

Title Page**The $\alpha 4\beta 1$ homing pathway is essential for ileal homing of Crohn's disease
effector T cells in vivo**

Dr Sebastian Zundler^{1*}, Anika Fischer MSc^{1*}, Daniela Schillinger MSc¹, Marie-Theres
Binder¹, Prof Raja Atreya¹, Dr Timo Rath¹, Dr Rocío Lopez-Pósadas¹, Dr Caroline J.
Voskens², Prof Alastair Watson³, Dr Imke Atreya^{1#}, Dr Clemens Neufert^{1#}, Prof
Markus F. Neurath^{1#}

¹ Department of Medicine 1, University of Erlangen-Nuremberg, Kussmaul Campus for
Medical Research & Translational Research Center, Erlangen, Germany

² Department of Dermatology, University of Erlangen-Nuremberg, Erlangen, Germany

³ Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, UK

* # Equal contribution

Correspondence to:

Markus F. Neurath, MD

Professor of Medicine

Medical Clinic 1, Friedrich-Alexander Universität Erlangen-Nürnberg

Ulmenweg 18, D-91054 Erlangen, Germany

phone: +4991318535000; fax: +4991318535209

Email: markus.neurath@uk-erlangen.de

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Abbreviations:

CCR2 – C-C chemokine receptor type 2

CFSE – carboxyfluorescein succinimidyl ester

DSS – dextran sodium sulphate

MAdCAM-1 – mucosal vascular addressin cell adhesion molecule 1

NTZ – natalizumab

Teff – effector T cell

Treg – regulatory T cell

VCAM-1 – vascular cell adhesion molecule 1

VDZ - vedolizumab

Abstract

Background: The precise mechanisms controlling homing of T effector (Teff) cells to the inflamed gut in Crohn's disease (CD) are still unclear and clinical outcome data from inflammatory bowel disease (IBD) patients treated with the anti- $\alpha 4\beta 7$ integrin antibody vedolizumab suggest differences between ulcerative colitis (UC) and CD.

Methods: Expression of homing molecules was studied with flow cytometry and immunohistochemistry. Their functional role was investigated in in vitro adhesion assays and in a humanized mouse model of T cell homing to the inflamed gut in vivo.

Results: Despite in vitro blockade of CD Teff adhesion to MAdCAM-1 and in contrast to previous observations in UC, anti- $\alpha 4\beta 7$ treatment did not result in reduced Teff cell homing to the gut in vivo. However, the integrin $\alpha 4\beta 1$ was expressed in higher levels on Teffs from CD patients compared with controls, while its expression in the peripheral blood declined and its expression in the intestine increased during the course of clinical vedolizumab treatment. Consistently, adhesion of CD Teffs to VCAM-1 was blocked by inhibition of $\alpha 4$ and $\alpha 4\beta 1$ in vitro. Moreover, in vivo homing of CD Teffs to the inflamed ileum was reduced by inhibition of $\alpha 4$ and $\alpha 4\beta 1$ integrins, but not $\alpha 4\beta 7$ integrins.

Conclusions: Our findings suggest that Teff cell homing to the ileum via the axis $\alpha 4\beta 1$ – VCAM-1 is an essential and non-redundant pathway in CD in vivo possibly affecting efficacy of clinical treatment with anti-adhesion compounds.

Keywords

Inflammatory bowel diseases – T cells – vedolizumab – natalizumab – gut homing

Introduction

The pathogenesis of inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) remains poorly understood. However, a crucial role of the immune system in the initiation and perpetuation of chronic intestinal inflammation is beyond question¹⁻³.

Lymphocyte homing from postcapillary high endothelial venules to the inflamed gut contributes to amplification of intestinal effector T lymphocyte (Teff) populations that outnumber increased regulatory T cells (Tregs)⁴. Subsequently, augmented cytokine signaling in effector T cells leads to further immune cell activation resulting in damage of intestinal structures and clinical symptoms like diarrhea, bleeding or pain⁵. Homing to the inflamed intestine is a tightly regulated multistep process that has only been rudimentarily uncovered. In general, lymphocytes establish contact with and roll along the endothelium through interaction of selectins with selectin ligands. This is followed by chemokine-induced cell activation and promotes firm adhesion to the endothelium by binding of integrins to respective addressins. Subsequently, lymphocytes may cross the endothelial wall by migrating para- or transcellularly to the lamina propria⁶. In the intestine, interaction of $\alpha 4\beta 7$ integrin with mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 is an important and gut-specific homing mechanism⁷. Consequently, therapeutic blockade of $\alpha 4\beta 7$ by the monoclonal antibody vedolizumab that was developed from the mouse anti-human $\alpha 4\beta 7$ antibody Act-1⁸ is successfully used for clinical treatment of patients with both UC and CD^{9,10}. Additionally, adhesion of $\alpha 4\beta 1$ to vascular cell adhesion molecule (VCAM)-1 has been identified as an important mechanism for gut homing of

lymphocytes but successful therapeutic inhibition with the anti- $\alpha 4$ antibody natalizumab¹¹ was abandoned due to severe cerebral viral infections probably resulting from concurrent blockade of lymphocyte homing to the central nervous system¹².

We have recently shown that vedolizumab decreases colonic homing of UC Teffs in a humanized mouse model in vivo and that CD Teff cells express $\alpha 4\beta 7$ similarly to UC Teff cells¹³. However, the functional implications for in vivo homing of Teff cells from CD patients have not yet been addressed. Moreover, clinical data suggest that vedolizumab might be more beneficial in UC than in CD¹⁴ but a molecular explanation is missing.

In the present study, we demonstrate that inhibition of $\alpha 4\beta 7$ -dependent homing of Teff cells from CD may be bypassed by compensatory homing to the ileum via $\alpha 4\beta 1$ integrin in a humanized mouse model in vivo. Our data indicate that due to an essential role of the $\alpha 4\beta 1$ integrin/VCAM-1 pathway for gut homing in CD, therapeutic interference with $\alpha 4\beta 7$ integrin might now work as well in this disease.

Materials and Methods:

IBD patients

Following informed written consent peripheral blood and gut samples from patients with CD (n = 116) and UC (n = 39) were collected according to the regulations of the local Ethics Committee at the Medical Clinic I of the University Hospital Erlangen. Controls (n = 49) came from blood and tissue specimens provided by healthy donors and tumor patients, respectively. IBD gut samples were from areas of active disease. Patients receiving vedolizumab were recruited during the induction phase of treatment and followed up for 14 +/- 0.5 weeks with periodic collection of blood samples.

Immunohistochemistry

Cryopreserved tissue sections were fixed in 4 % paraformaldehyde and sequentially blocked with avidin/biotin blocking reagent (Vector Laboratories) and protein-blocking reagent (Roth). Primary antibodies specific for CD4 (RPA-T4, BD), α 4 integrin (D2E1, Cell Signaling), β 1 integrin (JB1B, Abcam), α 4 β 7 integrin (Vedolizumab, Takeda), α 4 integrin (Natalizumab, Biogen) and CCR2 (polyclonal, Abcam) were used. The slides were subsequently incubated with fluorescent- or biotin-labeled secondary antibodies (Vectorlabs and Merck) followed by treatment with Dylight488- or Cy3-conjugated streptavidin (Biolegend), if applicable. Natalizumab was directly labelled with Alexa Fluor 647 (Thermo Fisher). Nuclei were counterstained with Hoechst reagent (molecular probes) and samples were analyzed by fluorescence and confocal

microscopy (DMI6000B and LSM SP8, Leica). Single and double positive cells in at least three high power fields were counted.

Cell isolation and in vitro treatment

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Pancoll (Pan Biotech). CD4⁺CD25⁻ Teff cells were isolated using the CD4⁺CD25⁺ Treg isolation kit (Miltenyi Biotec) according to the manufacturer's instruction. Where indicated, cells were cultured in RPMI 1640 medium (Gibco) with 10 % FCS (Pan Biotech) and 1 % penicillin/streptomycin (Biochrom) and treated with vedolizumab (Takeda) and natalizumab (Biogen) at the indicated concentrations.

Flow cytometry

For flow cytometric analyses, human cells were incubated with antibodies against CD4 (VioBlue, VIT4, Miltenyi Biotec), CCR2 (BV605, K036C2, Biolegend), α 4-integrin (FITC, MZ18-24A9, Miltenyi Biotec), β 7-integrin (PerCP/Cy5.5, FIB27, Biolegend), β 1-integrin (AF647, TS2/16, Biolegend) or FoxP3 (PE, 236A/E7, eBioscience). For intracellular staining, cells were treated with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Analyses were performed on an LSR Fortessa instrument (BD).

MAdCAM-1/VCAM-1 adhesion assay

Adhesion assays were performed on epoxy-coated glass slides (Neolab) as previously described^{13,15}. Wells were coated with Fc chimera of rhMAdCAM-1 and rhVCAM-1 (both 5 µg/mL, both 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 cells (purified CD4⁺ or CD4⁺CD25⁻ cells as indicated) in adhesion buffer were added for 90 minutes at 37 °C. After washing, adherent cells were counterstained with Hoechst and slides were analyzed by fluorescence and confocal microscopy. In blocking experiments, cells were treated with the indicated concentrations of vedolizumab and natalizumab or 100nM or the small molecule α 4 β 1 inhibitor BIO5192 (Tocris Bioscience).

Dynamic VCAM-1 adhesion assay

Ultra-thin glass capillaries (Vitrocom) were coated with Fc chimera of rhVCAM-1 (5 µg/ml) and subsequently blocked with 5 % BSA, one hour at 37° C each. Cells were labeled with CFSE (Life technologies) and treated as mentioned above. The glass capillaries were connected with plastic tubings and suspensions of 1.5 million cells/ml were perfused through the capillaries at a rate of 2.0ml/h by a perfusion pump (B. Braun). Analyses were performed by taking clips of 3 minutes length with time-lapse confocal microscopy. For quantification three sequential images at the beginning and the end of these 3 minutes were exported, colored in red, green and blue and subsequently merged in ImageJ. In the composite image, stationary cells appeared white while moving cells kept the assigned color. White cells at the beginning and the

end of the sequences were counted with and the difference (i.e. number of adhering cells in 3 minutes) was calculated.

Humanized mouse model of in vivo homing 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. Dextran sodium sulfate (DSS) colitis and adoptive transfer experiments were performed as recently described¹³. Briefly, two million Teff cells were labeled with CFSE and treated with 100 µg/mL vedolizumab or 100 µg/mL natalizumab overnight, where specified. Where indicated, mice were injected with 0.5mg of the $\alpha 4\beta 1$ inhibitor BIO5192 12h prior to adoptive transfer of cells. 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 (Leica). For in vivo imaging, the colon was longitudinally opened and the mucosa positioned on a glass transparent petri dish prior to intravital analysis with an SP8 confocal microscope (Leica).

For flow cytometric analyses, mice were sacrificed one hour 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.

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.

Ethical considerations

Patient material was obtained after informed written consent and according to the regulations and approval of the Ethics Committee of the University Erlangen-Nuremberg. Mice were housed and cared for according to the Guidelines for the Care and Use of Laboratory Animals and experiment were performed according to the approval of the Government of Lower Franconia.

Results

Vedolizumab blocks adhesion of CD Teff cells to MAdCAM-1 in vitro but does not reduce CD Teff cell homing to the inflamed colon in vivo

Recent data from our group have shown that binding of Teff cells from UC patients to MAdCAM-1 is blocked by vedolizumab in vitro and that vedolizumab treatment results in reduced in vivo Teff homing to the inflamed gut in a humanized mouse model¹³. However, the question whether similar observations can be made in Teff cells from CD patients remained unclear. We therefore set out to explore these interactions in CD. As expected, vedolizumab inhibited in vitro adhesion of overall blood CD4⁺ T cells and CD4⁺CD25⁻ Teff cells from both CD patients and controls to MAdCAM-1 coated glass slides, although effects in CD were not clearly dose-dependent (Suppl. Fig. 1, Fig. 1A). Next, we investigated $\alpha 4\beta 7$ inhibition of CD Teff cells by vedolizumab in the aforementioned humanized mouse, model in which human T cells are injected into the ileocolic artery of immunodeficient colitic mice. Colitis was induced with DSS prior to injection of CFSE labeled CD Teff cells and fluorescence dyes as described in the methods section.

By means of intravital confocal microscopy human CD lymphocytes that had homed to the colonic lamina propria could be observed. Unexpectedly and in contrast to our previous observations in UC¹³, however, no significant difference between untreated and vedolizumab-treated CD Teff cells was noticed microscopically or upon flow cytometric analysis of CFSE⁺ human cells within the LPMCs from the murine colon (Fig. 1B).

This was mirrored by the finding that unlike in UC¹³ the ratio of effector CD4⁺Foxp3⁻ and regulatory CD4⁺Foxp3⁺ T cells in the peripheral blood of CD patients receiving vedolizumab did not change over the course of therapy (Suppl. Fig. 1B). Taken together, these data suggested that in spite of efficient blockade of $\alpha 4\beta 7$ -mediated Teff cell adhesion to MAdCAM-1 and although CD and UC Teffs express similar levels of $\alpha 4\beta 7$ ¹³, homing via $\alpha 4\beta 7$ might be less functionally relevant for colonic in vivo homing of CD Teff cells to the inflamed gut in our humanized mouse model compared with UC Teff cells.

Increased expression of $\alpha 4\beta 1$ on peripheral and intestinal Teff cells from CD patients and reciprocal regulation of peripheral and intestinal $\alpha 4\beta 1$ expression under vedolizumab therapy

We reasoned that this finding could be due to a preferential or compensatory use of alternative homing pathways by CD Teff cells. Accordingly, we analyzed the expression of various alternative homing markers on Teff cells in the peripheral blood of CD patients. While we detected no differences in the expression of several homing markers like CD62L or PSGL-1 (Suppl. Fig. 2A), we found that the number of $\alpha 4\beta 1$ ^{high} Teff cells was significantly increased in CD compared with UC and controls. Similarly, more peripheral CCR2⁺ Teff cells were found in CD as compared to control patients (Fig. 2A).

We therefore further addressed these markers and assessed their intestinal expression by immunohistochemistry. Matching with higher expression in the peripheral blood the number of $\alpha 4\beta 1$ ⁺ and CD4⁺CCR2⁺ cells was significantly higher

in cryosections from inflamed CD compared with inflamed UC and control patients (Fig. 2B, Suppl. Fig. 2B).

To explore whether these molecules might be a potential bypass of $\alpha 4\beta 7$ -dependent homing, we measured the expression of $\alpha 4\beta 1$ and CCR2 in CD patients receiving clinical vedolizumab therapy. While CCR2 levels did not significantly change over the course of 14 weeks, we discovered that expression of $\alpha 4\beta 1$ distinctly declined over the observed course in almost all CD but not UC patients (Fig. 2C). This might be explained by peripheral contraction of $\alpha 4\beta 1$ -expressing cells in favor of compensatory enrichment in the intestinal lamina propria in CD, when $\alpha 4\beta 7$ -dependent homing is blocked. On the contrary, upregulation of CCR2 in CD patients does not seem to result in counter-regulatory increased CCR2-dependent homing in vedolizumab patients. Consistently, stainings of cryosections from biopsies of CD patients undergoing colonoscopy directly prior to initiation of therapy with vedolizumab or follow-up colonoscopy during the maintenance phase of vedolizumab treatment indicated that the number of $\alpha 4\beta 1$ -positive cells was increased in the latter group (Fig. 2D, Suppl. Fig. 2C). Moreover, we found that a significant portion of $\alpha 4\beta 7^+$ cells co-expresses $\alpha 4\beta 1$ (Suppl. Fig. 2D). As this was consistent with the idea that vedolizumab-induced blockade of the $\alpha 4\beta 7$ homing pathway might be circumvented by the $\alpha 4\beta 1$ pathway, we focused on $\alpha 4\beta 1$ and its ligand VCAM-1 in further steps.

Preferential ileal homing of CD Teff cells

Besides homing via alternative pathways we also considered that regarding the clinical distribution of CD it might be additionally relevant to study ileal homing with CD Teff cells. Accordingly, we determined ileal homing of Teff cells in vivo in our mouse model. First, we assessed the expression of VCAM-1, the receptor for $\alpha 4\beta 1$ in the ileum of the DSS treated mice used in our study by performing immunohistochemistry on ileal cryosections from DSS treated and untreated mice. We found that while expression in non-inflamed tissue was low, it was markedly upregulated in the ileum of DSS treated mice (Fig. 3A). Thus, although macroscopic signs of ileal inflammation are largely absent upon DSS treatment, this finding added to earlier reports fueling the notion that some features of inflammation extend beyond the colon to the ileum¹⁶⁻¹⁸ and, more specifically, suggested that our model is valid for the investigation of $\alpha 4\beta 1$ -dependent ileal homing as it served to induce the necessary ligand in the ileum. Moreover, upon adoptive transfer of untreated Teff cells from CD patients to the ileocolic artery of DSS-treated mice, intravital confocal microscopy demonstrated homed T cells in ileal villi (Fig. 3B), similarly supporting the applicability of our model for the exploration of in vivo homing to the ileum.

Next, we compared colonic and ileal homing after adoptive transfer of untreated CD Teff cells. We observed high amounts of human CD Teff cells in the murine ileum by in vivo microscopy, while colonic numbers were markedly lower (Fig. 3C). Quantification by flow cytometry of lamina propria mononuclear cells (LPMCs) isolated from the mice confirmed this observation, since a significant difference was noted between ileal and colonic CD Teff cell infiltrates. This difference was more pronounced in CD patients with small intestinal involvement (L1 and L3 phenotypes

according to the Montreal classification) than in patients without ileal involvement (L2 phenotype) (Suppl. Fig. 3).

To exclude that this was an effect related to our in vivo model, we performed similar experiments with untreated cells from healthy control donors. Here, colonic and ileal cell accumulation were not significantly different but tended towards higher colonic levels (Fig. 3D). This suggested that the effects seen with CD Teff cells were specific for the disease.

This was consistent with the notion that unaffected colonic homing of CD Teff cells upon vedolizumab treatment in our humanized mouse model might be explained not only by alternative homing via $\alpha 4\beta 1$ and VCAM-1 but also by a predominant role of ileal homing in CD.

Static and dynamic adhesion to VCAM-1 is blocked by natalizumab

Hence, we decided to study adhesion of $\alpha 4\beta 1$ to VCAM-1 more closely. VCAM-1-coated glass slides were incubated with Teff cells from CD patients that had been treated with different concentrations of vedolizumab and natalizumab in vitro (Fig. 4A, C). As expected, the anti- $\alpha 4$ antibody natalizumab dose-dependently reduced adherence of Teff cells to VCAM-1, while vedolizumab had a significant effect only at non-physiologically relevant concentrations¹⁹ matching with previous data that report some binding of $\alpha 4\beta 7$ to VCAM-1 as well^{20,21}.

However, these experiments only addressed static adhesion and might therefore not fully reflect the physiological situation where adhesion happens under the dynamic

flow conditions of the blood stream with shear stress. Thus, we refined our technique to investigate adhesion to VCAM-1 under flow by perfusing VCAM-1 coated ultra-thin glass capillaries with suspensions of CD Teff cells (Fig. 4B, Suppl. Video 1).

While virtually no cells adhered to uncoated glass capillaries, many untreated cells bound to VCAM-1-coated counterparts. Fitting to the observations under static conditions, adhesion of natalizumab treated cells was obviously and significantly reduced, while vedolizumab had no significant effect (Fig. 4C).

Pan- $\alpha 4$ but not $\alpha 4\beta 7$ inhibition blocks ileal homing of CD Teff cells in vivo

Consequently, we compared the implications of $\alpha 4\beta 7$ and $\alpha 4$ blockade for ileal homing in our humanized mouse model and transferred vedolizumab and natalizumab treated Teff cells from CD (Fig. 5A). Quantitative analyses revealed that while vedolizumab only had a marginal effect that missed statistical significance, natalizumab significantly reduced ileal numbers of CD Teff cells (Fig. 5B).

As natalizumab additionally blocks $\alpha 4\beta 1$, this further supports the concept that this molecule might be essential in CD to explain our findings for $\alpha 4\beta 7$.

Inhibition of $\alpha 4\beta 1$ but not $\alpha 4\beta 7$ blocks ileal homing of CD Teff cells in vivo

However, since natalizumab is a pan- $\alpha 4$ inhibitor these experiments were not suitable to directly address the role of $\alpha 4\beta 1$. Thus, we performed experiments using the specific $\alpha 4\beta 1$ inhibitor BIO5192.

In vitro adhesion assays demonstrated that this inhibitor reduces adherence of CD Teff cells to VCAM-1 to a level comparable with natalizumab (Fig. 6A). In our humanized mouse model, $\alpha 4\beta 1$ inhibition with or without vedolizumab significantly reduced in vivo homing to the inflamed ileum, while vedolizumab alone again failed to induce a significant effect (Figs. 6B, C).

Taken together, these findings suggested that blockade of $\alpha 4\beta 1$ might be sufficient to impede homing of CD Teff cells to the inflamed ileum while inhibition of $\alpha 4\beta 7$ – though preventing adhesion to MAdCAM-1 – might be compensated by increased homing via $\alpha 4\beta 1$.

Discussion

Although the precise role of intestinal T lymphocyte trafficking in IBD is still incompletely understood, both recent data from basic and translational research and clinical advances have underscored the importance of this process in the framework of chronic intestinal inflammation^{22–25}. After the episode of natalizumab, which was effective in CD but led to serious safety concerns^{12,26}, this has finally been clinically implemented by the monoclonal anti- $\alpha 4\beta 7$ integrin antibody vedolizumab.

The efficacy of vedolizumab in both UC and CD has been demonstrated in several trials^{9,10} and no increased risk for infections of other organs has been reported so far²⁷, thus supporting the concept of gut-specific inhibition of homing by this molecule^{7,22}. However, clinical observations also suggest that gut homing might be of differential relevance and might be differentially controlled in UC and CD as the main entities of IBD. In concrete terms, while it is beyond question that vedolizumab is effective in both diseases, several studies indicate that vedolizumab might be beneficial for a higher percentage of UC than CD patients or have a faster time to response in UC²⁸. In the GEMINI 2 phase III induction trial, a significantly higher proportion of CD patients receiving vedolizumab had a clinical remission compared with patients receiving placebo, but no significant effect was observed in the CDAI-100 response or in the change of CRP levels. In the maintenance study, higher rates of clinical remission and response compared with placebo were reported, yet, these differences only appeared very late in the one-year course of therapy and rates of durable clinical remission were similar¹⁰. In the GEMINI 3 trial, moreover, differences in clinical remission were only detectable at week ten but not at week 6²⁹. On the

contrary, vedolizumab met all primary endpoints in the UC GEMINI 1 induction trial, i.e. clinical remission, clinical response and mucosal healing. Moreover, it induced significantly higher rates of remission, response, mucosal healing, steroid-free and durable remission in the maintenance phase⁹. Although some open-label studies reported similar efficacy in both diseases^{30,31} later reports indicated higher percentages for remission, response and mucosal healing in UC compared with CD^{32,33} matching with our own clinical experience, and two recent meta-analyses found higher RR values for remission and response endpoints in UC than in CD^{14,34}. To date, the reason for these differences is not clear. We have recently introduced a humanized mouse model taking advantage of DSS-treated immunodeficient mice to study vedolizumab effects on homing of T lymphocytes from UC patients^{13,35}. Using this model we now addressed colonic and ileal homing of CD effector T lymphocytes and reveal that redundant homing via $\alpha 4\beta 1$ might be an explanation for the above mentioned observations.

While our first experiments confirmed that vedolizumab blocks $\alpha 4\beta 7$ -mediated adhesion to MAdCAM-1 in T lymphocytes from CD similarly to UC, we were astonished that we were not able to detect a significant effect in our humanized mouse model of homing to the inflamed colon. Compared with our previous data on UC, we only observed a modest decrease in colonic lymphocyte accumulation upon treatment with vedolizumab that did not reach statistical significance. We had also shown that the peripheral Treg population increases within the first weeks of vedolizumab treatment in UC patients resulting in a decreasing T_{eff}/Treg ratio, which potentially leads to suppression of systemic inflammation¹³. Similar analyses in CD patients treated with vedolizumab at our department did not show any change in the

ratio for Foxp3⁻/Foxp3⁺ CD4 T cells in the same period of time, thus further supporting the notion that effects of $\alpha 4\beta 7$ inhibition are not identical in CD and UC. The mechanistic consequence of this finding remains an object of speculation as it is not clear to what extent intestinal Treg and Teff pools and their imbalance in IBD depend on homing from the bloodstream and local induction or proliferation in the intestine^{4,36}. However, it underscores differences in $\alpha 4\beta 7$ -mediated homing and therapeutic $\alpha 4\beta 7$ blockade between UC and CD.

We considered three potential approaches towards an explanation for our in vivo findings of only non-significant vedolizumab effects:

First, lack of $\alpha 4\beta 7$ -dependent homing of CD Teff cells in our system. However, regarding the clear effect of vedolizumab on CD T lymphocytes in vitro, the previously observed effects of vedolizumab on UC T cell homing, the expression of murine MAdCAM-1 in DSS-treated immunodeficient mice and the ability of human $\alpha 4\beta 7$ to bind to murine MAdCAM-1¹³ it was very unlikely that vedolizumab would not inhibit interaction of $\alpha 4\beta 7$ with MAdCAM-1 and thus $\alpha 4\beta 7$ -dependent homing in our humanized in vivo model.

Second, compensatory homing via alternative receptors, i.e. a partly redundant role of $\alpha 4\beta 7$ in CD. Consequently, we analyzed the expression of alternative homing markers on Teff lymphocytes and, in fact, we found a significant upregulation of $\alpha 4\beta 1$ and CCR2 in the peripheral blood and the gut in CD but not in UC compared with controls. Sequential measurements of $\alpha 4\beta 1$ on Teff cells from CD patients treated with vedolizumab revealed a reduction of the peripheral expression in the course of treatment, while – reciprocally – immunohistochemistry of gut tissue suggested an intestinal increase. This was indeed compatible with a compensatory shift of $\alpha 4\beta 1^+$

Teff cells from the peripheral blood to the diseased intestine. A similar concept has earlier been established in a murine model of chronic ileitis, where single blockade of neither $\alpha 4\beta 7$ nor MAdCAM-1 resulted in disease protection. However, the authors could show that the combination of MAdCAM-1 and L-Selectin inhibition reduced disease severity and as $\alpha 4\beta 1$ and $\alpha 4\beta 7$ were frequently co-expressed they proposed that interference with one of these pathways might result in alternative homing via the other one³⁷. An earlier report from the same group pointed in a similar direction. Here, single blockade of the addressins ICAM-1, VCAM-1 or the integrin $\alpha 4$ had no effect on murine ileitis, while dual inhibition of ICAM-1 with VCAM-1 or $\alpha 4$ reduced colitis severity³⁸. In this context, it is also interesting to mention that alicaforsen, an antisense inhibitor of ICAM-1 did not show efficacy in CD, which might also be explained by redundant homing pathways³⁹.

Third, an only minor role of colonic homing in CD compared with UC. Addressing this possibility, we compared ileal and colonic homing of CD Teff lymphocytes and found that more of these cells homed to the ileum than to the colon. Given the prevalent disease distribution³ this seems not very surprising and, thus, we decided to study $\alpha 4\beta 1$ - and $\alpha 4\beta 7$ -dependent homing of cells from CD patients to the murine ileum.

In these experiments, we could demonstrate that – again – $\alpha 4\beta 7$ inhibition through vedolizumab caused a non-significant minor decrease in homing, while blockade of $\alpha 4$ with natalizumab and thus $\alpha 4\beta 7$ in combination with $\alpha 4\beta 1$ resulted in a marked reduction of homing. This was consistent with compensatory $\alpha 4\beta 1$ -dependent homing in the presence of anti- $\alpha 4\beta 7$ treatment. However, it did not answer the question whether $\alpha 4\beta 7$ might also work as an alternative pathway bypassing $\alpha 4\beta 1$ blockade. We thus treated mice with an $\alpha 4\beta 1$ inhibitor and compared the effects to

vedolizumab. Both $\alpha 4\beta 1$ inhibitor alone and in combination with vedolizumab resulted in a significant decrease of homed cells compared to no treatment or only vedolizumab treatment. This observation favors the idea that while $\alpha 4\beta 7$ blockade can be circumvented by $\alpha 4\beta 1$ -dependent homing, $\alpha 4\beta 1$ is essential for ileal homing of Teff cells in CD and its blockade cannot be evaded by compensatory use of the $\alpha 4\beta 7$ pathway (Fig. 7).

It is not finally clear, why this seems to be clinically relevant in CD but not in UC. While one reason might be the higher expression of $\alpha 4\beta 1$ in CD, another possibility is that $\alpha 4\beta 7$ circumvention by $\alpha 4\beta 1$ is not a specific feature of homing in CD but rather a feature of ileal compared with colonic homing. Another issue in this context might be time. As mentioned above, significant effects of vedolizumab in CD were more likely to be observed at later time points in clinical trials^{10,29}. Naturally, our model only examines short-term trafficking and is therefore not able to reveal long-term effects of the drugs tested. Thus, it is possible that vedolizumab exerts its effect in CD by constantly leading to slight reductions in homing that have to accumulate over time before active inflammation is measurably suppressed. Apart from this and regarding our data it seems essential to place effort in the identification of suitable biomarkers to predict the individual response to vedolizumab therapy like it was conceptually shown for adalimumab or etrolizumab^{24,40}.

Taken together, our data show for the first time that human Teff cells may circumvent inhibition of one integrin by homing to the inflamed gut via another one in vivo and thereby evade targeted anti-integrin therapy. These findings emphasize the need for refinement of current and development of future therapies interfering with intestinal trafficking.

Conflict of interest:

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Author contributions:

SZ, AF, DS and MB performed the experiments. SZ, RA, TR, RLP, CV, AW, CN, IA and MFN provided clinical samples, protocols, reagents or designed experiments; SZ, AF, DS, MTB, CN, IA and MFN analyzed and interpreted the data; SZ drafted the

manuscript; all authors critically revised the manuscript for important intellectual content.

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Figure legends:

Figure 1: Homing of CD Teff cells to the inflamed murine colon is not significantly affected by vedolizumab

(a) Adhesion of control and CD Teff cells to MAdCAM-1-coated glass slides. Left panels: Representative adhesion assays showing adhered cells from control and CD upon treatment with different concentrations of vedolizumab as indicated. Right panels: Pooled statistics (n = 5-7).

(b) In vivo homing of CD Teff cells to the inflamed colon. Left panels: Representative intravital microscopy of the murine colon upon adoptive transfer of human cells. Green: human cells (→), red: murine vessels, blue: murine cells. Right upper panels: Representative flow cytometry displaying the relative proportion of colonic CFSE⁺ human cells in a control mouse without transfer and mice after transfer of CD Teff cells treated with or without vedolizumab. Right lower panels: Pooled statistics (n = 13).

Figure 2: Increased expression of $\alpha 4\beta 1$ in CD patients and reciprocal peripheral vs. intestinal regulation under vedolizumab therapy

(a) Flow cytometric analyses of $\alpha 4\beta 1$ and CCR2 expression on peripheral CD4⁺CD25⁻ Teff cells in controls (n = 20), CD (n = 31) and UC (n = 9). Left upper panels: Representative plots showing the percentage of $\alpha 4\beta 1^{\text{high}}$ cells. Left lower panels: Representative plots showing the percentage of CCR2⁺ cells. Right upper panel: Quantitative analysis and statistics of $\alpha 4\beta 1^{\text{high}}$ Teff cells in controls, CD and

UC. Right lower panel: Quantitative analysis and statistics of CCR2⁺ T eff cells in controls, CD and UC.

(b) Immunohistochemistry of $\alpha 4\beta 1$ (left) and CCR2 (right) expression in the human intestine in controls (n = 5-7), CD (n = 6-7) and UC (n = 3-7). Left upper panels: Representative stainings of $\alpha 4$ and $\beta 1$. Right upper panels: Representative stainings of CD4 and CCR2. Scale bars: 25 μ m. Left lower panels: Quantification and statistical analysis of the number of $\alpha 4\beta 1$ -double positive cells in controls, CD and UC. Right lower panels: Statistical analysis of the number of CD4⁺CCR2⁺ cells in controls, CD and UC.

(c) Relative peripheral $\alpha 4\beta 1$ and CCR2 expression on Teff cells over the course of Vedolizumab therapy. Peripheral blood from CD (left panels, n = 8) and UC patients (right panels, n = 6) receiving vedolizumab was analyzed by flow cytometry for $\alpha 4\beta 1$ and CCR2 expression at recruitment and after 14 +/- 0.5 weeks. Values were normalized to the first value.

(d) Immunohistochemistry of $\alpha 4\beta 1$ expression in the intestine of CD patients directly prior to vedolizumab therapy (n = 4) and during the maintenance phase of treatment (n = 5). Slides were stained with vedolizumab and natalizumab and single natalizumab-positive cells were considered $\alpha 4\beta 1$ ⁺. BT – before therapy, MP – maintenance phase.

Figure 3: CD Teff cells preferentially home to the ileum of humanized mice in vivo

(a) Immunohistochemistry of VCAM-1 in ileal cryosections from mice treated with DSS or not. Representative images from one out of three independent experiments are shown. Scale bars: 25 μ m.

(b) Representative images of intravital confocal microscopy from the murine ileum after adoptive transfer of CD4⁺CD25⁻ T cells from CD patients. Scale bars: 100 μ m and 25 μ m (inserts).

(c) Left upper panels: Representative flow cytometry plots comparing ileal and colonic accumulation of CD Teff cells. Left lower panels: Quantitative flow cytometry of CFSE⁺ cells from CD patients the ileal and colonic lamina propria of DSS treated mice after adoptive transfer (n = 10). Right panels: Representative images of in vivo microscopy comparing CD Teff cell homing to the inflamed ileum and colon as indicated. Scale bars: 75 μ m and 25 μ m (inserts). Green: CFSE (\rightarrow), red: Texas Red Dextran, blue: Hoechst.

d) Quantitative flow cytometry of CFSE⁺ cells from control donors in the ileal and colonic lamina propria of DSS treated mice after adoptive transfer (n = 7).

Figure 4: Static and dynamic adhesion to VCAM-1

(a) Adhesion of CD Teff cells to VCAM-1 coated glass slides. Representative adhesion assays showing adhesion to VCAM-1 upon treatment with different concentrations of vedolizumab (VDZ) and natalizumab (NTZ) as indicated. Scale bars: 50 μ m and 25 μ m (inserts).

(b) Dynamic adhesion of CFSE-labeled CD Teff cells to VCAM-1 coated glass capillaries perfused with a perfusion pump. Representative sequential images at the indicated relative time points. Coating of the capillary and treatment of the cells is specified on the left. White circles connected by white lines highlight positions where cells newly adhere to the capillary, red circles connected by red lines show positions where cells pass the field of view without adhering. Also cf. Suppl. Video 1.

(c) Quantification of adhesion assays. Left panel: Pooled statistics ($n = 5$) of static adhesion assays showing the number of cells adherent to VCAM-1-coated glass slides. Right panel: Pooled statistics ($n = 3$) of dynamic adhesion assays showing the number of cells adhering to VCAM-1 over three minutes. Values were normalized to untreated cells.

Figure 5: Pan- $\alpha 4$ but not $\alpha 4\beta 7$ inhibition impedes ileal homing of CD Teff cells in vivo

(a) Representative intravital confocal microscopy upon transfer of CD4⁺CD25⁻ cells from CD and treatment as indicated. Green: human cells (\rightarrow), red: murine vessels, blue: murine cells. Scale bars: 100 μ m and 25 μ m (inserts).

(b) Flow cytometric quantification of ileal homing upon treatment with natalizumab (NTZ) and vedolizumab (VDZ). Left panels: Representative plots. Right panels: Pooled statistics ($n = 5$).

Figure 6: $\alpha 4\beta 1$ inhibition is essential for ileal homing of CD Teff cells in vivo

(a) Adhesion of CD Teff cells to VCAM-1 coated glass slides. Left panels: Representative adhesion assays showing adhesion to VCAM-1 upon treatment with different concentrations of natalizumab (NTZ) or $\alpha 4\beta 1$ inhibitor as indicated. Scale bars: 50 μ m and 25 μ m (inserts). Right panels: Pooled statistics (n = 5).

(b) Representative intravital confocal microscopy upon transfer of CD4⁺CD25⁻ cells from CD and treatment as indicated. Green: human cells (→), red: murine vessels, blue: murine cells. Scale bars: 100 μ m and 25 μ m (inserts).

(c) Flow cytometric quantification of ileal homing upon treatment with vedolizumab (VZD) and/or $\alpha 4\beta 1$ inhibitor. Left panels: Representative plots. Right panels: Pooled statistics (n = 8).

Figure 7:

Schematic model of $\alpha 4\beta 7$ - and $\alpha 4\beta 1$ -dependent homing and interference with vedolizumab in CD (left side) and UC (right side). In CD, blockade of $\alpha 4\beta 7$ via vedolizumab is circumvented by ileal homing via $\alpha 4\beta 1$, which is expressed in increased levels. In UC, vedolizumab-induced inhibition of $\alpha 4\beta 7$ leads to significant reduction of colonic homing.

Supplementary Figure 1:

(a) Adhesion of control and CD CD4⁺ T cells to MAdCAM-1-coated glass slides. Upper panels: Representative adhesion assays showing adhered cells from control

and CD upon treatment with different concentrations of vedolizumab as indicated.

Lower panels: Pooled statistics (n = 6-9).

(b) Flow cytometric quantification of the Foxp3⁻/Foxp3⁺ ratio in peripheral blood CD4⁺ T cells from patients receiving vedolizumab therapy visiting our department at week 0 (visit 1), 2 (visit 2) or 6 (visit 3) of treatment.

Supplementary Figure 2:

(a) Flow cytometric quantification and statistical analyses of CD62L, PSGL, CXCR3, CCR4 and CCR8 expression on peripheral CD4⁺CD25⁻ Teff cells in controls (n = 7-15), CD (n = 7-18) and UC (n = 4-10)

(b) Negative controls for the stainings shown in Fig. 2B. Scale bars: 25µm.

(c) Representative immunohistochemistry stainings of cryosections from CD patients before treatment (BT) with vedolizumab or in the maintenance phase (MP) of therapy with natalizumab (NTZ) and vedolizumab (VDZ). Examples of double positive cells (i.e. α4β7⁺) are marked with orange arrows, examples of single NTZ⁺ cells are marked with white arrows. Scale bars: 25µm and 12,5µm (inserts).

(d) Left panel: Representative flow cytometry of β1 integrin expression on α4β7⁺ CD4⁺Foxp3⁻ T cells. Right panels: Quantitative flow cytometry indicating the percentage of β1⁺ cells among α4β7⁺ CD4⁺Foxp3⁻ T cells in control patients (n = 5), CD (n = 5) and UC (n = 2).

Supplementary Figure 3:

Stratification of the data shown in Fig. 3B according to the localization category of the Montreal classification.

Supplementary Video 1:

Representative 3 minute-clips from dynamic adhesion assays with CD Teff cells in control or VCAM-1 coated capillaries and treatment with vedolizumab or natalizumab as indicated in fast motion. The blue arrow (left lower corner) indicates the direction of the cell stream. Newly adhering cells are marked with white arrows.

Supplementary Table 1: Clinical data

		Control	CD	UC
Blood	Number	38	101	32
	Age (Ø)	29	38	46
	Female %	79	53	44
	HBI (Ø)		7.0 (1 – 24)	
	Mayo c.s. (Ø)			2.1 (0 – 6)
	Biologicals %		64 *	72 *
	Immunosuppressants %		26*	25 *
	* some patients with both types of medication			
Gut	Number	11	18	7
	Age (Ø)	63	41	48
	Female %	55	56	43
	area of active disease %		100	100
	histologic severity**		2.5	2.4
	Biologicals %		15	14
	Immunosuppressants %		45	0

**** 1 – low grade, 2 – moderate grade, 3 – high grade**