## Online Supplementary Methods

### Isolation of human peripheral blood mononuclear cells (PBMCs), PBMC subsets and granulocytes

PBMCs were isolated using density gradient centrifugation with Pancoll (PAN-Biotech). MACS isolation of $CD4^+$ T cells (CD4 MicroBeads, human/ $CD4^+$ T Cell Isolation Kit, human, Miltenyi Biotec) was performed according to the manufacturer's instructions.

Granulocytes were isolated from the cell pellet obtained after density gradient centrifugation. After removing PBMCs as well as remaining plasma and Pancoll reagent, cells were resuspended in 1 % Dextran 500 (Roth) in PBS and erythrocytes left to sediment for 30 min at room temperature. Supernatants containing granulocytes were transferred into fresh tubes and the remaining erythrocytes were lysed with hypotonic lysis (1 min 0.2 M NaCl, 1 min 1.6 M NaCl).

### Fluorescence-activated cell sorting (FACS)

FACS was used to isolate $CD4^+CD127^{low}CD25^{high}$ $T_{Reg}$ cells and $CD4^+CD127^{high}CD25^{low}$ $T_{Eff}$ cells. To this end, PBMCs isolated from leukocyte cones were stained with following fluorochrome-conjugated extracellular antibodies: CD3 (VioGreen, REA613/REAL104, Miltenyi Biotec), CD4 (VioBlue, VIT4/REAL103 Miltenyi Biotec), CD25 (PE/Cy7/FITC, BC96, Biolegend; PE-Vio770, REAL128, Miltenyi Biotec) and CD127 (APC, A019D5, Biolegend; APC/Vio770, REA614 or APC, REAL102, Miltenyi Biotec). Isolation of T cells binding or not binding vedolizumab was performed using fluorescently labelled vedolizumab at a concentration of 10 µg/ml and following

fluorochrome-conjugated extracellular antibodies: CD4 (FITC/VioGreen, VIT4, Miltenyi Biotec), CD45RO (BV510, clone, Biolegend), CD25 (PE/Cy7, BC96, Biolegend), CD127 (VioBright FITC, REA614, Miltenyi Biotec), CD49d (VioBlue, MZ18-24A9, Miltenyi Biotec), integrin beta 7 (PE, FIB27, Biolegend). Living cells were gated as negative for fixable viability dye efluor780 (eBioscience).

FACS was performed on MoFlo Astrios EQ1, MoFlo Astrios EQ2 (Beckman Coulter) and FACS Aria II SORP (BD Bioscience) instruments. Detachment of REAlease antibodies after isolation was performed using REALease support kit (Miltenyi Biotec) according to the manufacturer's instructions.

**Lamina propria mononuclear cell (LPMC) isolation from human biopsies**

Human intestinal biopsies from IBD patients were obtained during colonoscopy following informed written consent according to the approval of the Ethics Committee of the Friedrich Alexander University Erlangen-Nürnberg (249_13 B). LPMC isolation was performed using the Lamina propria dissociation kit (Miltenyi Biotec) according to manufacturer's instructions followed by density gradient centrifugation with Percoll (GE Healthcare). After isolation, cells were either incubated with 50 ng/ml PMA, 1 µM ionomycine and 1 µg/ml brefeldin A for 4 h at 37°C or directly stained for flow cytometry.

**Cell culture and *in vitro* stimulation**

For stimulation with $MnCl_2$, PBMCs were resuspended at a concentration of 10 Mio cells/ml in adhesion buffer (150 mM NaCl + 1 % HEPES) ± 1 mM $MnCl_2$ for 1 minute at room temperature. For stimulation with anti-CD3/CD28, PBMCs were cultured in 48-

well-plates (Sarstedt) at a concentration of 1 Mio cells/ml over night at 37°C. Stimulation with PMA (1 µg/ml) and ionomycine (1 µM) was performed with 1 Mio cells/ml in 48-well-plates for 4 h at 37°C. For *in vitro* treatment with vedolizumab, cells were cultured in 48-well plates at a concentration of 1 Mio cells/ml with the indicated vedolizumab concentrations for 1 h at 37°C. For the detection of interleukin expression, cells were stimulated with 50 ng/ml PMA, 1 µM ionomycine and Golgi export blocked using 1 µg/ml brefeldin A for 4 h at 37°C.

**Fluorescence microscopy of vedolizumab binding to $T_{Reg}$ and $T_{Eff}$ cells**

For imaging of vedolizumab binding to $T_{Reg}$ and $T_{Eff}$ cells, FACS-isolated cells were incubated with fluorescently labelled vedolizumab and integrin beta 7 antibody (PE, FIB27, Biolegend) or adequate isotype controls for 30 min at 4°C after removal of REAlease antibodies, then fixed using 4 % PFA. Nuclei were stained using Hoechst 33342 (ThermoFisher Scientific). Cells were embedded in Mowiol (Sigma Aldrich) and subsequently imaged using a fluorescence microscope (DM600B, Leica).

**$T_{Reg}$ cell suppression assay**

To analyse the suppressive function of FACS-isolated $T_{Reg}$ cells, sorted $T_{Reg}$ and $T_{Eff}$ cells were stained with CellTrace FarRed (Invitrogen) according to the manufacturer's instructions. FarRed$^+$ $T_{Eff}$ cells were incubated with $T_{Reg}$ Suppression Inspector (Miltenyi Biotec) in RPMI 1640 (+ 10 % FBS, + 1 % Pen/Strep, Gibco) for 6 days at 37°C with or without unstained $T_{Reg}$ cells according to the manufacturer's instructions. FarRed$^+$ $T_{Reg}$ cells were incubated without $T_{Eff}$ cells as a control. After incubation, cells were harvested and stained with Fixable Viability Dye efluor 506 (Invitrogen). The

portion of proliferating cells was determined by analysis of CellTrace FarRed dilution by flow cytometry.

**Transmigration assays**

For the analysis of the impact of vedolizumab on MAdCAM-dependent transmigration of $T_{Reg}$ and $T_{Eff}$ cells, MACS-isolated $CD4^+$ T cells were stained with fluorochrome-conjugated extracellular antibodies against CD4 (VioBlue, VIT4, Miltenyi Biotec), CD25 (PE/Cy7, BC96, Biolegend) and CD127 (APC/Vio770, REA614, Miltenyi Biotec) and resuspended in X-Vivo15 medium (Lonza) with 1 mM $MnCl_2$ and different concentrations of vedolizumab (0, 0.4, 2, 10 and 50 µg/ml) at a concentration of 2 Mio cells/ml. 160,000 cells were seeded in duplicates into inserts of a 3 µm transwell plate (Corning) that were previously coated with 5 µg/ml rhMAdCAM-1 for 1 h. Inserts were placed into wells filled with X-Vivo15 medium + 100 nM rhCCL25 (ImmunoTools) and the plate was incubated for 4 h at 37°C. Subsequently, inserts were discarded and the number of transmigrated $T_{Reg}$ and $T_{Eff}$ cells was quantified by flow cytometry.

**Immunohistochemistry**

Cryosections of colon biopsies from IBD patients before and under treatment with vedolizumab were fixed with 4 % PFA, permeabilised with 0.1 % Triton-X in PBS and subsequently blocked with Avidin-Biotin Block (Vector Laboratories) and Roti-ImmunoBlock (Carl Roth) with 5 % BSA and 20 % goat serum. Primary antibodies targeting CD4 (1:200, 13B8.2, Novus Biological), Foxp3 (1:100, 236A/E7, eBioscience and 1:100, FJK-16s, eBioscience) and integrin β1 (1:1000, JB1B, abcam) were incubated over night at 4°C or for 2 h at room temperature. Goat anti-rabbit AF488

(1:200, Invitrogen), goat anti-mouse Cy3 (1:200, Biolegend) and goat anti-rat AF647 (1:200, Biolegend) were used as secondary antibodies, respectively, and incubated for 1 h at room temperature. Sections treated with the secondary antibodies alone served as control. Nuclei were counter-stained using Hoechst 33342 (ThermoFisher Scientific). Slides were embedded with Vectashield Mounting Medium (Vector Laboratories) and imaged using the fluorescence microscope DM600B (Leica) and the confocal microscope SP8 (Leica). Data analysis and quantification were performed with Fiji (v1.51n, National Institutes of Health).

**Mice**

B6.129S7-Rag1$^{tm1Mom}$ (Rag1$^{-/-}$) mice were housed in individually ventilated cages with a regular day-night cycle. For the induction of colitis, age- and sex-matched mice were given 1.5-2.5 % DSS in drinking water for 7 days. All experiments involving animals were approved by the Government of Lower Franconia, Germany.

**Humanised *in vivo* model of T cell homing to the inflamed gut**

Quantification of cell homing *in vivo* was performed using a humanised mouse model previously established in our lab [1]. FACS-purified $T_{Reg}$ and $T_{Eff}$ cells were stained with CellTrace FarRed (Invitrogen) at 37°C for 15 min and adjusted to a concentration of 1-2 Mio cells/100 µl in PBS. Cells were treated with 10 µg/ml vedolizumab or with human IgG Isotype control (Invitrogen). B6.129S7-Rag1$^{tm1Mom}$ mice were anesthetised by intraperitoneal injection of ketamine/xylazin. Fluorescently labelled cells were injected into the ileocolic artery of the mice and left to circulate for 1 h. Thereafter, mice were sacrificed and lamina propria mononuclear cells (LPMCs) were isolated from the colon

using the Lamina Propria Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions followed by density gradient centrifugation with Percoll (GE Healthcare). Fluorescent human cells homed to the murine colon were quantified by flow cytometry.

For intravital microscopy, cells were stained with CellTrace Yellow (Invitrogen) for 15 min at 37°C, treated with vedolizumab or isotype control and injected into the ileocolic artery together with Hoechst 33342 (ThermoFisher Scientific) and 10 µg of anti-mouse CD31 antibody (AF647, MEC13.3, Biolegend). After an incubation period of 15 minutes, the 2 cm of the proximal part of the colon were opened longitudinally, cleaned from faeces and placed onto a coverslip. Cell migration was monitored over 45 min using a confocal microscope (SP8, Leica). Finally, mice were perfused with 15 ml 5 mM PBS/EDTA followed by 4 % PFA and a colon piece was post-fixed overnight and then further processed for lightsheet microscopy.

**Lightsheet microscopy**

Preparation of samples for lightsheet microscopy was performed as described elsewhere [2]. In short, fixed colon tissue was washed with PBS and then dehydrated using an ascending ethanol series of 50, 70 and 2x 100 % ethanol for 2 h each at 4°C. Thereafter, tissue was cleared using ethyl cinnamate (Sigma) for at least 24 h at room temperature and imaged using an UltraMicroscope II (LaVision, BioTec.). 3D reconstruction of colon tissue and quantification of accumulated human cells was performed with Imaris Image Analysis software 9.0.2 (Bitplane).

**Bioinformatic analysis of single cell RNA sequencing data**

Single cell analysis was performed in Python v3.6.13 using Scanpy v1.7 [4]. All cells with a mitochondrial content higher than 20 % were filtered out. Normalisation was achieved through size factor correction using Scran v1.14.6 in R [5]. In order to cluster the data, Uniform Manifold Approximation and Projection (UMAP) [6] was applied to the data for dimension reduction and consecutively the Leiden algorithm was applied for community detection [7].

To define $T_{Reg}$ cell clusters, we subclustered all clusters and subsequently performed a ranking of gene expression in each respective subcluster, as defined in the Scanpy documentation. We manually selected the $T_{Reg}$ cell clusters, by calculating an overlap score using the following 8 prominent $T_{Reg}$ cell marker genes in humans, using a cutoff of at least 3 of these markers to define the $T_{Reg}$ cell subcluster: *FOXP3, DUSP4, IL2RA, IL2RB, CTLA4, IKZF2, IKZF4, RTKN2*. All $T_{Reg}$ cell subclusters were pooled into one comprehensive $T_{Reg}$ cell subcluster, divided by the samples $VDZ^+$ and $VDZ^-$. Differential expression analysis was conducted using the R package MAST v1.12. Additionally, a python script was written to count the amount of cells present per gene for a certain cluster or specified group based on a previously published example [8].

The entire analysis workflow is available on Github as a Jupyter notebook file (see accession codes).

For the analysis of the publicly available single cell RNA sequencing dataset GSE162335, the same pipeline was used as described above. After selecting the T cell cluster we defined $T_{Reg}$ cells in this LPMC-dataset as cells expressing at least 3 out of 8 key regulatory genes (*FOXP3, DUSP4, IL2RA, IL2RB, CTLA4, IKZF2, IKZF4, RTKN2)*.

**Statistics**

Statistical analyses were performed using Prism 8 software (GraphPad). The following statistical test were used and are specified in the figure legends: When comparing two groups, two-tailed Student's t-test, paired Student's t-test with or without Welch correction, or Mann-Whitney test were used as applicable. When comparing more groups, One-Way ANOVA, Mixed effects analysis, Kruskal-Wallis test or Two-Way ANOVA were used as applicable. For relative data, One sample t test or Wilcoxon test were used as applicable. If indicated, single outliers were detected by the Grubbs' test or ROUT ($\alpha = 0.05$). For contingency analysis, Chi-Square and Fisher's exact test were performed. Results are displayed as box plots indicating median and interquartile range with whiskers indicating minimum and maximum. Correlations were calculated by the Spearman or Pearson test as indicated. $P < 0.05$ was considered statistical significant in all tests with asterisks indicating the following levels of significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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