Blockade of $\alpha E\beta 7$ integrin controls accumulation of $CD8^+$ and Th9 lymphocytes from IBD patients in the inflamed gut in vivo

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Short title: $\alpha E\beta 7$ controls $CD8^+$ and Th9 accumulation in IBD

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Abstract

Objective Therapeutically targeting lymphocyte adhesion is of increasing relevance in IBD. Yet, central aspects of the action of anti-adhesion compounds are incompletely understood. We investigated the role of αEβ7 and α4β7 integrins and their blockade by vedolizumab and etrolizumab for trafficking of IBD T lymphocytes in an in vivo model of homing to and retention in the inflamed gut.

Design We explored integrin expression in IBD patients by flow cytometry and immunohistochemistry, while regulation of integrins was studied in T cell cultures. The functional relevance of integrins was assessed by adhesion assays and a recently established humanized mouse model in DSS-treated immunodeficient mice.

Results High expression of αEβ7 was noted on $CD8^+$ and $CD4^+$ Th9 cells, while α4β7 was expressed on $CD8^+$, Th2 and Th17 cells. TCR stimulation and TGF-β were key inducers of αEβ7 on human T cells, while butyric acid suppressed αEβ7. In comparison to α4β7 blockade via vedolizumab, blockade of β7 via etrolizumab surrogate antibody superiorly reduced colonic numbers of $CD8^+$ and Th9 cells in vivo after 3 hours, while no difference was noted after 0.5 hours. AEβ7 expression was higher on $CD8^+$ T cells from IBD patients under vedolizumab therapy.

Conclusion AEβ7 is of key relevance for gut trafficking of IBD $CD8^+$ T cells and $CD4^+$ Th9 cells in vivo and mainly retention might account for this effect. These findings indicate that blockade of αEβ7 in addition to α4β7 may be particularly effective in intestinal disorders with expansion of $CD8^+$ and Th9 cells such as IBD.

Keywords

IBD; gut homing; in vivo microscopy; T cells; vedolizumab; etrolizumab

What is already known about the subject?

- The α4β7 integrin antibody vedolizumab blocks gut homing of regulatory and effector $CD4^+$ T cells and is successfully used for clinical therapy in IBD
- αEβ7 integrin is believed to mediate retention of lymphocytes in or near the epithelium via interaction with E-Cadherin
- The β7 integrin antibody etrolizumab targets α4β7 and αEβ7 integrins and is currently evaluated in phase III trials

What are the new findings?

- αEβ7 and α4β7 are differentially expressed on human T lymphocytes both in the peripheral blood and the intestine
- TCR stimulation and TGF-β treatment increase αEβ7 expression especially on $CD8^+$ lymphocytes
- Etrolizumab surrogate antibody blocks adhesion of T lymphocytes to MAdCAM-1 and E-Cadherin in vitro
- <u>In a recently established humanized mouse model, etrolizumab surrogate antibody is more potent than vedolizumab in reducing accumulation of human $CD8^+$ and $CD4^+$ Th9 cells in the inflamed gut, probably due to additional inhibition of αEβ7-mediated retention</u>

How might it impact on clinical practice in the foreseeable future?

β7 inhibition by antibodies such as etrolizumab might offer additional benefits for the treatment of IBD compared with α4β7 inhibition

Introduction

A dysguided inflammatory response within the intestinal lamina propria is believed to be a cornerstone of the pathogenesis of inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease (CD) [1–3]. In particular, $CD4^+$ and $CD8^+$ T lymphocytes are critically involved in the responsible immunologic network by secreting pro-inflammatory cytokines, orchestrating the function of other immune cells and causing direct damage to cellular or extracellular elements of the intestinal wall [4–6].

Naïve T-lymphocytes are primed to become gut homing lymphocytes in the mucosa associated lymphoid tissue (MALT). Here, dendritic cells (DCs) not only function as antigen presenting cells, but also induce the expression of α4β7 integrin via production of retinoic acid [7,8]. After reentering the circulation thus primed lymphocytes are able to access the intestinal lamina propria in a complex homing process [9] that critically involves the interaction of α4β7 integrin with its endothelial receptor mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), which is exclusively expressed on postcapillary venules in the intestine [10].

Blockade of α4β7 integrin-mediated gut homing with vedolizumab has greatly increased the options in clinical therapy of patients with IBD [11,12]. Mechanistically, we have recently shown, that vedolizumab reduces colonic homing of both effector and regulatory $CD4^+$ T lymphocytes [13]. However, the impact of vedolizumab on $CD8^+$ T cells and cytokine-producing $CD4^+$ T lymphocyte subsets has not been addressed so far. Moreover, lymphocyte homing can potentially be mediated by other homing molecules like α4β1 integrin as well [14] suggesting that vedolizumab may not suppress homing of all lymphocyte subsets. Consistent with this concept vedolizumab does not induce or maintain remission in a significant portion of patients [11,12].

Several other compounds interfering with the gut homing process are currently under development. One of them is the monoclonal humanized rat anti-β7 integrin antibody etrolizumab, which has recently successfully been tested in a phase II study [15]. As anti-β7 antibody, it not only interferes with the interaction of α4β7 with MAdCAM-1, but also inhibits binding of αEβ7 to its receptor E-Cadherin. As E-Cadherin is mainly

expressed on epithelial cells, its ligand αEβ7 is believed to mediate epithelial retention of homed gut lymphocytes [16] such as intraepithelial lymphocytes (IEL) [17]. Moreover, a decrease in the number of αE^{+} cells in the intestinal crypt epithelium was associated with a therapeutic response to etrolizumab treatment [15]. Yet, functional in vivo studies assessing the importance of αEβ7 for trafficking of lymphocytes are lacking and the abilities of α4β7 versus β7 blockade to reduce lymphocyte accumulation in IBD have not been compared to date. Here, we explored homing and retention of human IBD $CD4^{+}$ and $CD8^{+}$ T cells upon α4β7 blockade via vedolizumab and β7 blockade via etrolizumab surrogate antibody in a humanized mouse model of colitis. Our findings indicate that etrolizumab has marked additional effects on $CD8^{+}$ and Th9 trafficking compared with vedolizumab and might be a useful tool for future clinical therapy in IBD.

Methods

IBD patients

Peripheral blood and gut samples from patients with CD (n = 120) and UC (n = 74) were collected after informed written consent according to the approval of the local Ethics Committee. Blood and tissue specimens from healthy donors and tumor patients served as controls (n = 61), respectively. Gut samples of IBD patients came from areas of active disease. Blood from patients receiving vedolizumab was collected during (i.e. infusions at weeks 0, 2 and 6) or after (i.e. later infusions from week 14 on) the induction phase of vedolizumab therapy. Supplementary table 1 summarizes the patients' clinical data.

Cell isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Pancoll (Pan Biotech). $CD4^+$ or $CD8^+$ cells were isolated with immunomagnetic beads (Miltenyi Biotec). Where indicated, cells were cultured in RPMI 1640 medium (Gibco) with 10 % FCS (Pan Biotech) and 1 % penicillin/streptomycin (Biochrom) or X-Vivo medium (Lonza) with 1 % penicillin/streptomycin and stimulated with precoated anti-human CD3 (OKT3, eBioscience) and 1 µg/mL anti-human CD28 (CD28.2, BD) antibodies.

Where indicated, cells were treated with the following recombinant human cytokines for 72 hours: IL-1β, IL-2, IL-4, IL-6, IL-7 (all from Immunotools), IL-9 (Peprotech), IL-12 (all 10 ng/mL), IFN-γ (100 ng/mL, both from Immunotools) and TGF-β (20 ng/mL, R&D Systems). Moreover, cells were treated with CCL-25 (Immunotools), retinoic acid (Cayman Chemical), butyric acid (Roth), isobutyric acid (abcr), formic acid (Merck) and propionic acid (Roth).

For some CCL-25 stimulation experiments, $CD4^+CCR9^+$ and $CD8^+CCR9^+$ cells were purified by FACS (FACS Aria, BD).

Flow cytometry

For flow cytometric analyses, human cells were incubated with antibodies against CD4 (APC/Cy7,RPA-T4), CD8 (FITC, RPA-T8; PE, HIT8a; AF647, SK1), β7 integrin (PerCP/Cy5.5 and PE, FIB27), αE integrin (PE/Cy7, Ber-Act8), IFN-γ (AF700, 4S.B3), IL-4 (AF488, 8D4-8), IL-9 (AF647, MH9A4), IL-17A (BV605, BL168, all from Biolegend), α4 (VioBlue, MZ-18-24A9, Miltenyi), Foxp3 (PE, 236A/E7, eBioscience) and appropriate isotype-matched control antibodies. In some experiments, vedolizumab (Takeda) and etrolizumab surrogate antibody (FIB504, Genentech) were directly labeled with AF488 and AF647, respectively (Thermo Fisher). For intracellular staining cells were treated with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Analyses were performed on LSR Fortessa instruments (BD). For identification of T cell subsets PBMCs were restimulated with PMA and ionomycin (Sigma Aldrich) together with transport inhibition by brefeldin A (Biochemica) for four hours.

Immunohistochemistry

Cryosections were fixed with 4 % paraformaldehyde and blocked with avidin/biotin blocking reagent (Vector Laboratories) and protein-blocking reagent (Roth). The sections were incubated with antibodies against CD4 (RPA-T4), CD8 (RPA-T8, both BD; polylonal, abcam), E-Cadherin (36/E, BD), Foxp3 (236A/E7, eBioscience), αE integrin (ab129202, Abcam), CD11c (BU15, AbD Serotec), CD69 (FN50, BD), CD123 (6H6, eBioscience), CD141 (Qbend/40, AbD Serotec), vedolizumab and etrolizumab surrogate followed by fluorescent- or biotin-labeled secondary antibodies (Vectorlabs and Merck). If applicable, slides were treated with Dylight488- or Cy3-conjugated streptavidin (Biolegend). Nuclei were counterstained with Hoechst reagent (molecular probes) and samples were analyzed by fluorescence confocal microscopy (LSM SP8, Leica). Single and double positive cells in at least three high power fields were counted.

MAdCAM-1/E-Cadherin adhesion assay

Adhesion assays were performed as described previously [13,18] on epoxy-coated glass slides (Neolab). Wells were coated with Fc chimera of rhMAdCAM-1, rhE-Cadherin or rmE-Cadherin (all 5 μg/mL, all from R&D) in 150 mM NaCl with 20 mM

HEPES (AMRESCO) at 37 °C overnight followed by blocking with 5 % BSA at 37 °C for two hours. 200,000 purified $CD4^+$ or $CD8^+$ cells in adhesion buffer [18] were added for 90 minutes at 37 °C. After washing, adherent cells were counterstained with Hoechst. In blocking experiments, cells were preincubated with 5 µg/mL anti-αE integrin (Ber-Act8, Biolegend), 100 µg/mL vedolizumab or 5 µg/mL of the etrolizumab surrogate antibody rat anti-human/mouse β7 integrin for two hours. Etrolizumab is the humanized version of the latter antibody with the identical antigen recognition site, but is only available in clinical trials at the moment and could thus not be used. Mouse anti-human IgG1 and rat anti-human/mouse IgG2a (both Biolegend) were used as isotype controls for vedolizumab and etrolizumab surrogate, respectively, where indicated. Finally, slides were analyzed by fluorescence and confocal microscopy and adherent cells in at least three high power fields per condition were counted. Values are presented normalized to the respective untreated condition.

Proliferation and apoptosis assay

Human PBMCs were stained with CellTrace Violet cell proliferation kit (Life technologies) and stimulated for 72 hours. Afterwards, cells were stained with Annexin V (FITC, Biolegend), Propidiumiodide (eBioscience) and antibodies against CD4 and CD8 before flow cytometric analysis.

Humanized mouse model of in vivo homing and retention to the inflamed gut

NSG (NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ) and RAG1-deficient (B6 $RAG1^{-/-}$) mice lacking native lymphocytes were housed in individually ventilated cages and used for experiments according to the approval of the Government of Lower Franconia. Dextran sodium sulfate (DSS) colitis and adoptive transfer experiments were performed as recently described [13]. Two million cells of the indicated type were labeled with CFSE (Life technologies) and treated with 5 µg/mL anti-αE integrin, 100 µg/mL vedolizumab or 5 µg/mL FIB504 overnight, where specified. Mice were anesthetized with ketamine/xylazine by intraperitoneal injection. Subsequently, Hoechst dye was injected to the tail vein for murine cell staining. CFSE-labeled cells and Texas Red Dextran (Life technologies) for vessel staining were injected into the ileocolic artery guided by a stereomicroscope (Zeiss). For in vivo imaging, the colon

was longitudinally opened and the mucosa positioned on a glass transparent petri dish for intravital analysis with an SP8 confocal microscope (Leica).

For flow cytometric analyses, mice were sacrificed 0.5 or 3 hours after adoptive transfer and T cell enriched lamina propria mononuclear cells (LPMCs) were isolated using the lamina propria isolation kit (Miltenyi Biotec). The $CFSE^+$ fraction was quantified by flow cytometry. For further characterization of $CD4^+$ cells LPMC aliquots were stained for αEβ7 and the transcription factors T-bet (BV605, 4B10, Biolegend), ROR-γt (PE, REA278, Miltenyi), PU.1 (AF647, 7C6B05, Biolegend).

Statistics

Statistical differences were identified using ANOVA or student’s t-test where applicable in Graph Pad Prism (Graph Pad Software). Levels of significance are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Graphs display means with SEM indicated by error bars.

Results

AEβ7 and α4β7 integrins are differentially expressed on T cell subsets in the peripheral blood of IBD patients

Although it is known that αEβ7 integrin is expressed on a subset of peripheral T cells [17], no quantitative analysis of αEβ7 expression in IBD has been performed so far. Moreover, αEβ7 and α4β7 expression on cytokine-producing lymphocyte subsets is largely unknown. We therefore measured the expression of αEβ7 and α4β7 on $CD4^+$ and $CD8^+$ T cells in patients with UC, CD and control donors. We found a significantly higher expression of αEβ7 and α4β7 on $CD8^+$ than on $CD4^+$ T cells regardless of the group analyzed (Fig. 1A, Suppl. Fig. 1A+B). Furthermore, α4β7 levels on UC $CD8^+$ T cells were found to be significantly higher than on CD $CD8^+$ T cells suggesting a particular relevance of this integrin for $CD8^+$ T cell homing in the former disease. To define the potential differential target cells for vedolizumab and etrolizumab in the peripheral blood more clearly, we analyzed α4 expression on $αEβ7^+$ cells and found that many of these cells also bore α4 (Suppl. Fig. 1C), thus formally expressing both α4β7 and αEβ7, although co-expression of α4β1 and αEβ7 might also lead to this picture. Therefore, we directly labeled vedolizumab and an etrolizumab surrogate antibody with the identic antigen recognition site and used these antibodies in flow cytometry. While the majority of cells was stained by both labelled vedolizumab and etrolizumab, we were able to detect a small subset of T lymphocytes that was targeted by etrolizumab but not vedolizumab and this subset was significantly larger in $CD8^+$ than in $CD4^+$ cells (Suppl. Fig. 1D).

Next, we quantified the expression of αEβ7 on $CD4^+$ T cell subsets (Fig. 1B, Suppl. Figs 1E, F). While Treg cells and overall Teff cells had low levels of αEβ7, the expression was markedly increased on Th17 and Th9 cells with the highest levels on Th9 cells. No differences between UC, CD and controls were noted. With regard to the expression of α4β7, Th2 and Th17 cells showed particularly high expression, while levels on Treg, Th1 and Th9 cells were lower. With the exception of a higher α4β7 expression on Th9 cells from CD than controls, α4β7 levels on the different subsets in UC, CD and controls were similar (Suppl. Fig. 1G).

In CD8$^+$ T cell subsets [19], we detected no significant differences of α4β7 levels between different groups (Suppl. Figs. 1H, I). However, IL-9 secretion was correlated with a high expression of αEβ7, while Tc2 cells expressed particularly low levels of αEβ7. Additionally, we found that Tc17 cells from CD patients expressed more αEβ7 than Tc17 cells from controls and UC. Taken together, these findings supported the notion that αEβ7 and α4β7 are differentially expressed on various T cell subsets suggesting that anti-adhesive therapies may not equally block adhesion of all lymphocyte subsets in IBD.

High expression of αEβ7 on intestinal CD4$^+$ and CD8$^+$ T lymphocytes in IBD

In subsequent studies, we explored the expression of αEβ7 on intestinal lymphocytes in IBD by immunohistochemistry. We observed that both in CD and UC the percentage expression of αEβ7 on CD4$^+$ T cells is higher than in controls and exceeds 10 %, therefore being markedly higher than in the peripheral blood (Figs 2A, D, Suppl. Fig. 2A). However, the expression of αEβ7 on Foxp3$^+$ cells in the lamina propria was unchanged in samples from CD and UC compared with controls (around 5 %; Figs 2B, D, Suppl. Fig. 2B), suggesting that mucosal Treg cells might be a population with particularly low expression of αEβ7.

The αEβ7 expression on CD8$^+$ cells in the gut markedly differed between CD8$^+$ cells in the lamina propria and the epithelium (Figs 2C, D, Suppl. Fig. 2C). However, the expression did not significantly vary between controls, UC and CD. Overall, αEβ7 was expressed by around 40 % of the CD8$^+$ lymphocytes, thus on a remarkably greater fraction than in the peripheral blood. Again, the differential expression of αEβ7 on CD4$^+$ and CD8$^+$ lymphocytes highlighted the idea that therapeutically blocking this integrin might have different effects on lymphocyte subpopulations.

To further explore the nature of these CD8$^+$αEβ7$^+$ cells in the intestine, we performed additional stainings with the T resident memory (TRM) cell marker CD69 [20]. We found that around 40 % of the CD103$^+$ cells also expressed CD69, roughly equaling the frequency of CD8 expression among CD103+ cells (Suppl. Fig. 2D) and thus suggesting that the majority of CD8$^+$αEβ7$^+$ T lymphocytes are TRM cells. Moreover, we sought to exclude that dendritic cells (DCs) add to the CD8$^+$αEβ7$^+$ population as both CD8 and αEβ7 are potential markers of DC subsets [21]. As expected, we

identified αEβ7^{+} DCs using the pan-DC marker CD11c [22] (Suppl. Fig. 2E). However, almost no CD11c^{+} cell co-expressed CD8 (Suppl. Fig. 2F). To additionally cover DC subsets with lower CD11c expression, we also used CD123 and CD141 as markers for plasmocytoid DCs and type II classical DCs, respectively [22]. In these subsets, the co-expression of αEβ7^{+} was very low (Suppl. Figs. 2G, H). Thus, if at all, the contribution of CD8^{+}αEβ7^{+} DCs to the overall CD8^{+}αEβ7^{+} population seems infinitesimal and the vast majority of these cells are T lymphocytes.

The high expression of αEβ7 on CD4^{+} and CD8^{+} lymphocytes in the intestinal mucosa compared with the peripheral blood was consistent with the idea that αEβ7 might be induced on both CD4^{+} and especially CD8^{+} lymphocytes upon reaching the intestinal mucosa. However, we also considered that this finding might be due to accumulation of these cells following preferential homing. To address this, we performed another series of stainings with vedolizumab and etrolizumab surrogate antibody (Suppl. Fig. 2I). As expected, virtually all vedolizumab^{+} cells were also etrolizumab^{+} but in contrast to the peripheral blood a relevant single etrolizumab^{+} population could be identified. As this is markedly different from the pattern in the peripheral blood (Suppl. Fig. 1D), it strongly suggested that indeed regulation of integrin expression is responsible for the differential integrin levels in the peripheral blood and the intestine. Moreover, these experiments proved that targeting β7 instead of α4β7 increases the number of target cells not only in the blood but also in the gut.

TGF-beta and butyric acid are key regulators of αEβ7 expression on CD4+ and CD8+ T lymphocytes

To further elucidate the mechanisms controlling αEβ7 upregulation in the intestinal mucosa, we studied αEβ7 expression in response to various stimuli in T cell cultures. Here, we noted that TCR stimulation alone or together with anti-CD28 caused a marked upregulation of αEβ7 on CD4^{+} and CD8^{+} T cells (Fig. 3A, Suppl. Fig. 3A).

In following experiments, we assessed whether cytokines might affect αEβ7 expression. With the exception of TGF-β, however, none of the cytokines tested had any effect on αEβ7 levels. Interestingly, the potential of TGF-β to induce αEβ7 was clearly higher in CD8^{+} than in CD4^{+} T cells (Fig. 3B). When CD4^{+} lymphocyte subsets

were analyzed, we observed that TGF-β was not able to induce αEβ7 expression on Th9 cells (Suppl. Fig. 3B), the subset with the highest intrinsic αEβ7 levels. This might be explained by the role of TGF-β in Th9 differentiation [23] in the way that high levels of αEβ7 on Th9 cells are a result of prior contact with TGF-β and can therefore not be further boosted.

As earlier murine studies reported that TGF-β simultaneously downregulates α4β7 [24] and the effect of α4β7-inducing retinoic acid on αEβ7 expression has not been tested so far, we further dissected the impact of these stimuli on the expression of both markers on human T cells (Suppl. Fig. 3C-F). We could demonstrate that TGF-β indeed reduces α4β7 expression on $CD8^+$ but not $CD4^+$ cells, while retinoic acid had no significant effect on αEβ7 levels.

Furthermore, we assessed the effect of CCL-25, which has been shown to induce αEβ7 in murine $CD8^+$ lymphocytes [25], but could not observe a similar effect in neither overall $CD4^+$ and $CD8^+$ nor FACS-purified $CD4^+CCR9^+$ and $CD8^+CCR9^+$ cells (Suppl. Fig. 3G).

Finally, we incubated $CD4^+$ and $CD8^+$ T cells with short-chain fatty acids derived from microbes in the human gut [26] (Fig. 3C). While isobutyric acid and formic acid had no effects, low concentrations of propionic acid significantly increased αEβ7 expression on $CD4^+$ but not $CD8^+$ T cells. However, we detected a significant and dose-dependent down-regulation of αEβ7 expression on both $CD4^+$ and $CD8^+$ T cells upon treatment with butyric acid, a key inducer of anti-inflammatory Treg cells.
Collectively, these results showed that stimulation of T cells via the TCR, butyric acid and TGF-β are key regulators of αEβ7 expression. Moreover, TGF-β and TCR stimulation trigger a much stronger induction of αEβ7 expression on $CD8^+$ than on $CD4^+$ T lymphocytes. This is further supporting the notion that αEβ7 is especially important for the adhesion of $CD8^+$ cells.

Etrolizumab surrogate antibody blocks adhesion of IBD T cells to E-Cadherin and MAdCAM-1 more effectively than vedolizumab

To investigate the interaction of α4β7 and αEβ7 with their ligands in vitro, we performed a series of adhesion assays with plates coated with E-Cadherin and/or MAdCAM-1. First, we employed the etrolizumab surrogate antibody FIB504 (etro-s),

which blocks β7 integrin, in two concentrations corresponding to the reported effective etrolizumab levels in human serum [27]. This antibody blocked the adhesion of both $CD4^+$ and $CD8^+$ T cells from IBD patients and control donors to E-Cadherin (Fig 4A, Suppl. Fig. 4E). Moreover, we tested an anti-αE antibody and observed that this antibody was also able to block the adhesion of $CD4^+$ and $CD8^+$ cells to E-Cadherin (Suppl. Fig. 4A).

Next, we compared how vedolizumab and etrolizumab-s antibodies block the adhesion of lymphocytes to MAdCAM-1. Adhesion of $CD4^+$ as well as $CD8^+$ T cells from IBD patients and control donors was similarly blocked by both antibodies (Fig 4B; Suppl. Figs 4B, E). Additionally, more untreated $CD8^+$ T cells bound to E-Cadherin and MAdCAM-1 compared with $CD4^+$ cells matching which preferential α4β7 expression on $CD8^+$ T cells (Suppl. Figs 4A, B). Subsequently, we coated slides with E-Cadherin together with MAdCAM-1. The number of adherent $CD4^+$ and $CD8^+$ cells was significantly reduced after treatment with etrolizumab-s compared with vedolizumab (Fig 4C; Suppl. Fig. 4C). In preparation of our in vivo experiments, we then coated slides with human and murine E-Cadherin. Both $CD4^+$ and $CD8^+$ cells from UC and controls bound to murine E-Cadherin in lower numbers compared with human E-Cadherin. However, binding still occurred to a considerable extent of around 70 % (Suppl. Fig. 4D) showing a marked interaction between human receptor and murine ligand. In conclusion, our data show that etrolizumab-s blocks the adhesion of human lymphocytes to both MAdCAM-1 and E-Cadherin and suggest that cell adhesion is reduced upon etrolizumab-s treatment compared with vedolizumab when both ligands are present.

Superior reduction of colonic IBD $CD8^+$ and Th9 cell accumulation in vivo upon treatment with etrolizumab-s compared with vedolizumab by additional inhibition of retention

We next explored the functional relevance of the above mentioned interactions in vivo by using a humanized mouse model for analysis of T cell accumulation in the inflamed gut [13]. We thus induced DSS colitis in immunodeficient mice prior to adoptive transfer of human T cells to the ileocolic artery (Fig. 5A). Intravital confocal microscopy showed that CFSE-labeled $CD4^+$ and $CD8^+$ UC and control cells could be

detected in the murine lamina propria and near the epithelium (Suppl. Fig. 5A). Moreover, z-stacks showed the positioning of human T cells in close proximity to epithelial cells and confirmed extravasation (Fig. 5B, Suppl. Fig. 5B).

Further studies confirmed that E-Cadherin is present throughout the colon of DSS-treated mice in a pattern comparable to IBD patients (Supp. Fig. 5C). We continued our studies by treating $CD4^+$ T cells from UC patients with vedolizumab, anti-CD103 or both before adoptive transfer to DSS-treated mice. As determined by in vivo confocal microscopy and flow cytometry of LPMCs isolated 3h after transfer, we found that anti-CD103 treatment alone was not able to reduce homing of UC $CD4^+$ cells and the combination of anti-CD103 with vedolizumab and vedolizumab alone yielded an equal reduction of colonic UC $CD4^+$ cells (Suppl. Fig. 6A). Yet, in a similar series of experiments with $CD8^+$ cells, combined blockade of α4β7 and αEβ7 through vedolizumab and anti-CD103 was followed by a decrease of colonic UC $CD8^+$ cells compared with sole α4β7 inhibition by vedolizumab (Fig. 5D, Suppl. Fig. 6B). Consistently, anti-CD103 treatment also had significant effects. These results suggested that, while additional blockade of αEβ7 together with α4β7 might not affect the colonic localization of peripheral UC $CD4^+$ cells due to their low αEβ7 expression, higher levels of αEβ7 on UC $CD8^+$ cells might go along with a further reduction of colonic accumulation after combined blockade of both adhesion molecules.

Before using etrolizumab-s in vivo we excluded the possibility that this antibody directly impacts proliferation, apoptosis or necrosis of $CD4^+$ and $CD8^+$ T cells (Suppl. Fig. 5D). Subsequently, we found that both vedolizumab and etrolizumab-s similarly decreased the number of colonic UC $CD4^+$ T cells (Fig. 5C). $CD8^+$ T cells, however, were found in lower numbers upon etrolizumab-s treatment compared with vedolizumab treatment, supporting the notion that combined α4β7 and αEβ7 blockade might be of special relevance for $CD8^+$ T cells (Fig. 5E).

As αEβ7 expression is not uniformly distributed among different $CD4^+$ T subsets, we reasoned that etrolizumab-s might still additionally affect certain $CD4^+$ T subsets. Hence, we stained LPMCs for αEβ7 and markers of Th1, Th9 and Th17 cells, respectively (Figs. 5F, G, Suppl. Figs. 6C, D). Analysis of these markers on $CFSE^+$ cells demonstrated that $\alpha E\beta 7^+$ cells as well as Th1, Th9 and Th17 cells were enriched among untreated human cells that had entered the lamina propria compared with aliquots from the respective donor before transfer. When comparing

vedolizumab and etrolizumab-s treated cells in the lamina propria, we found that the proportion of $\alpha E\beta 7^{+}$ cells was reduced upon etrolizumab-s treatment matching with its additional blockade of αEβ7. Concerning the $CD4^{+}$ subsets, numbers of Th9 but not Th1 and Th17 cells were reduced with etrolizumab-s compared with vedolizumab. Although limited by the low number of cells analyzed, these findings prompted the conclusion that while the overall $CD4^{+}$ T cell infiltrate was not reduced after etrolizumab-s compared with vedolizumab treatment, etrolizumab-s reduced the accumulation of Th9 cells.

To analyze the effect that is accountable for these finding in more detail, we compared colonic accumulation of UC $CD8^{+}$ cells 0.5 and 3 hours after adoptive transfer (Fig. 6). Interestingly, the effect of vedolizumab and etrolizumab-s was similar after 0.5h. After 3h, however, we observed similar differences as above. This is in line with the view that both compounds similarly inhibit α4β7-mediated homing, while – subsequently – etrolizumab possesses an additional and longer-lasting mechanism, which is easily reconcilable with the concept of inhibition of retention.

Taken together, these results suggested that combined α4β7 and αEβ7 blockade in UC T cells through etrolizumab therapy results in a superior reduction of distinct lymphocyte subsets in the colon compared with sole α4β7 blockade through vedolizumab.

To get an estimate of the potential clinical relevance of this finding, we studied the expression of αEβ7 on peripheral blood lymphocytes from IBD patients treated with vedolizumab (Figs. 7A-C). Interestingly, we observed that no changes in the expression of αEβ7 on $CD4^{+}$ Teff and Treg cells occurred when comparing samples obtained during the induction phase with samples obtained during later stages of treatment. However, we noticed a significant increase in the expression of αEβ7 but not α4β7 (Fig. 7D) on $CD8^{+}$ T cells in the maintenance compared with the induction phase suggesting that these T cells might possibly develop αEβ7-dependent compensatory strategies to ensure their colonic positioning despite suppression of gut homing via vedolizumab-induced blockade of α4β7.

Discussion

Although clinical treatment with the monoclonal anti-α4β7 antibody vedolizumab is available for therapy of IBD patients for some years now, the knowledge about the factors that control the colonic homing and retention of lymphocytes is still fragmentary [28]. Particularly, this is the case for $CD8^+$ T cells, a lymphocyte subset that has been largely overlooked in the past due to the prominent role of $CD4^+$ T cells [4,29]. However, $CD8^+$ T cells are thought to be not only important effector cells but also crucial mediators of pathologic immune responses in a number of immunologically mediated diseases [19,30]. Moreover, there is evidence that $CD8^+$ T cells are also implicated in the events leading to human and murine colitis [5,6]. Using an in vivo model for IBD T cell trafficking in humanized mice, we provide evidence here that blockade of β7 is more effective in suppressing colonic accumulation of $CD8^+$ T cells than α4β7 blockade suggesting a crucial regulatory role of αEβ7. Similar findings were obtained for Th9 cells, a recently identified $CD4^+$ T cell subset with important pathogenic function in UC [31], but not for total $CD4^+$ T cells. These results provide new insights into the molecular factors that control $CD4^+$ and $CD8^+$ T cell homing and retention in IBD patients and suggest new avenues for therapy.

Interestingly, little attention has been paid to αEβ7 in the framework of IBD since its discovery [16] and functional in vivo data for human cells have not been reported so far. We uncovered that both αEβ7 and α4β7 are differentially expressed on blood and intestinal $CD4^+$ and $CD8^+$ T cells in IBD patients with higher expression on $CD8^+$ T cells. The potential of TGF-β and TCR stimulation to induce αEβ7 expression was much higher in $CD8^+$ than in $CD4^+$ T cells, possibly leading to this finding. This observation led to the hypothesis that differential expression of adhesion markers might also concern $CD4^+$ T lymphocyte subsets in IBD. Indeed, according studies revealed that Th9 cells bear particularly high levels of αEβ7, while Th2 and Th17 cells had the highest expression of α4β7. Similarly, Tc9 cells expressed the highest levels of αEβ7 among $CD8^+$ T cells. Collectively, these results were consistent the idea that clinical therapy with α4β7 or β7 blockers differentially affects distinct T lymphocyte subsets and might therefore have specific strengths and weaknesses in targeting the adhesion of these populations. This could be of potential therapeutic

relevance as there is fundamental evidence that different T cell subsets and their signature cytokines are of varying importance in individual patients [4,6,28–31]. Compatibly, it is postulated that therapy will prospectively be assigned on the basis of biomarkers reflecting such immunologic profiles [35].

Differential expression of αEβ7 on $CD4^+$ and $CD8^+$ cells was demonstrated in the peripheral blood and the intestine of controls and IBD patients. Moreover, we show that both in the blood and the gut more cells can be targeted with anti-β7 than with anti-α4β7 antibodies. Matching with earlier reports [36] the expression of αEβ7 was found to be higher in the intestine than in the peripheral blood and we provide further evidence [37] that this is majorly due to regulation of expression and not frequency shifts following homing. Stimulation experiments demonstrated that mainly TGF-β and TCR stimulation may be responsible for this increase of αEβ7 on intestinal lymphocytes, while TGF-β simultaneously downregulates α4β7 on $CD8^+$ cells. In conclusion, these stimuli might induce an integrin profile that allows targeting of these cells with etrolizumab but not vedolizumab. Moreover, butyric acid, a key inducer of anti-inflammatory Treg cells in the colon [26], markedly suppressed αEβ7 expression on both $CD4^+$ and $CD8^+$ T cells. Thus, it seems possible that commensal bacteria in close proximity to intestinal epithelial cells might affect αEβ7 levels via butyric acid production.

Further stainings indicated that while DCs do not appreciably contribute to the population of $CD8^+αEβ7^+$ cells, the majority of these cells belongs to the compartment of $CD69^+$ TRM cells. As several authors have highlighted [20,38–40], a subset of mainly $CD8^+$ lymphocytes develops into resident cells that may immediately react to translocated antigens with cytokine secretion and thus coordinate prompt defense against infectious agents but might also contribute to the dysregulated immune response in IBD [41]. In conclusion, it is likely that TRM cells are a main target of anti-β7 antibodies in the intestine. To investigate the functional relevance of αEβ7 and α4β7 for colonic positioning in vivo, we used a recently described humanized mouse model [13] where DSS colitis is induced in immunodeficient mice prior to adoptive transfer of labeled human lymphocytes to the ileocolic artery. A prerequisite for assessing αEβ7 and α4β7 on human lymphocytes in this model is the interaction of these integrins with their respective murine ligands E-Cadherin and MAdCAM-1. Similarly to the described binding of human α4β7 to murine MAdCAM-1

[13], we found that human αEβ7 mediates adhesion to murine E-Cadherin in spite of reduced adhesion compared with human E-Cadherin. Moreover, confocal in vivo imaging after adoptive transfer of human cells demonstrated that UC T cells can be found in close proximity of the murine epithelium, thus allowing an interaction of human αEβ7 with murine E-Cadherin in vivo.

In humanized mice, we showed that the number of UC $CD4^+$ $\alpha E\beta 7^+$ and Th9 cells is reduced upon β7 blockade with the etrolizumab surrogate antibody FIB504, which shares the identical antigen recognition site with etrolizumab, in comparison with the α4β7 blocker vedolizumab. Thus, β7 blockade via the former antibody targets $CD4^+$ subsets with high αEβ7 expression in UC, although the overall $CD4^+$ T cell population was not affected. This is particularly interesting as we found that the expression of α4β7 on Th9 cells is relatively low suggesting that a considerable portion of these cells might be able to access the intestine despite α4β7 blockade with vedolizumab. Additionally targeting the epithelial retention of Th9 cells by etrolizumab might therefore narrow this "gap" and may be clinically relevant, as an expansion of these cells has been demonstrated in IBD patients and as IL-9 blockade was effective in a murine model of colitis induced by the hapten reagent oxazolone [31].

Studies using cell transfer of human T cells to DSS-treated mice showed that α4β7 blockade via vedolizumab not only reduces the colonic homing of UC $CD4^+$ but also $CD8^+$ T cells. In addition, β7 blockade with etrolizumab-s or treatment with vedolizumab together with an anti-CD103 antibody further reduced the number of $CD8^+$ T cells but not $CD4^+$ T cells. Given our data on the expression and regulation of αEβ7 on peripheral $CD4^+$ and $CD8^+$ cells this may reflect the higher expression of αEβ7 on $CD8^+$ T cells. Consistently, anti-CD103 antibody treatment had significant effects on T cell homing and retention in UC $CD8^+$ T cells rather than $CD4^+$ cells highlighting the functional relevance of αEβ7 for the former cells.

This is in line with data from two murine studies: While Annacker et al. reported that αE plays no role for the pathogenic and beneficial potential of effector and regulatory $CD4^+$ lymphocytes, respectively, but rather has an important regulatory role in DCs in the T cell transfer model of colitis [42], Lúdvíksson et al. have shown that pan-lymphocyte inhibition of αE significantly ameliorated disease in a T cell-dependent colitis model [43].

Different suggestions have been made regarding the concrete function of αEβ7 for cell trafficking. While it is known that αEβ7 interacts with the epithelial ligand E-Cadherin and many authors have thus proposed that αEβ7 is responsible for retention of homed lymphocytes within or near the epithelium [16,44], αEβ7 has also been shown to bind to intestinal endothelial cell lines independently of E-Cadherin [45]. Moreover, the number of lamina propria lymphocytes is reduced in αE-deficient mice [46], leading to the possibility of additional direct functions for αEβ7 in gut homing through an alternative, yet unknown, ligand [47]. However, our reported findings do not provide evidence for an alternative homing via αEβ7. Importantly, although limited by the restricted observation period achievable in our model, our data provide for the first time functional in vivo evidence that αEβ7 is indeed implicated in the retention of human UC T cells.

Interestingly, clinical data from UC patients treated with vedolizumab showed that more αEβ7, but not α4β7 is found on blood $CD8^+$ T cells after the induction phase of vedolizumab therapy. This suggests that lymphocytes might use alternative molecules such as αEβ7 to ensure their localization within the intestine despite α4β7 inhibition and internalization [48] by vedolizumab further supporting the concept that blocking αEβ7 together with α4β7 may increase the effects of anti-adhesion therapy.

Taken together, our data suggest a key regulatory role of αEβ7 for $CD8^+$ rather than global $CD4^+$ T cell trafficking. Nevertheless, αEβ7 seems to be important in specified $CD4^+$ subsets, namely Th9 cells (Figure 7D). Additional therapeutic targeting of αEβ7 on $CD8^+$ and Th9 cells with antibodies such as etrolizumab might therefore open new avenues for clinical treatment of IBD by increasing and extending the impact of sole α4β7 inhibition.

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Disclosure:

The etrolizumab surrogate antibody FIB504 was provided by Genentech, San Francisco, CA. The company was neither involved in the conception and conduction of the study nor in the analysis and interpretation of the results. M.F.N. has served as an advisor for Abbvie, MSD, Boehringer, Takeda, Pentax and Giuliani.

Abbreviations:

CCL-25 – C-C motif ligand 25

CFSE – Carboxyfluorescein succinimidyl ester

DSS – dextran sodium sulfate

IFN-γ – interferon γ

IL - interleukin

MAdCAM-1 – mucosal vascular addressin cell adhesion molecule 1

MALT – mucosa associated lymphoid tissue

LPMCs – lamina propria mononuclear cells

PBMCs – peripheral blood mononuclear cells

TCR – T cell receptor

TGF-β – transforming growth factor β

Author Contributions:

SZ, DS and AF performed experiments; SZ, RA, RLP, AW, CN, IA and MFN provided clinical samples, protocols, reagents or designed experiments; SZ, CN, IA and MFN analyzed and interpreted the data; SZ and MFN drafted the manuscript; all authors critically revised the manuscript for important intellectual content.

Figure legends

Figure 1: Differential expression of αEβ7 and α4β7 on T cell subsets in the peripheral blood

(A) Left panels: Representative flow cytometric analyses of αEβ7 (upper panels) and α4β7 expression (middle panels) on $CD4^+$ and $CD8^+$ blood lymphocytes from patients with UC, CD and control donors along with isotype control stainings. Numbers indicate the percentage of $αEβ7^+$ and $α4β7^+$ cells. Right panels: Pooled statistics of αEβ7 expression (upper panels) and α4β7 expression (lower panels) on peripheral blood $CD4^+$ and $CD8^+$ T cells from patients with UC (n = 13), CD (n = 24) and control donors (n = 13).

(B) Flow cytometry of αEβ7 and α4β7 expression on $CD4^+Foxp3^+$ (Treg), $CD4^+Foxp3^-$ (Teff), $CD4^+IFN\text{-}γ^+$ (Th1), $CD4^+IL\text{-}4^+$ (Th2), $CD4^+IL\text{-}9^+$ (Th9) and $CD4^+IL\text{-}17A^+$ (Th17) cells from the peripheral blood of patients with UC (n = 6-15), CD (n = 30-44) and control donors (n = 11-21).

Figure 2: Differential expression of αEβ7 and α4β7 on IBD T cell subsets in the lamina propria

(A) - (C) Representative immunofluorescent stainings of cryosections from patients with UC, CD and control donors (n = 15-21) for CD4 *(A)*, Foxp3 *(B),* CD8 *(C)* and CD103 (αE integrin, *A*-*C*) along with control stainings *(A)*. Scale bars: 25µm (upper rows), 10µm (lower rows).

(*D*) Quantitative analysis of the frequency of αEβ7 expression on intestinal $CD4^+$, $Foxp3^+$ and total, lamina propria and intraepithelial $CD8^+$ T cells as indicated.

Figure 3: Expression of αEβ7 is differentially regulated in T cell subsets

(A) Left panels: Representative flow cytometric analyses of αEβ7 expression on $CD4^+$ and $CD8^+$ T cells from the peripheral blood upon stimulation with anti-CD3 antibodies. Right panels: Flow-cytometric quantification of αEβ7 expression on $CD4^+$

and $CD8^+$ cells upon stimulation with anti-CD3 antibodies. Values were normalized to the respective value before stimulation.

(B) Flow cytometry of αEβ7 expression on $CD4^+$ and $CD8^+$ T cells upon stimulation with the indicated cytokines for 72 hours together with anti-CD3/anti-CD28 antibodies (n = 5-15).

(C) Flow cytometry of αEβ7 expression on $CD4^+$ and $CD8^+$ T cells upon treatment with short chain fatty acids for 72 hours together with anti-CD3/anti-CD28 antibodies (n = 8-37).

Figure 4: Adhesion of $CD4^+$ and $CD8^+$ IBD T cells to MAdCAM-1 and E-Cadherin is blocked by etrolizumab surrogate antibody

(A) Upper panels: Representative adhesion assays showing the adhesion of $CD4^+$ and $CD8^+$ T cells from patients with UC and control donors to slides coated with the indicated ligands and control conditions without ligand. Scale bars: 25µm and 10µm (inserts). Lower panels: Quantitative analysis of the adhesion of $CD4^+$ and $CD8^+$ T cells from patients with UC and control donors to E-Cadherin upon treatment with different concentrations of etrolizumab-s.

(B+C) Quantitative analysis of the adhesion of $CD4^+$ and $CD8^+$ T cells from patients with UC and control donors to MAdCAM-1 (B) and E-Cadherin + MAdCAM-1 (C) upon treatment with vedolizumab or etrolizumab-s.

Figure 5: Etrolizumab surrogate antibody is more potent than vedolizumab in blocking the in vivo trafficking of UC $CD8^+$ and Th9 cells

(A) Illustration of adoptive transfer. After anesthesia, laparotomy and exposure of the ileocecal region, the ileocolic artery was punctured (left image) and CFSE-labelled human T cells were injected. Here, ink is injected to demonstrate successful puncture (right image).

(B) 3D-reconstruction from a representative z-stack obtained during intravital confocal microscopy highlighting an extravasated human cell (green in white circle). Blue: murine cells. Red: murine vessels.

(C+E) Upper panels: Representative confocal in vivo microscopy images of murine colon after adoptive transfer of UC $CD4^+$ *(C)* and $CD8^+$ T cells *(E)* to the ileocolic artery. Treatment with blocking antibodies is indicated. Scale bars: 25µm (upper row) and 10µm (lower row). Left lower panels: Representative flow cytometry of murine LPMCs 3h after adoptive transfer of UC $CD4^+$ *(C)* and $CD8^+$ T cells *(E)*. Numbers denote the $CFSE^+$ cell fraction. Right lower panels: Flow-cytometric quantification of $CFSE^+$ cells in the murine lamina propria 3h after adoptive transfer of UC $CD4^+$ *(C)* or CD8+ T cells *(E)* in the presence or absence of integrin blockers, as indicated. Data were pooled from 5 independent experiments.

(D) Left panel: Flow-cytometric quantification of CFSE+ cells in the murine lamina propria 3h after adoptive transfer of UC CD8+ cells upon treatment with vedolizumab and anti-CD103 as indicated (n = 7). Right panel: Flow-cytometric comparison of the colonic accumulation of untreated UC CD4+ and CD8+ T cells.

(F) Flow-cytometric comparison of the relative portion of Th1 and Th9 cells among $CFSE^+$ purified $CD4^+$ T cells before and after transfer (n = 5-6). BT – before transfer. AT – after transfer.

(G) Flow-cytometric comparison of the relative portion of Th1 and Th9 cells among $CFSE^+$ purified $CD4^+$ T cells in the murine lamina propria upon vedolizumab or etrolizumab-s treatment (n = 4-5).

Figure 6: Time course of vedolizumab and etrolizumab-s effects – evidence for in vivo blockade of retention

Upper panels: Representative intravital confocal images of murine colon at the indicated timepoints after adoptive transfer of UC CD8+ T cells treated with vedolizumab and etrolizumab-s as indicated. Scale bars: 50µm. Lower panels: Flow-cytometric quantification of $CFSE^+$ cells in the murine lamina propria 0.5h (left) and 3h (right) after adoptive transfer of UC CD8+ T cells in the presence or absence of integrin blockers, as indicated (n = 3).

Figure 7: Vedolizumab treatment leads to higher $\alpha E\beta 7$ expression on blood IBD $CD8^+$ cells

(A-C) Representative flow cytometric data and quantitative analyses of αEβ7 expression on peripheral $CD4^{+}Foxp3^{-}$ *(A)*, $CD4^{+}Foxp3^{+}$ *(B)* and $CD8^{+}$ *(C)* T cells during or after the induction phase of vedolizumab therapy as indicated.

(*D*) Representative flow cytometric data and quantitative analyses of α4β7 on peripheral $CD8^{+}$ T cells during or after the induction phase of vedolizumab therapy.

Figure 8: Model for the control of colonic localization of IBD T lymphocytes by α4β7 and αEβ7

Cells may extravasate to the lamina propria via interaction of α4β7 with MAdCAM-1. Possibly, an alternative αEβ7-dependent pathway via an unknown ligand might exist. After homing to the intestine, interaction of αEβ7 with epithelial E-Cadherin allows retention of T lymphocytes in or near the epithelium. Vedolizumab blocks the interaction of α4β7 with MAdCAM-1, which particularly affects $CD8^{+}$, Th2 and Th17 cells, while inhibition of αEβ7 by etrolizumab is of special relevance for $CD8^{+}$ and Th9 cells.

Supplementary Figure 1

(A) Exemplary gating strategy for flow cytometric assessment of α4β7 and αEβ7. Upper row (from left to right): After excluding doublets, lymphocytes were selected in the FSC-A/SSC-A plot and subsequently CD4$^+$ and CD8$^+$ populations were identified. On these populations, α4β7 and αEβ7 expression was determined (lower row).

(*B*) Flow cytometry of αEβ7 on peripheral blood lymphocytes was repeated with the fluorochromes PE for β7 and PE/Cy7 for αE with excitement at 561nm to obtain a particularly bright signal. Left panels: Representative dot plots. Right panel: Pooled statistics of samples from control donors (n = 12), CD (n = 19) and UC (n = 9) patients.

(*C*) Flow cytometric analysis of α4 expression on CD4$^+$αEβ7$^+$ and CD8$^+$αEβ7$^+$ cells. Left panels: Representative density plots. Right panel: Pooled statistics (n = 13-24).

(*D*) Flow cytometry of peripheral blood lymphocytes with directly labeled vedolizumab and etrolizumab-s. Upper panels: Representative stainings from control (n = 10), UC (n = 10) and CD (n = 27) patients along with isotype control stainings. Lower panels: Pooled statistics of the frequency of vedolizumab$^+$etrolizumab-s$^+$ double positive cells and vedolizumab$^-$etrolizumab-s$^+$ single positive cells among CD4$^+$ and CD8$^+$ cells as wells percentage of vedolizumab-etrolizumb-s$^+$ single positive cells among total etrolizumab-s$^+$ cells.

(E) Left panels: Representative flow cytometric cytokine stainings for IFN-γ, IL-4, IL-9 and IL-17A in CD4$^+$ cells from the peripheral blood from patients with CD, UC and control donors along with negative control stainings. To induce cytokine production, cells were stimulated with anti-CD3/anti-CD28 overnight before restimulation with PMA/ionomycin and transport inhibition by brefeldin A for four hours. Right panels: Quantification of cytokine-positive CD4$^+$ cells in the peripheral blood from patients with CD, UC and control donors.

(*F*) Representative dot plots showing the expression of αEβ7 (upper panels) and α4β7 (lower panels) on the gated CD4$^+$ subsets of Treg (Foxp3$^+$), Teff (Foxp3$^-$), Th1 (IFN-γ$^+$), Th2 (IL-4$^+$), Th9 (IL-9$^+$) and Th17 (IL-17A$^+$) cells.

(*G*) Flow cytometry of αEβ7 and α4β7 expression on CD4$^+$Foxp3$^+$ (Treg), CD4$^+$Foxp3$^-$ (Teff), CD4$^+$IFN-γ$^+$ (Th1), CD4$^+$IL-4$^+$ (Th2), CD4$^+$IL-9$^+$ (Th9) and CD4$^+$IL-

$17A^+$ (Th17) cells from the peripheral blood of patients with UC (n = 6-15), CD (n = 30-44) and control donors (n = 11-21).

(H+ I) Flow cytometry of αEβ7 and α4β7 expression on $CD8^+IFN\text{-}\gamma^+$ (Tc1), $CD8^+IL\text{-}4^+$ (Tc2), $CD8^+IL\text{-}9^+$ (Tc9) and $CD8^+IL\text{-}17A^+$ (Tc17) cells from the peripheral blood of patients with UC (n = 10), CD (n = 19) and control donors (n = 13).

Supplementary Figure 2

(A) Absolute numbers of $CD4^+$ and/or $CD103^+$ single- or double-positive cells in the immunohistochemistry stainings shown in Fig. 2A+D.

(B) Absolute numbers of $Foxp3^+$ and/or $CD103^+$ single- or double-positive cells in the immunohistochemistry stainings shown in Fig. 2B+D.

(C) Absolute numbers of $CD8^+$ and/or $CD103^+$ single- or double-positive cells in the immunohistochemistry stainings shown in Fig. 2C+D.

(D) Left panels: Representative immunohistochemistry stainings of frozen cryosections from control, UC and CD patients (n = 12) for CD69 and CD103. Right upper panel: Frequency of $CD103^+$ cells among $CD69^+$ cells. Right lower panels: Frequency of $CD8^+$ (left) and $CD69^+$ (right) cells among $CD103^+$ cells.

(E) Left panels: Representative immunohistochemistry stainings of frozen cryosections from control, UC and CD patients (n = 12) for CD11c and CD103. Right panel: Frequency of $CD103^+$ cells among $CD11c^+$ cells.

(F) Left panels: Representative immunohistochemistry stainings of frozen cryosections from control, UC and CD patients (n = 12) for CD8 and CD11c. Right panel: Frequency of $CD8^+$ cells among $CD11c^+$ cells.

(G) Left panels: Representative immunohistochemistry stainings of frozen cryosections from control, UC and CD patients (n = 15) for CD123 and CD103. Right panel: Frequency of $CD103^+$ cells among $CD123^+$ cells.

(H) Left panels: Representative immunohistochemistry stainings of frozen cryosections from control, UC and CD patients (n = 15) for CD141 and CD103. Right panel: Frequency of $CD103^+$ cells among $CD141^+$ cells.

(I) Left panels: Representative immunohistochemistry stainings of frozen cryosections from control, UC and CD patients (n = 12) with vedolizumab and etrolizumab-s. Examples of etrolizumab single-positive and vedolizumab/etrolizumab-s double-positive cells are marked with white and orange arrows, respectively. Right panels: Absolute number of vedo$^+$etro-s$^+$ double positive cells and vedo$^-$etro-s$^+$ single positive cells and frequency of etro-s single positive cells among total etro-s$^+$ cells.

Supplementary Figure 3

(*A*) Quantitative flow cytometry of αEβ7 expression on CD4$^+$ and CD8$^+$ cells upon stimulation with anti-CD3 and/or anti-CD28 antibodies (n = 4-8). Values were normalized to the respective value before stimulation.

(*B*) Flow cytometry of αEβ7 expression on Treg (CD4$^+$Foxp3$^+$), Teff (CD4$^+$Foxp3$^-$), Th1 (CD4$^+$IFN-γ$^+$), Th2 (CD4$^+$IL-4$^+$), Th9 (CD4$^+$IL-9$^+$) and Th17 (CD4$^+$IL-17A$^+$) cells upon stimulation with the indicated cytokines for 72 hours together with anti-CD3/anti-CD28 antibodies (n = 5-15).

(*C*) Left panels: Representative flow cytometric analyses of integrin α4, β7 and αE positivity of CD4$^+$ and CD8$^+$ cells following incubation with (red lines) or without (blue lines) TGF-β together with anti-CD3/CD28 antibodies for 72 hours. Right panels: Mean fluorescence intensity of α4, β7 and αE on CD4$^+$ and CD8$^+$ cells following incubation with or without TGF-β together with anti-CD3/CD28 antibodies for 72 hours (n = 8).

(*D*) Representative dot plots and flow cytometric quantification of α4β7 (left panels) and αEβ7 expression (right panels) on CD4$^+$ and CD8$^+$ cells following incubation with or without TGF-β together with anti-CD3/CD28 antibodies for 72 hours (n = 8).

(*E*) Left panels: Representative flow cytometric analyses of integrin α4, β7 and αE positivity of CD4$^+$ and CD8$^+$ cells following incubation with (red lines) or without (blue lines) retinoic acid (RA) together with anti-CD3/CD28 antibodies for 72 hours. Right panels: Mean fluorescence intensity of α4, β7 and αE on CD4$^+$ and CD8$^+$ cells following incubation with or without RA together with anti-CD3/CD28 antibodies for 72 hours (n = 8).

(*F*) Representative dot plots and flow cytometric quantification of α4β7 (left panels) and αEβ7 expression (right panels) on $CD4^+$ and $CD8^+$ cells following incubation with or without RA together with anti-CD3/CD28 antibodies for 72 hours (n = 8).

(G) Upper panels: Flow cytometry of αEβ7 expression on $CD4^+$ and $CD8^+$ T cells upon treatment with different concentrations of CCL-25 together with anti-CD3/anti-CD28 antibodies for 72 hours (n = 11). Lower panels: Flow cytometry of αEβ7 expression on FACS-purified $CD4^+CCR9^+$ and $CD8^+CCR9^+$ T cells upon treatment with different concentrations of CCL-25 together with anti-CD3/anti-CD28 antibodies for 72 hours (n = 4)

Supplementary Figure 4:

(A) Left and middle panel: Quantitative analysis of the adhesion of $CD4^+$ and $CD8^+$ cells from patients with UC and control donors to E-Cadherin upon treatment with the anti-CD103 antibody used for the experiments shown in Fig. 5D and Suppl. Fig. 6A+B (n = 5). Right panel: Comparison of the adhesion of untreated $CD4^+$ and $CD8^+$ cells to E-Cadherin (n = 7).

(*B*+*C*) Upper panels: Representative adhesion assays showing the adhesion of $CD4^+$ and $CD8^+$ T cells from patients with UC and control donors to slides coated with the indicated ligands and control conditions without ligand. Scale bars: 25µm and 10µm (inserts). Lower panels: Comparison of the adhesion of untreated $CD4^+$ and $CD8^+$ cells to the indicated ligands (n = 7-10).

(*D*) Left panels: Representative adhesion assays showing the adhesion of $CD4^+$ and $CD8^+$ T cells from patients with UC and control donors to slides coated with human and murine E-Cadherin and control conditions without ligand. Scale bars: 25µm and 10µm (inserts). Right panels: Quantitative analysis of the adhesion of $CD4^+$ and $CD8^+$ cells to human and murine E-Cadherin (n = 5).

(*E*) Quantitative analysis of the adhesion of CD4+ and CD8+ cells from patients with CD to E-Cadherin (upper panels), MAdCAM-1 (middle panels) and E-Cadherin + MAdCAM-1 (lower panels) upon treatment with vedolizumab and etrolizumab-s as indicated (n = 3-4).

Supplementary Figure 5

(A) Intravital confocal microscopy of murine colon after adoptive transfer of CFSE-labeled $CD4^+$ and $CD8^+$ T cells to the ileocolic artery. Additionally, Hoechst dye and Texas Red Dextran were injected to stain murine cells (blue) and vessels (red), respectively. Human cells (green) can be observed within the murine lamina propria and near the epithelium. Scale bars: 50µm and 10µm (inserts).

(B) Upper row: Representative confocal in vivo images from a z-stack at the indicated relative positions highlighting an area containing an extravasated cell (white circle). Scale bars: 50µm. Lower row: 3D reconstruction of the z-stack showing single channels and the merged image.

(C) Representative immunofluorescent stainings of murine (left panels) and human (right panels) cryosections for E-Cadherin along with a control staining of murine colon. Scale bars: 25µm and 10µm (magnifications). Murine samples come from DSS-treated NSG mice, human samples from UC and CD patients as indicated.

(D) Proliferation, apoptosis and necrosis of $CD4^+$ (upper panels) and $CD8^+$ cells (lower panels) upon treatment with the indicated concentrations of etrolizumab surrogate antibody for 72 hours together with anti-CD3, anti-CD28 and IL-2 (n = 8).

Supplementary Figure 6

(*A*) Upper panels: Representative confocal in vivo microscopy images of murine colon after adoptive transfer of UC $CD4^+$ T cells to the ileocolic artery. Treatment with blocking antibodies is indicated. Scale bars: 25µm (upper row) and 10µm (lower row). Middle panels: Representative flow cytometry of murine LPMCs 3h after adoptive transfer of UC $CD4^+$ and $CD8^+$ T cells. Numbers denote the $CFSE^+$ cell fraction. Lower panels: Flow-cytometric quantification of $CFSE^+$ cells in the murine lamina propria 3h after adoptive transfer of UC $CD4^+$ T cells in the presence or absence of integrin blockers, as indicated. Data were pooled from 7 independent experiments.

(*B*) Upper panels: Representative confocal in vivo microscopy images of murine colon after adoptive transfer of UC $CD8^+$ T cells to the ileocolic artery. Treatment with blocking antibodies is indicated. Scale bars: 25µm (upper row) and 10µm (lower row). Middle panels: Representative flow cytometry of murine LPMCs 3h after adoptive

transfer of UC $CD4^+$ and $CD8^+$ T cells. Numbers denote the $CFSE^+$ cell fraction. Pooled statistics are shown in the left panel of Fig. 6D.

(*C*) Comparison of the relative portion of αEβ7 and Th17 cells among $CFSE^+$ purified $CD4^+$ T cells before and after transfer (n = 5).

(*D*) Comparison of the relative portion of αEβ7 and Th17 cells among $CFSE^+$ purified $CD4^+$ T cells in the murine lamina propria upon vedolizumab or etrolizumab-s treatment (n = 3-4).

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