELs express much higher levels of cytotoxicity-associated genes (Figures 5 and S3G). These differences highlight the importance of cross-validation of observations between pre-clinical and clinical studies to inform our mechanistic understanding of therapies in IBD. It is also critical to continue to validate observations, such as the relative roles of α4β7 and αEβ7 in integrin-mediated homing and retention made in model systems directly in human clinical studies wherever possible, with particular attention to new methodologies that can increase our mechanistic understanding.

Although anti-integrin therapies have been suggested to block trafficking of lymphocytes into the intestinal mucosa, data from patients treated with anti-integrins has been mixed. One study showed a reduction in intestinal CD4+ T cells in patients with CD treated with natalizumab (anti-α4) using immunohistochemistry. More recently, patients with UC and those with CD underwent a cell-labeling and imaging approach before and after vedolizumab (anti-α4β7) treatment, and no change in T cell migration to the intestinal mucosa was observed. In the same study, no change in T cell frequency was observed when evaluated by immune cell phenotyping by flow cytometry after 14 weeks of vedolizumab treatment. Vedolizumab treatment in HIV+ patients with IBD resulted in an increase in T cells in the ileum as well as a striking decrease in the size of lymphoid aggregates. Although HIV-1 tropism for mucosal homing complicates the interpretation, the decrease in lymphoid aggregate size, which may be more dependent on α4β7, instead of mucosal T cells, is notable.

In the phase III cohort of patients with CD assessed here, as well as in a previous phase II study in patients with UC, etrolizumab reduced expression of T cell genes in intestinal mucosa after 14 and 10 weeks, respectively. We applied the same methodology to a second vedolizumab study in patients with UC and confirmed that no change of T cell-associated gene sets was observed 12 weeks after treatment. Possible explanations for this negative finding may be the reduced dynamic range for measuring gene-expression changes inherent to microarrays in comparison with RNA-seq or that blockade of α4β7-dependent cell entry by vedolizumab may take longer than 12 weeks to have a noticeable effect on colonic T cells. However, our analysis showing T cell genes do not decrease after vedolizumab treatment is consistent with the Zeissig et al. study and, together with our murine data, suggests the reduction of cytotoxic IEL genes by etrolizumab may be attributable to the additional blockade of αEβ7-dependent tissue retention, which may contribute to greater or faster inhibition of inflammation during induction therapy with etrolizumab compared with vedolizumab. Future clinical studies with head-to-head comparison of etrolizumab and vedolizumab will be required to fully evaluate the differences between these therapeutic approaches.

The reduction in CD8+ cytotoxic IELs observed only in etrolizumab-treated patients suggests a potential effect of etrolizumab on the dysregulated mucosal immune response in IBD.
T cells have been associated with prognostic outcomes in IBD, and the role of IELs, which are predominantly CD8+, in inflammation is beginning to be better appreciated. CD8+ cytotoxic IELs are closely associated with the intestinal epithelium and, under normal conditions, can quickly provide defense against pathogen dissemination because of their resting expression of cytotoxic genes, which enables them to respond without proliferation. Upon stimulation, rapid IFN-γ secretion by CD8+ cytotoxic IELs augments T cell cytotoxicity against infected epithelial cells and increases barrier permeability. Additionally, gut-resident CD8+ T cells express high levels of NK receptors, such as NKG2D and KLRD1, which enable recognition of stressed epithelial cells. Two recent studies of CD8+ T cells from healthy volunteers and patients with UC using single-cell (sc)RNA-seq identified αEβ7 effector TNF-α/IFN-γ+CD8+ T cells. It has long been recognized that the frontline role of cytotoxic IELs is not without risks of aberrant activation. and recent data show that chronic inflammation in celiac disease, a gastrointestinal disorder triggered by immune response to dietary gluten, induces a pathogenic phenotype in IELs characterized by increased IFN-γ production and expression of CCL4. Whether similar effects of chronic inflammation on cytotoxic IEL populations occur in IBD will require further investigation but may soon yield to scRNA-seq techniques able to probe CD8+ cellular heterogeneity.

In conclusion, we demonstrate that integrins α4β7 and αEβ7 have cooperative roles in CD8+ T cell accumulation in the gut mucosa in a murine model of T cell trafficking. These data highlight an underappreciated role for αEβ7 in T cell retention at the epithelial barrier through T cell-epithelial interactions that inhibit S1PR1-dependent tissue egress and migration to mLN. Key differences between human and mouse αEβ7 expression on CD4+ T cell lineages, particularly regulatory T cells, were identified. Finally, we observed a significant reduction in expression of gene sets associated with CD8+ cytotoxic IELs after treatment with anti-β7 etrolizumab therapy in a large phase III study of patients with CD. Although recent data from phase III UC studies of etrolizumab showed mixed efficacy in top-line clinical efficacy, clinical studies of etrolizumab in CD are on-going.

**Limitations of the study**

Recognizing that mouse models have limitations in their ability to recapitulate human biology is important to acknowledge. Here, we report data using mouse-surrogate antibodies to dissect migration to, and retention of, lymphocytes in the intestinal mucosa, which show a role for α4β7 and αEβ7 in T cell trafficking, suggesting that the therapeutic potential in IBD may be greater if both integrin heterodimers are blocked. Because of the technical challenges of photo-conversion, a key component of the experimental model, we focused on the small intestine because cell retention in the colon could not be extensively investigated. In addition, possible depletion effects of murine surrogate antibodies have not been rigorously tested and could be mitigated by use of Fcγ receptor (FcGR)-deficient mice as recipients in experiments. Patient data from a clinical study of etrolizumab, which blocks both α4β7 and αEβ7, provide correlative data on the effectiveness of β7 blockade in reducing intestinal CD8 T cell populations analogous to our observations in murine models. There are limitations in the interpretation of human data based on sample size numbers, particularly given the number of paired samples in subanalyses based on treatment group and anatomic location, which will require validation in on-going etrolizumab trials.

**STAR+METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100381.

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**AUTHOR CONTRIBUTIONS**

B.D. performed experiments and analyzed data, J.A.H. analyzed data; R.I., K.-H.S., and A.G. performed experiments; A.N. processed patient samples; J.E., L.D.O., A.S., and Z.M. analyzed data; J.G., A.H., M.D., M.S., and W.A.F. were responsible for enrollment and acquisition of patient samples; A.C., Z.S., A.H., Y.S.O., S.T., and J.M. designed and executed the clinical studies; B.D., J.A.H., M.E.K., and T.Y. designed experiments and wrote the manuscript; all authors were involved in manuscript editing and finalization.
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DECLARATION OF INTERESTS

All authors, except J.G., A.H., M.D., M.S., and W.A.F., are current or past employees of Genentech, a member of the Roche group, and may hold Roche stock or stock options.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**       |        |            |
| APC anti-mouse CD45 (clone 30-F11) | BioLegend | Catalog #: 103112 |
| PE/Cy7 anti-mouse TCRζ (clone H57-597) | BioLegend | Catalog #: 109222 |
| APC/Cy7 anti-mouse TCRβ2 (clone B20.1) | BioLegend | Catalog #: 127818 |
| PerCP/Cy5.5 anti-mouse CD8 (clone 53-6-7) | BioLegend | Catalog #: 100734 |
| APC/Cy7 anti-mouse CD4 (clone GK1.5) | BioLegend | Catalog #: 100414 |
| BV510 anti-mouse CD62L (clone MEL14) | BioLegend | Catalog #: 104441 |
| BUV395 anti-mouse CD44 (clone 1M7) | BD Bioscience | Catalog #: 740215 |
| APC/Cy7 anti-mouse MHCII (clone M5/114.15.2) | BioLegend | Catalog #: 107628 |
| PE/Cy7 anti-mouse CD11c (clone N418) | BioLegend | Catalog #: 117318 |
| FITC anti-mouse/human CD11b (clone M1/70) | BioLegend | Catalog #: 101206 |
| PE anti-mouse FOXP3 (clone MF14) | BioLegend | Catalog #: 126404 |
| FITC anti-mouse IFN-γ (clone XMG1.2) | BioLegend | Catalog #: 505806 |
| PE anti-mouse IL-17A (clone TC11-18H10.1) | BioLegend | Catalog #: 506904 |
| APC anti-mouse TNF-α (clone TN3-19.12) | BioLegend | Catalog #: 506108 |
| PerCP/Cy5.5 anti-mouse CD103 (clone M290) | BD Bioscience | Catalog #: 563637 |
| APC anti-mouse CD103 (clone 2E7) | BioLegend | Catalog #: 121414 |
| Anti-Laminin antibody produced in rabbit | Sigma-Aldrich | SKU no: L9393 |
| Rat anti-mouse E-cadherin (clone ECCD2) | Thermo Fisher | Catalog #: 13-1900: RRID: AB_2533005 |
| Rabbit polyclonal anti-Epcam | Abcam | ab71916 |
| Goat anti-mouse LYVE-1 (R&D systems) | R&D systems | Catalog #: AF2125 |
| TruStain FcX (anti-mouse CD16/32 Antibody (clone 93) | BioLegend | Catalog #: 101320 |
| BV786 anti-human CD45 (clone HI30) | BioLegend | Catalog #: 304048 |
| Alexa Fluor 700 anti-human CD8 (clone HIT8a) | BioLegend | Catalog #: 300920 |
| PE/Cy7 anti-human CD4 (clone RPA-T4) | BioLegend | Catalog #: 300512 |
| PerCP/Cy5.5 anti-human CD103 (clone Ber-ACT8) | BioLegend | Catalog #: 350226 |
| PE anti-human/mouse lT (clone RIBS04) | BioLegend | Catalog #: 321204 |
| BV605 anti-human TCRγ (clone iP26) | BD Bioscience | Catalog #: 745088 |
| BV711 anti-human CD25 (clone M-A251) | BioLegend | Catalog #: 356138 |
| Pacific Blue anti-human FOXP3 (clone 259D) | BioLegend | Catalog #: 320216 |
| APC anti-human/mouse Helios (clone 22F6) | BioLegend | Catalog #: 137222 |
| FITC anti-human IFN-γ (clone B27) | BioLegend | Catalog #: 506504 |
| PE anti-human IL-17A (clone N49-4653) | BD Bioscience | Catalog #: 560486 |
| APC anti-human TNF-α (clone MAB11) | BioLegend | Catalog #: 502912 |
| PE/Cy7 anti-human CD49a (clone TS2/7) | BioLegend | Catalog #: 328312 |
| PE anti-human IL-15Rx (clone JM7A4) | BioLegend | Catalog #: 330208 |
| BV605 anti-human NKG2D (clone 1D11) | BioLegend | Catalog #: 320832 |
| BV650 anti-human IL-7Rx (clone A019D5) | BioLegend | Catalog #: 351326 |
| Pacific Blue anti-human KLRL1 (clone HP-3G10) | BioLegend | Catalog #: 339914 |
| Alexa Fluor 647 anti-human/mouse Granzyme B (clone GB11) | BioLegend | Catalog #: 515406 |
| FITC anti-human Granzyme A (clone CB9) | BioLegend | Catalog #: 507204 |
| PE anti-human Perforin (clone B-D48) | BioLegend | Catalog #: 353304 |
| InVivomAb anti-mouse IL-10R (clone 1B1.3A) | Bio X Cell | Catalog #: BE0050 RRID: AB_1107611 |
| InVivomAb anti-mouse CD103 (Clone M290) | Bio X Cell | Catalog #: BE0026 RRID: AB_1107570 |

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### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| anti-E-cadherin antibody (Clone ECCD-2) | Invitrogen | Catalog # 13-1900 |
| anti-β7 (clone FIB504) | This paper | RRID:AB_2892125 |
| anti-α4β7 (clone DATK32) | This paper | RRID: AB_2892121 |

#### Bacterial and virus strains

**Helicobacter hepaticus** Fox et al.
- ATCC
- Strain no: 51448

#### Biological samples

- **Diverticulitis or ulcerative colitis tissue samples (surgical resections)**
  - Mayo Clinic
  - Mayo Clinic Institutional Review Board (IRB 10-006628)
- **Healthy or IBD patient intestinal tissue biopsies**
  - Stanford
  - Stanford Institutional Review Board (IRB protocol 28427)
- **Healthy intestinal tissue biopsies**
  - UCSF
  - UCSF Institutional Review Board (IRB 10-00263 and 10-01218)

#### Chemicals, peptides, and recombinant proteins

| Chemicals, peptides, and recombinant proteins | Source | SKU |
|-----------------------------------------------|--------|-----|
| Ovalbumin (257-264) chicken | Sigma-Aldrich | SKU no: S7951 |
| Cholera toxin from *Vibrio cholerae* | Sigma-Aldrich | SKU no: C8052 |
| FTY720 | Sigma-Aldrich | SKU no: SML0700 |

#### Critical commercial assays

- **Foxp3 / Transcription Factor Staining Buffer Set**
  - eBioscience
  - Catalog number: 00-5523-00
- **BD Cytofix/Cytoperm Fixation/Permeabilization kit**
  - BD Bioscience
  - Catalog number: 554714
- **Cell Activation Cocktail (with Brefeldin A)**
  - BioLegend
  - Catalog number: 423304
- **CD8+ T Cell Isolation Kit, mouse**
  - Miltenyi Biotec
  - Order no: 130-104-075
- **Lamina Propria Dissociation Kit, mouse**
  - Miltenyi Biotec
  - Order no: 130-097-410
- **Zombie NIR™ Fixable Viability Kit**
  - BioLegend
  - Catalog number: 423106
- **Chromium Single Cell 3’ GEM, Library & Gel Bead Kit v3**
  - 10x Genomics
  - PN-100075
- **Chromium Single Cell B Chip Kit**
  - 10x Genomics
  - PN-100153
- **Chromium i7 Multiplex Kit**
  - 10x Genomics
  - PN-120262
- **Bioanalyzer High Sensitivity DNA kit**
  - Agilent Technologies
  - Part number: 5067-4626 & 5067-4627
- **Kapa Library Quantification Kit**
  - Roche Diagnostics
  - Catalog number: 07960409001

#### Deposited data

- **Bulk RNA-sequencing of Crohn’s disease clinical trial samples**
  - This paper
  - GEO: GSE152316
- **Bulk RNA-sequencing of sorted colonic CD103+ and CD103- T cells**
  - This paper
  - GEO: GSE152320
- **Single cell RNA-sequencing of healthy colonic immune cells**
  - This paper
  - GEO: GSE152290
- **RNA-sequencing of individual sorted colonic T cells**
  - This paper
  - GEO: GSE152306
- **RNA-sequencing of colonic biopsies from vedolizumab-treated patients**
  - Arjis et al.39
  - GEO: GSE73661
- **Human reference genome NCBI build 38, GRCh38**
  - Genome Reference Consortium
  - [https://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/](https://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/)

#### GENCODE (v27)

- **GENCODE Project**
  - [https://www.gencodegenes.org/human/release_27.html](https://www.gencodegenes.org/human/release_27.html)

#### Experimental models: Organisms/strains

- **Mouse: C57BL/6**
  - Charles River
  - Strain code: 027
- **C57BL/6NTac**
  - Taconic Biosciences
  - Model #: B6-F
- **Tg(Tg(CAG-KiKGR)33Hadj/J-KiKGR)33Hadj/J**
  - The Jackson Laboratory
  - Stock No: 013753
- **C57BL/6-Tg(TcraTcrb)1100Mjb/J**
  - The Jackson Laboratory
  - Stock No: 003831
- **B6.Cg-Gt(Rosa)26Sor(1H-CAG-tdTomato-H2B)J**
  - The Jackson Laboratory
  - Stock No: 007914
- **B6.129P2(Cg)-Cdhd1tmCly/J**
  - The Jackson Laboratory
  - Stock No: 016933

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tangsheng Yi (yi.tangsheng@gene.com).

Materials availability
All unique/stable reagents generated in this study are available from the lead contact, Tangsheng Yi, with a completed Materials Transfer Agreement.

Data and code availability
All RNA-sequencing data generated during this study are available at the Gene Expression Omnibus (GEO) under accession number GSE152321. Vedolizumab treatment data were downloaded from GEO using accession number (GEO: GSE73661).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
All of the mice used in this study were bred and maintained at Genentech under specific pathogen-free conditions, and were cared for in accordance with institutional guidelines with review and approval by the Genentech Institutional Animal Care and Use Committee. Female C57BL/6 wild-type mice were from Charles River Laboratories or Taconic Biosciences, and were used at age 6-8 weeks for studies. KikGR transgenic mice (Tg(CAG-KikGR)33Hadj/J, 013753), and E-cadherin-mCFP mice (B6.129P2(Cg)-Cdh1tm1Cle/J, 016933) were obtained from the Jackson Laboratories and bred at Genentech. OT1-TCR transgenic mice (Tg(TcraTcrb)1100Mjb/J, 003831), and LSL.tdTomato mice (B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, 007914) were obtained from the Jackson Laboratories and were used for breeding. CD4.Cre (B6.Cg-Tg(CD4-cre)1CviN9, 4196) mice were obtained from Taconic and were used for breeding. KikGR-OT-1 and TdTomato-OT-1 transgenic mice were generated at Genentech by crossing OT-1-TCR transgenic mice with KikGR transgenic mice or LSL.tdTomato mice, respectively. Female KikGR, E-cadherin-mCFP, KikGR-OT-1 and TdTomato-OT-1 transgenic mice were used at age 6-8 weeks for studies.

Patient samples
Analysis of patient samples included three distinct cohorts independent of the etrolizumab clinical study NCT02394028. Biopsies from healthy subjects undergoing routine flexible sigmoidoscopy or endoscopy as part of their clinical care at UCSF (IRB 10-00263 and 10-01218; UCSF Institutional Review Board) were processed for single cell analysis or flow cytometry as described below. IBD patients undergoing routine endoscopy as part of their clinical care at Stanford were enrolled in a prospective, observational registry study (IRB 28247; Stanford Institutional Review Board) to donate biopsies for flow cytometry. For single cell
analysis of sorted αE⁺ and αE⁻ T cells, tissue samples from patients undergoing intestinal resections as part of their clinical care at the Mayo Clinic. into an observational Review Board. All patients provided consent in accordance with Institutional Review Board or institutional as noted above. Patient characteristics are shown in Table S1.

**Etrolizumab clinical study in CD**

Moderately to severely active CD patients with a CDAI of 220-480 and a centrally read SES-CD score of ≥ 7 (≥ 4 for isolated ileitis) who had previously been treated with corticosteroids, immunosuppressants or anti-TNF were enrolled in an exploratory cohort of 350 patients within the BERGAMOT etrolizumab study (ClinicalTrials.gov: NCT02394028). Briefly, patients underwent endoscopy as part of their screening visit and baseline biopsies were taken in the ileum or the colon for analysis prior to randomization. Patients were then randomized into placebo or etrolizumab cohorts at a 1:4 ratio with subcutaneous administration at 0, 2, 4, 8 and 12 weeks. Following the induction period of 14 weeks, patients underwent a second ileocolonoscopy and follow-up biopsies were obtained from the ileum or colon and placed directly into RNAlater (QIAGEN). Biopsy samples were scored as being from active or inactive segments using the cut-off of colonic SES-CD ≥ 7 for colonic biopsies and ileal SES-CD ≥ 4 for ileal biopsies. Patient characteristics are shown in Table S2.

**METHOD DETAILS**

**Blocking antibodies for murine studies**

Anti-IFN-γ (FIB504) and anti-αEβ7 (DATK32) antibodies were ordered from ATCC and their sequences were individually cloned from hybridomas. Antigen-binding domain sequences from the parent hybridomas were fused to mouse IgG1 Fc in a pRK expression vector and transfected into HEK293 cells. Transfected supernatants were purified on a HiTrap column (GE healthcare) with Mabselect Sure resin (GE healthcare) with a phosphate-buffered saline (PBS) loading buffer. Antibodies were eluted with 0.1 M citrate (pH 3.0) and neutralized with 3 M Tris, pH 8.0, to a final pH of ~7.0 prior to dialysis against PBS, pH 7.2. Each antibody was run on a Superdex 200 10/300 GL size exclusion column (SEC) (GE Healthcare) using PBS, pH 7.2, load buffer at a flow rate of 1 mL/min (30 cm/h) to remove any aggregates. Pooled fractions were filtered using a 0.2 mm filter and the final antibody preparation was assessed by analytical SEC carried out with a TSK-GEL, Super SW3000, 4.6 mm × 30 cm, 4 mm (Tosoh Bioscience) column using a Dionex Ultimate 3000 system (Thermo Fisher Scientific) to confirm ~95% homogeneity of monomeric antibody. Anti-αE blocking antibody (clone number M290) was from BioXcell and anti-E-cadherin antibody (clone number ECD-2) was from Invitrogen.

**Flow cytometry antibodies and staining**

Cell suspension was washed twice in staining buffer (PBS with 2% of fetal bovine serum), blocked with TruStain FcX (mouse CD16/32, Biolegend) for 5mins at room temperature, and stained with indicated fluorescence conjugated antibodies below for 30mins. For intracellular staining, cells were fixed, permeamibled and stained using the Foxp3/Transcription Factor Staining Kit according to the manufacturer’s instructions (eBioscience). For intracellular cytokine staining, cells were stimulated with the Cell Activation Cocktail with Brefeldin A (BioLegend) for 5 hours, followed by fixation, permeabilization and staining using the Cytofix/ Cytoperm kit (BD Biosciences). Fluorochrome-conjugated anti-mouse antibodies used are as follows: CD45 (30-F11), TCRβ (H57-597), CD8 (53-6.7), CD4 (GK1.5), CD62L (MEL14), CD44 (1M7), MHCII (M5/114.15.2), CD11c (N418), CD11b (M1/70) IFN-γ (XM1;2). IL-17A (TC11-19H10.1), TNF-α (TN3-19.12), CD103 (M290, 2E7), Zombie NIR (live/dead, Biolegend). Fluorochrome-conjugated anti-human antibodies used are as follows: CD45 (HI30), CD8 (HIT8a), CD4 (RPA-T4), CD103 (Ber-Act8), β7 (FIB504), TCRζβ (IP26), CD25 (M-A251), Foxp3 (259D), Helios (22F6), IFN-γ (B27), IL-17A (N49-4653), TNF-α (Mab11), CD49a (TS2/7), IL-15Rα (JM7A4), NKG2D (1D11), IL-7Rα (A019D5), KLKB1 (HP-3G10), Granzyme B (GB11), Granzyme A (CB9), Perforin (B-D48).

**Murine surgical photoconversion models of T cell trafficking**

Photoconversion of mLN or specified intestinal segment was performed during survival surgeries carried out in a sterile environment under proper anesthesia. Following shaving of the surgical site, a small abdominal incision of ~1 cm allowed the cecum to be gently extruded to localize mLN, colon, and/or small intestine. A piece of sterile foil with a 5-mm hole was used to surround the tissue of interest, e.g., mLN, and limit exposure of surrounding tissue to the photoconverting light. For ileum photoconversion, regions with Peyer’s patches (PPs) was avoided. A silver LED (Prizmatix) with an aperture polymer optical fiber (1.5 mm core diameter) and a lens focusor was used as the violet light source (415 nm) to treat the target tissue as indicated. Each exposed area was treated with violet light for a total period of 5 minutes then extruded organs were returned to the abdominal cavity and the incision was sutured closed. Anti-integrin antibodies (250 µg/mouse) were administered intraperitoneally (iP) 3 hours prior to photoconversion and analysis was performed 16 hours post photoconversion.

**T cell adoptive transfer and oral antigenic challenge**

KikGR⁺ OT-1⁺ green fluorescent CD8⁺ T cells were enriched from splenocytes using the MACS CD8⁺ T cells isolation kit (Miltenyi Biotech) according to the manufacturer’s instructions. Each C57BL/6 wild-type recipient mouse received 5 × 10⁶ KikGR⁺ OT-1⁺ T cells intravenously (IV) and were then orally challenged with 150 µg Cholera toxin:OVA (Sigma) at a 1:25 molar ratio the following
day. Three days post-challenge, 250 μg of anti-integrin antibodies were administered IP, the mice were rested overnight (16 hours) then sacrificed for analysis. Alternatively, anti-integrin antibodies (250 μg/mouse) were administered IP 3 hours prior to photoconversion of small intestine and analysis was performed 16 hours post photoconversion.

Isolation and quantification of murine IEL and LP lymphocytes
Intestinal IEL and LP lymphocytes were prepared using the MACS lamina propria dissociation kit (Miltenyi Biotech). Briefly, the colon or small intestine was cut into 1-cm pieces following removal of PPs, then incubated in Hank’s Balanced Salt Solution (HBSS) (Ca²⁺ and Mg²⁺ free) containing EDTA and DTT to isolate IELs. Following removal of IELs, the remaining tissue was transferred to HBSS (with Ca²⁺ and Mg²⁺) supplemented with collagenase to release LP lymphocytes. Isolated IEL and LP lymphocytes were washed in PBS and counted prior to staining for flow cytometry. For photoconversion studies, the percentage of KikR⁺ cells within the live TCRβ⁺CD8⁺ cell gate was normalized to the percent of KikR⁺ non-migratory DCs in the mLN to account for differences in photoconversion efficiency between experiments.

Intra-vital two-photon microscopy live imaging
T cell adoptive transfer and oral antigenic challenge was performed as described above but using tdTomato⁺ OT-1⁺ CD8⁺ T cells adoptively transferred into E-cadherin-mCFP recipient mice to enable intravital imaging. Intravital microscopy was performed on anesthetized mice with the ileum exposed and opened 1 cm longitudinally along the anti-mesenteric border. The incision site was placed far away from the vasculature to ensure continuous blood supply. The mucosal surface was placed against a customized stage with a moistened PBS pad to prevent dehydration. The tissue was immobilized on a stage using a plastic ring with pinholes and a coverslip was placed on top of the mucosal surface. The luminal side of the ileum was imaged using a two-photon laser-scanning microscope (Ultima In Vivo Multiphoton Microscopy System; Bruker Technologies) using a 16X objective (Olympus) and dual Ti:sapphire lasers (MaïTai and Insight lasers, Spectra-Physics) tuned to 890 nm and 1020 nm. Analysis was performed using Imaris 9.2.1 (Bitplane; an Oxford Instruments company).

Immunofluorescence and confocal microscopy
For confocal microscopy, T cell adoptive transfer and oral antigenic challenge was performed as described above. Upon sacrifice of recipient mice, the ileal portion of the small intestine was excised and prepared as a “swiss roll.” Tissues were fixed in 4% paraformaldehyde for 6-8 hours or in Cytofix/Cytoperm Buffer (BD Bioscience) for 2-4 hours for lymphatics staining, and then dehydrated in 30% sucrose overnight prior to embedding in optimal cutting temperature (OCT) freezing media. Cryosections (8-10 μm) were stained with the following primary antibodies: Laminin (Sigma-Aldrich, L9393), E-cadherin (Thermo fisher, ECCD2), EpCAM (Abcam) and LYVE-1 (R&D systems). All images were acquired using a Leica TCS SPE upright confocal microscope. Frequency of tdTomato⁺ cells in LYVE1⁺ lymphatics was blindly quantified from 100 villi across tissue sections and a mean percentage of 100 villi was used for each individual mouse.

Helicobacter hepaticus anti-IL10R induced colitis model
Female C57BL/6 mice (Taconic) at 5-6 weeks old were treated weekly with anti-IL10R antibody (1 mg/ mouse administered IP) (BioXcell). Oral administration of ~1.2 × 10⁹ colony-forming units (CFU) Helicobacter hepaticus (ATCC 51448) was performed on two consecutive days as previously described, and mice were monitored three times per week for signs of colitis, such as watery stool. Following onset of disease symptoms, colitic mice were sacrificed and cells were isolated from the intestine as described above.

Preparation of cells from patient tissue samples for flow cytometry
Patient resection samples from the Mayo clinic and patient colonic biopsies from UCSF were shipped on ice in RPMI 1640 containing 10% FBS and antibiotics. Frozen biopsies from Stanford were thawed in a 37°C water bath immediately prior to processing. For resection tissue, the mucosa layer was separated from the serosal layer and cut into small pieces. Tissue samples were washed in HBSS (w/o Mg²⁺) and then transferred to 37°C prewarmed digestion buffer (DMEM/F12 50:50, 10% FBS, 15mM HEPES, PenStrep, 0.35mg/mL Collagenase D, 0.5mM DTT) and incubated in a 37°C water bath for 10 minutes with agitation by shaking every 5 minutes. Cells released from the tissue into the supernatant were removed and transferred to the wash buffer (DMEM/F12 50:50, 10% FBS, 15mM HEPES, PenStrep). Following three rounds of digestion, pooled cells were spun down, counted and stained as described above for flow cytometry analysis or sorting.

Preparation of tissue samples for RNA-seq
CD45⁺TCRβ⁺ TCRγ⁺αE⁺ and αE⁺ single cells were sorted directly into RLT lysis buffer (QIAGEN) with β-mercaptoethanol. RNA was isolated from sorted cells using the QIAGEN RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Tissues were digested into single cell suspension and sorted by FACS into 3 populations: CD45⁺Epcam⁺, CD45⁺Epcam⁻, and CD45⁻Epcam⁻. Sort time was capped at 2 hours. Sample processing for single-cell RNA-seq was done using Chromium Single Cell 3’ Library and Gel bead kit v3 following manufacturer’s guide (10x Genomics). The cell density and viability of single-cell suspension were determined by Vi-CELL XR cell counter (Beckman Coulter). All of the processed samples had a very high percentage of viable cells. The cell
density was used to infer the volume of single cell suspension needed in the reverse transcription (RT) master mix, aiming to achieve ~6,000 cells per sample. cDNAs and libraries were prepared following the manufacturer’s user guide (10x Genomics). Libraries were profiled by Bioanalyzer High Sensitivity DNA kit (Agilent Technologies) and quantified using Kapa Library Quantification Kit (Kapa Biosystems). Each library was sequenced in one lane of HiSeq4000 (Illumina) following the manufacturer’s sequencing specification (10x Genomics).

Biopsies from the etrolizumab clinical study were thawed, removed from RNAlater, homogenized with 0.1 mm glass beads and RNA was purified using QIAGEN All-prep 96 kits. Quantity and quality of the RNA was assessed with a NanoDrop 8000 (Thermo Scientific) and Bioanalyzer, respectively. Sequence libraries were prepared from 0.1 μg of RNA using TruSeq Stranded Total RNA Library Prep kit (Illumina) according to the manufacturer’s instructions. Size of the libraries was confirmed using 4200 TapeStation and High Sensitivity D1K screen tape (Agilent Technologies) and their concentration was determined by a qPCR-based method using a Library Quantification Kit (KAPA). Initially, 387 libraries were multiplexed and sequenced on Illumina HiSeq4000 to generate 30M single end 50 base pair reads. Samples (n = 255) with a minimum of 20 million high quality reads were determined to be acceptable for further analysis, while samples below that threshold (n = 132) underwent additional library preparation and sequencing using a NovaSeq 6000 S2 flow cell (100 cycles), with a total read length of 1x50bp. Samples with little or poor quality RNA (n = 29) were excluded from further sequencing and analysis. Analysis of the study combined results from both sequencing runs.

**Single cell RNA-seq analysis**

Single cell RNA-seq data collected using 10x Genomics were processed as previously described, with the following modifications: only exonic counts were used for determining gene expression counts, and barcodes with ≥ 2000 exonic UMIs with < 8% of UMIs coming from mitochondrial genes were considered to contain viable cells. For downstream analyses, the Seurat package (v 3.0) was used with default values to normalize transcript counts, and CCA was used to align expression data across individual donors. Graph-based clustering identified groups of cells at relatively low resolution corresponding to broad cell types in the CD45+ sorted population. We repeated the procedure within the T cell population to identify subtypes of T cells. A Wilcoxon test was used to identify gene expression markers for each cell type. If < 30 genes with ≥ 1.5-fold higher expression were found in a cluster, it was merged with the most similar cluster of cells. In this way, we defined broad subsets of T cells that were manually annotated using marker gene lists specific to each cell cluster. The most differential genes for each cluster, including genes with an FDR < 0.01, were used to define signatures of at most 50 genes for each T cell subtype. For full length RNA-seq of individual sorted T cells, wrench was used to normalize the raw counts, which were then transformed using voom and differential expression was determined using the limma package.

**Bulk RNA-seq analyses**

For bulk RNA-seq analysis, we used custom scripts written in the R programming language and packages from the Bioconductor project. Raw RNA-seq reads were processed using the HTSeqGenie R package, as previously described. Briefly, RNA-seq data was aligned to the reference human genome (GRCh38) and reads that uniquely matched to exons of gene models present in the GENCODE basic annotation set (v. 27) were counted. Protein coding genes and immune (immunoglobulin, TCR) constant regions were included in the analysis. Genes were included in the analysis if they had > 10 reads in > 31 samples. For all differential expression analyses, we again used the voom transformation method coupled with limma-based linear modeling and adjusted p values for multiple testing using the Benjamini-Hochberg procedure, unless otherwise stated. Signature scores were calculated as detailed in using the GSDecon package (https://github.com/JasonHackney/GSDecon).

Etrolizumab-specific changes were calculated as a log2-fold change in gene expression (count per million, CPM) values between week 14 and screening biopsy samples from the same bowel segment. A t test was used to determine the significance of the log2-fold between etrolizumab-treated samples and placebo-treated samples. Only genes that were significant in the transcriptome-wide analysis were considered in this analysis. The same method was used to assess changes in T cell signature scores.

Patient metadata was included as co-variates in the analysis of biopsies from patients enrolled in the clinical study of etrolizumab. To identify gene expression moderated by etrolizumab treatment, we used a linear model that included treatment arm (etrolizumab v. placebo), prior anti-TNF incomplete response, biopsy location (colon v. ileum), time of biopsy (screen v. post-induction) and the interaction effect between treatment arm and visit. Genes were considered significantly moderated by etrolizumab if they had an absolute fold change > 1.5, at an FDR of < 0.05 in the combined colonic and ileal analysis, and were nominally significant (unadjusted p value < 0.05) in both the colonic- and ileal-only analyses. Samples were included in the analysis of etrolizumab treatment effects if RNA-seq data was available for both time points and if the biopsy was taken from an endoscopically active region at the screening visit. To determine differential gene expression in active v. inactive bowel segments, we used a linear model that included covariates for prior anti-TNF incomplete response and anatomic location of the biopsy. Only screening visit biopsies were included in this analysis.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical methods and software packages used are described in detail in the Method details. Statistical analyses for both bulk and single cell RNA-sequencing were performed using R software (version 3.5.1) and R packages as described in the Methods details and legends for Figures 5 and 6. Exclusion criteria for analyses are described in the Method details.
GraphPad Prism was utilized for statistical analysis on Figures 1, 2, 3, 4, 5, and 6. Statistical details of experiments can be found in figure legends, including the statistical tests used and value and definition of n.

Differences were considered to be statistically significant when $p < 0.05$. For biological experiments, sample sizes were determined based on previous experience with similar experiments.

**ADDITIONAL RESOURCES**

Further information about sample preparation, data collection, or data processing is described in the Method details and can also be directed to the lead contact. Web resources containing the clinical trial design and enrollment criteria can be found in [http://ClinicalTrials.gov](http://ClinicalTrials.gov). Registry number for BERGAMOT study is NCT02394028.