

Methods (Supplemental File)

Lamina propria mononuclear cells (LPMC) isolation from human biopsies

Intestinal biopsies were obtained during routine colonoscopy following the patients' informed written consent and according to the approval of the ethics committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg (249_13 B). LPMCs were isolated using a Lamina Propria Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions followed by density gradient centrifugation with Percoll (GE Healthcare).

Isolation of peripheral blood mononuclear cells (PBMCs) from human donors

Peripheral blood from patients with IBD and non-IBD controls was obtained following the patients' informed written consent and according to the approval of the ethics committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg (135_20B, 426_20B). PBMCs were isolated using density gradient centrifugation with Lymphocyte Separation Media (anprotech). Where indicated, CD4⁺ or regulatory CD4⁺ T cells were isolated using the CD4⁺ or CD4⁺CD25⁺ Regulatory T Cell Isolation Kits (Miltenyi Biotec), respectively.

***In vitro* T cell stimulation and incubation with cytokines**

Cells were cultured in RPMI medium containing GlutaMAX (Gibco), 10% fetal bovine serum (FBS, good forte, PAN Biotech) and 1% Penicillin/Streptavidin (P/S, Thermo Fisher Scientific) at 37°C with and atmosphere containing 5% CO₂. Stimulation was performed in plates precoated with anti-human CD3 antibodies (OKT3, eBioscience)

and cells were additionally treated with anti-human CD28 antibodies (CD28.2, BD Biosciences). Where mentioned, recombinant human IL-3, IL-6 or IFN- γ (all 100 ng/ml, Immunotools) were added overnight or for 24h.

Mice

Il3ra knockout (*Il3r^{-/-}*) mice were generated by Cyagen BioSciences on a C57BL/6 background using the Cas9-system for depletion of the target region in exon 3 of *Il3ra* (Suppl. Fig. 4 A). C57BL/6 (*Il3r^{+/+}*) mice were used as controls. B10.129S2(B6)-*Il3<tm1Tyb>/J* (*Il3^{-/-}*), C57BL/10ScSnJ (*Il3^{+/+}*) and B6.129S7-Rag1<tm1Mom>/J (*Rag1^{-/-}*) mice were purchased from the Jackson Laboratory. All mice were housed in individually ventilated cages and used for experiments according to approval of the Government of Lower Franconia.

The genotype of *Il3r^{-/-}* mice was analyzed by PCR according to the manufacturers' protocol with the forward 5'-CTCCCAAACCTTACTTCCTGTCAC-3' and reverse primer 5'-GATCATGGGCTTTGCATAAATCTG-3' (both eurofins genomics) and KAPA2G Fast ReadyMix polymerase mix (Sigma-Aldrich) at an annealing temperature of 60°C. The targeted allele can be found at ~320 bp, while the wildtype allele shows at ~1216 bp (see Suppl. Fig. 4B).

Cell isolation from mouse tissues

LPMCs from mouse colon or small intestine were isolated using a Lamina Propria Dissociation Kit (Miltenyi Biotec) followed by density gradient centrifugation with Percoll (GE Healthcare). Lymphocytes from peripheral (pLN) and mesenteric lymph nodes (mLN), thymus and spleen were isolated by mashing the tissue through 40 μ m

cell strainers. Red blood cells from the spleen were lysed by hypotonic ammonium-chloride-potassium (ACK) lysis. Mouse blood was obtained from the buccal vein using MiniCollect LH Lithium Heparin tubes (greiner bio-one). For isolation of cells from the peritoneum, 5 ml of PBS were injected i.p. into sacrificed mice and re-drawn after five minutes of abdominal massage.

CD4⁺ and naïve CD4⁺ T cells were isolated from spleens with the CD4⁺ or naïve CD4⁺ T Cell Isolation Kits (Miltenyi Biotec), respectively. Purity of T cell isolates > 95% was confirmed by flow cytometry using the antibodies anti-CD3 (PE/Cy7, 17A2), anti-CD4 (FITC, GK15), anti-CD62L (APC, MEL-14, all Biolegend).

T cell transfer colitis model

Naïve (CD4⁺CD62L⁺) splenic T cells were isolated from splenocytes and 5×10^5 - 1×10^6 cells per mouse were injected into the peritoneum of *Rag1*^{-/-} mice. Colonoscopy was performed under anesthesia with isoflurane with a mini-endoscopy system (Karl Storz). Endoscopic signs of intestinal inflammation were scored using the MEICS score as previously described [1]. Where mentioned, the presence of reactive oxygen species was assessed with the *in vivo* imaging system (IVIS) Lumina II (Caliper Life Sciences) 10 minutes after intraperitoneal injection of L-012 (100 µl of 20 mMol solution/mouse; Fujifilm Wako Pure Chemical Corporation).

Oxazolone colitis model

Mice were sensitized on the shaved abdominal skin with 3% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, dissolved in acetone/olive oil (1:1, v:v)); Sigma-Aldrich) and challenged with an enema containing 0.3% oxazolone (dissolved in

ethanol/H₂O (1:1, v:v)) at day 3. Colonoscopy and IVIS were performed as described above and mice were sacrificed at day 5.

For *in vivo* overexpression of Il-3, a minicircle-based vector was constructed by cloning a codon-optimized full-length Il-3 cDNA construct into the SpeI and Sall sites of pMC-Hep. DNAs [2]. Vectors were propagated in E.coli K12 and plasmid DNA was isolated using Qiagen Plasmid-Maxikits followed by endotoxin removal (Miraclean endotoxin Removal Kit, MirusBio Madison). 5 µg of DNA were administrated in Krebs-Ringer solution to Bl/6 mice via hydrodynamic tail vein injection.

***In vivo* cell trafficking studies and *in vivo* microscopy**

In vivo gut homing assays were performed as previously described [3]. Briefly, *Rag1*^{-/-} mice were given 1.5-2% DSS in drinking water for 7 days to induce colitis. CD4⁺ splenocytes from the indicated donor mice were stained with CellTrace FarRed for flow cytometry or CellTrace Yellow (both Invitrogen) for *in vivo* microscopy. Up to 6*10⁶ fluorescently labelled cells were injected into the ileocolic artery of the *Rag1*^{-/-} recipient mice and left to circulate for 1h.

For *in vivo* microscopy 10 µg of anti-CD31 antibodies (AF647, MEC13.3, Biolegend) and 50 µl Hoechst 33342 (ThermoFisher Scientific) were additionally injected together with cells in total volume of 100 µl into the ileocolic artery. After 15 minutes, the proximal part of the colon was opened longitudinally and placed onto a coverslip for intravital microscopy with a confocal SP8 microscope (Leica).

LPMCs were isolated and used for flow cytometry to quantify CellTrace⁺ cells in the lamina propria.

For *in vivo* egress assays after intraperitoneal (i.p.) transfer, 5×10^6 donor thymocytes from *Il3r^{+/+}* and *Il3r^{-/-}* mice labeled with CellTrace CFSE and CellTrace FarRed (Invitrogen), respectively, were injected i.p. into *Rag1^{-/-}* mice. After 72h, peritoneal cells, peripheral blood cells and splenocytes were isolated and analyzed for the presence of CFSE⁺ and FarRed⁺ cells by flow cytometry.

Recirculation studies in T cell transfer colitis

T cell transfer colitis in *Rag1^{-/-}* was induced by injection of naïve CD4⁺T cells from *Il3r^{-/-}* and *Il3r^{+/+}* donors as described above. At day 14, mice were treated with i.v. injection of 1 µg anti- $\alpha 4\beta 7$ integrin antibody (DATK32, Bio X Cell) together with 15 µg Ozanimod (in 4% DMSO, Cayman Chemical) or 1 µg anti-rat IgG isotype together with 4% DMSO each in a total volume of 100 µl phosphate-buffered saline. After 16 hours mice were sacrificed and cells from mLN and spleen were isolated for flow cytometry. The Treg index was calculated as the frequency of CD25⁺Foxp3⁺ cells gated on CD3⁺CD4⁺ cells on treatment with DATK32 and ozanimod per on placebo treatment.

Proliferation assays

For the analysis of the proliferation of *Il3r^{-/-}* and *Il3r^{+/+}* lymphocytes, mouse thymocytes were stained with CellTrace CFSE (Invitrogen) and cultured and stimulated using the T cell activation/expansion kit, mouse (Miltenyi Biotec). Cells were analyzed by flow cytometry prior to stimulation and on days 1 to 7 of incubation.

Treg suppression and polarization assays

Suppression of *Il3r^{-/-}* and *Il3r^{+/+}* Treg on non-Treg lymphocytes was performed as described in [4]. In short, isolation of Tregs and non-Tregs was performed using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit, mouse (Miltenyi Biotec). Non-Tregs were stained with CellTrace CFSE (Invitrogen), Tregs were added in Treg:non-Treg concentrations of 1:3, 1:6, 1:12 and without Tregs, stimulated using the T cell activation/expansion kit, mouse (Miltenyi Biotec), and additionally treated with or without recombinant mouse Il-3 (100 ng/ml, Immunotools). Cells were analyzed by flow cytometry after 45h of incubation.

Treg-polarization was performed by stimulating *Il3r^{-/-}* and *Il3r^{+/+}* CD4⁺ splenocytes using the T cell activation/expansion kit, mouse (Miltenyi Biotec) with or without additional stimulation with recombinant mouse Il-2 and recombinant human TGFβ (both 5 ng/ml, both Immunotools). Cells were analyzed by flow cytometry for CD3⁺CD4⁺Foxp3⁺ cells after 42h of incubation.

Flow cytometry

For flow cytometry of mouse cells antibodies against the following antigens were used: CD3 (BV605, 17A2; APC/Cy7, 145-2C11; PacificBlue, 17A2; PE/Cy7, 17A2; PerCP/Cy5.5, 145-2C11), CD4 (APC/Cy7, GK1.5; PacificBlue, GK1.5; PE, GK1.5; BV421, GK1.5; FITC, GK1.5; AF647, GK1.5), CD11a/CD18 (APC, H155-78), CD25 (APC, PC61; PE, PC61; FITC, 3C7), CD29 (PE/Cy7, HMβ1-1), CD49d (FITC, R1-2), CD62L (APC, MEL-14), CD123 (PE, 5B11), CD127 (PE/Cy7, A7R34), CD275 (PerCP/Cy5.5, RUO), CCR1 (FITC, S15040E), CCR6 (BV421, 29-2L17), CCR7 (PE/Dazzle 594, 4B12), CCR9 (PE/Cy7, CW-1.2), Gata3 (AF488, 16E10A23), Foxp3

(PacificBlue, MF-14, all Biolegend), CD4 (VioGreen, GK1.5), CD45R (VioBlue, RA3-6B2), CCR5 (APC/Vio770, REA354), CCR7 (PE, 4B12), CXCR5 (APC, REA215), Rorgt (APC, REA278, all Miltenyi Biotec), Eomes (PerCP/eFl1710, Dan11mag), Foxp3 (APC, FJK-16s) and Tbet (PE, eBio4B10, all eBioScience). The antibody against the active conformation of CD49d (SG31) was a kind gift of John F. Kearny (University of Alabama, Birmingham, AL, USA) and labelled with the Alexa Fluor 647 antibody labelling kit (Invitrogen).

Human cells were fluorescently labelled with antibodies against CD4 (APC/Cy7, A161A1), CD25 (FITC, M-A251), CD123 (BV421, 6H6, all Biolegend) and Foxp3 (PE, 236/E7, eBioScience).

Viability was assessed with Fixable Viability Dye eFluor 506 or 780 (eBioScience). Fixation and permeabilization for intracellular staining were performed using the Foxp3/Transcription Factor Fixation/Permeabilization Set (eBioScience).

Isotype or fluorescence minus one (FMO) stainings were used as controls.

Measurements were performed on LSR Fortessa (BD Bioscience), MACSQuant 10 and MACSQuant 16 (Miltenyi Biotec) flow cytometers. Data were analyzed with FlowJo software v10.8.1 (Tree Star).

Lightsheet microscopy

For lightsheet microscopy, *Rag1*^{-/-} mice with colitis after transfer of naïve CD4⁺ *Il3r*^{-/-} or *Il3r*^{+/+} lymphocytes were perfused with wheat germ agglutinin CF770 (biotium) to stain blood vessels. After scarification, mesenteric lymph nodes (mLNs) were obtained and fixed overnight in 1:4 diluted Cytofix Fixation Buffer (BD BioScience) at 4 °C. MLNs were then stained with antibodies against Lyve-1 (eFluor570, eBioScience), CD4

(AF647, GK1.5, Biolegend) and CD25 (FITC, PC61, Biolegend) overnight at room temperature. After dehydration in ascending ethanol dilutions of 50, 70 and 100 % (twice) ethanol for 2 h each at 4 °C, mLNs were cleared using ethyl cinnamate (Sigma-Aldrich) at room temperature. Imaging was performed on an UltraMicroscope II (LaVision, Biotec). 3D reconstruction was performed using Imaris Image Analysis software v9.9 (Bitplane).

Dynamic adhesion assays

Dynamic adhesion assays were performed as previously described [5]. Briefly, cells were stained with CellTrace CFSE (invitrogen) and then perfused through miniature glass capillaries (Vitrocom) using a peristaltic pump (Schenchen). Capillaries were previously coated with VCAM-1 (rh (5 µg/ml) from Biolegend, rm (10 µg/ml) from R&D systems) or MAdCAM-1 (rh (5 µg/ml) and rm (10 µg/ml) from R&D systems) or buffer (150 mM NaCl, 20 mM HEPES) only (control) and unspecific binding sites were blocked with 10% fetal bovine serum (FBS). After a perfusion period of 3 minutes, capillaries were washed with adhesion buffer (pH 7.4; 150 mM NaCl, 10 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂) and the number of bound cells was analyzed using an inverted microscope (DMI8, Leica). The sum of eight pictures was taken for quantitative analysis. Additionally, up to seven pictures of these pictures were overlaid using Fiji software v1.52n (NIH) for visualization.

Transmigration assays

Transmigration assays with human regulatory T cells were performed as previously described [5]. Cells were incubated with or without 100 ng/ml rh IL-3 (ImmunoTools)

for 30 minutes at 37°C. After incubation, cells were resuspended in X-Vivo medium (Lonza) and 2×10^5 cells loaded into the upper wells of a 3 μ m transmembrane plate (Corning). The lower well contained each X-Vivo medium only, 100 ng/ml rh CCL19 or rh CCL21 (ImmunoTools). Plates were incubated for 4h at 37°C and the number of transmigrated cells in the lower wells was analyzed by flow cytometry.

Mouse transmigration assays were performed with isolated thymocytes. 2×10^5 cells were loaded into the upper wells of 3 μ m transmembrane plates. Lower wells contained X-Vivo medium with or without 10 ng/ml rm Ccl19 (ImmunoTools). Plates were incubated for 4h at 37°C and the number of transmigrated cells in the lower wells was analyzed using flow cytometry. The transmigration index was calculated as the difference of migrated cells towards rm CCL19 to migrated cells towards control per transmigrated cells towards control.

RNA-Isolation, cDNA Transcription and quantitative polymerase chain reaction (qPCR) analysis

RNA-isolation from tissues and cells was performed using the NucleoSpin RNA Kit for RNA isolation (Machery Nagel). RNA was transcribed into complementary DNA (cDNA) following the protocol of the AffinityScript Kit (Agilent). QPCR analysis was performed using SybrSelect MasterMix (Thermo Fisher Scientific) and Quantitect Primer Assay for human *IL3*, *IL10*, *IL13*, *IL18* and *TGFB* and mouse *Il3* (Qiagen). Mouse *Il3r* was analysed using the following two primer pairs with SybrSelect MasterMix (Thermo Fisher Scientific): (1) forward 5'-TCCTGGCATCCCACTCTTCA-3' and reverse 5'-CTGTTGTCAAGGGAGGTGG-3' and (2) forward 5'-TCAGATCTGGCTGCGGTCCG-3' and reverse 5'-CGGGGTCAGCCCAGACAAAG-3' (all euofins genomics). Mouse and human *HPRT* Quantitect Primer Assays (Qiagen)

were used for housekeeping controls. Plates were measured using the real-time PCR system CFX Connect (BioRad).

ELISA assays

For ELISA analysis of mouse samples, blood serum was collected or colon LPMCs and splenocytes were incubated with the T cell activation/expansion kit, mouse (Miltenyi Biotec) for 48h and supernatants were obtained for analysis. IL-3 secretion was quantified using the IL-3 Mouse ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

The ELISA analysis of human samples, blood serum was obtained from patients with IBD and healthy controls. Dilution and quantification of IL-3 was performed according to the manufacturer's instructions using the human IL-3 ELISA Kit (abcam).

Rac1-Activation and G-Actin/F-Actin Assay

Protein from mouse thymocytes and stimulated human T cells treated with or without IL-3 was isolated and processed according to the instructions of the Active Rac1 Pull-Down and Detection Kit (Thermo Fisher Scientific) and G-Actin/F-Actin In Vivo Assay Biochem Kit (Cytoskeleton Inc), respectively. Samples were run on 4-20% gradient Mini-Protean TGX stain-free gels (BioRad) and WesternBlot was analyzed using an Amersham Imager R (Thermo Fisher Scientific). Quantification of Rac1 activity was calculated as quotient of Rac1 band intensity through GAPDH control band intensity, normalized to the mean of *Il3*^{+/+} values.

Immunofluorescence staining of mouse and human cryosections and cells

Cryosections of human biopsies from IBD patients and non-IBD controls were fixed with 4 % PFA. Peroxidases were blocked with 30 % H₂O₂ in methanol/H₂O. Sections were permeabilised with 0.1 % Triton-X in PBS and blocked using the Avidin/Biotin Blocking Kit (Vector Laboratories), ROTI ImmunoBlock (Carl Roth) and 20% goat serum (PAN-Biotech). Primary antibodies targeting CD4 (1:100, RPA-T4, Biolegend or 1:200, 13B8.2, Novus Biologicals), CD14 (1:50, 61D3, eBioScience), CD19 (1:50, HIB19, BD Bioscience), Foxp3 (1:100, 236A/E7, eBioScience) and IL-3 (1:50, OTI3B11, Novus Biologicals) were incubated overnight at 4°C or for 2 h at room temperature. Goat anti-mouse AF488 (1:200, abcam), goat anti-rabbit AF488 (1:200, polyclonal, eBioScience) and goat anti-mouse Cy3 (1:200, polyclonal, abcam) were incubated for 1 h at room temperature as secondary antibody and the TSA Cy3 Kit (PerkinElmer) were used for detection according to the manufacturers' protocol. Sections treated with secondary antibodies alone served as control. Nuclei were counterstained with Hoechst 33342 (ThermoFisher Scientific) and slides were embedded with Vectashield Mounting Medium (Vector Laboratories).

Mouse cryosections were handled accordingly using primary antibodies targeting CD4 (1:100, RM4-5, Biolegend), Foxp3 (NRRF-30, 1:100, Invitrogen), MPO (1:200, abcam) and EpCAM (1:1000, EPR20533-266, abcam). Goat anti-rat AF647 (1:200, abcam), goat anti-rabbit Cy3 (1:200, Millipore), goat anti-rat Biotin (1:200, Biolegend) with Streptavidin Cy3 (1:200, Biolegend) or TSA Cy3 Kit (PerkinElmer) and donkey anti-rabbit Biotin (1:200, Jackson) with Streptavidin Cy3 (1:200, Biolegend) served as for secondary detection. TUNEL-staining was performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Merck). Microscopy was performed using a fluorescence microscope (DM600B, Leica) and a confocal microscope (SP8, Leica).

Blinded observers quantified the number of cells expressing the respective markers using Fiji software v1.52n (NIH).

Fluorescence staining of human CD4⁺ PBMCs was performed in cell suspension, but otherwise followed the same protocol. Staining with phalloidin reagent (1:1000, iFluor488, abcam) was performed for 40 min at room temperature. After staining of the nuclei with Hoechst 33342 (ThermoFisher Scientific), cells were embedded in Mowiol (Sigma Aldrich) and imaged using a confocal microscope (SP8, Leica). Data analysis and quantification was performed using Fiji software v1.52n (NIH). To normalize phalloidin signal to the amount of cells per sample, the mean of the quotient of the raw integrated density (rawintden) of phalloidin signal per picture through the Hoechst signal of 5 pictures per sample was calculated.

Mouse thymocytes cells were blocked with 10% donkey serum (RhoA, Cdc42) or 10% horse serum (Rac1) instead of goat serum. Cells were incubated with primary antibodies against RhoA (1:500, 67B9, Cell Signaling), Rac1 (1:500, C7H2, Cytoskeleton) or Cdc42 (1:200, 11A1, Cell Signaling) or associated isotype controls overnight at 4°C. As secondary antibody anti-rabbit biotin (1:200) or anti-mouse biotin (1:200) with the TSA Cy3 Kit (PerkinElmer) were used. After nuclei staining with Hoechst 33342 (ThermoFisher Scientific), cells were embedded in Mowiol (Sigma Aldrich) and imaged using a confocal microscope (SP8, Leica). Data analysis and quantification was performed using Fiji software v1.52n (NIH). A fixed binary threshold in the Hoechst channel was used to determine the area covered by cells in the pictures. Subsequently, the integrated density of Cy3 signal was normalized to this cell area.

H/E staining of mouse colon paraffin-embedded sections

Mouse colon tissue was fixed in ROTI Histofix (4% formaldehyde, Carl Roth) overnight, dehydrated and embedded in paraffin. Sections of the paraffin-embedded colon tissue were stained with hematoxylin and eosin (H&E). Following bright-field microscopy (DM6000B, Leica) inflammation was assessed with the scoring system described by Erben et al. by a blinded observer [6].

Real-time deformability cytometry (RT-DC) analysis

RT-DC measurements were performed using an AcCellerator instrument (Zellmechanik Dresden) as described previously [7]. Just before the measurement, cells were resuspended in CellCarrier B (Zellmechanik Dresden) and loaded into a 1 mL syringe attached to a neMESYS syringe pump (Cetoni). The syringe was connected to a microfluidic chip made of polydimethylsiloxane (PDMS) bonded on cover glass (Zellmechanik Dresden). A second syringe with sheath fluid (pure CellCarrier B) was connected to the chip. PEEK-tubing (IDEX Health & Science) was used to connect the syringes to the chip. Chips with a channel constriction of 20 × 20 μm square cross section were used for human cells; chips with a 15 × 15 μm square cross section were used for mouse cells. For human samples, the total flow rate was 0.06 μL/s, consisting of sheath flow rate 0.045 μL/s and sample flow rate 0.015 μL/s. For murine samples, the total flow rate was 0.024 μL/s, consisting of sheath flow rate 0.018 μL/s and sample flow rate 0.006 μL/s. We applied a gate for minimal cross-sectional area (15 μm²) and for the area ratio (1.00-1.08). Measurement temperature was 25°C. Images were acquired at a frame rate of 1600 fps with an included inverted Zeiss microscope (AxioObserver). Cells were detected in a region of interest of 250 × 80 pixels, and morphological and mechanical parameters were acquired in real time

using Shapeln software 2.2.2.4 (Zellmechanik Dresden). If a chip was clogged during measurement, the measurement was stopped, a new chip was used in a second measurement and the measurements were joined using DC-Kit software 0.12.6 (Zellmechanik Dresden).

To determine the deformability of CD3+CD4+CD25+ T cells from the lamina propria, fluorescence after staining with anti-CD3 (FITC, 17A2, Biolegend), anti-CD4 (AF647, GK1.5, Biolegend) and anti-CD25 (PE, PC61, Biolegend) antibodies was measured with FluorescenceModule (Zellmechanik Dresden) during RT-DC measurements using Shapeln software 2.2.2.4 (Zellmechanik Dresden). For fluorescence analysis axes were set to logarithm, x-axis was always set to maximum [a.u.], y-axis was set to width [μs]. The positive population was then taken with a polygon filter.

Cell images were analysed using ShapeOut 2 software 2.10.0 (Zellmechanik Dresden). Following box filters were used: Area: 25-100 μm^2 (human) or 20-45 μm^2 (murine); aspect ratio of bounding box: 0.5-2.0; deformation: 0.0054-0.08361 (only human); inertia ratio of convex contour: 1.00-76.23, inertia ratio of raw contour: 1.0-119.2; porosity: 1.0-1.050. The calculation of deformation, a measure of how much the cell shape deviates from circularity, was obtained from the image using the projected area (A) and cell contour length calculated from the convex hull (l): $Deformation = (1 - 2\sqrt{(\pi A)}) / l$. The Young's modulus, a measure of cell stiffness (inverse deformability) was calculated using a look-up table derived from simulations based on the on the numerical [8] and analytical [9] models.

Atomic force microscope (AFM) analysis of cell deformability

Nanoindentation measurements were carried out using an AFM Nanowizard 4 (Bruker JPK) mounted on an optical inverted microscope (Axio Zoom, Carl Zeiss). AFM-based

measurements were performed on non-adherent cells using a wedged cantilever to correct for the 10° tilt [10]. Wedged cantilevers were prepared using UV curing glue that was applied to an Arrow™ TL1 tip less cantilever (Nanoworld) with a nominal spring constant of 0.08 N/m. A 20X objective allowed the precise positioning of the cantilever on top of a single spherical cell. All mechanical measurements were carried out at 37°C in CO₂-independent medium (Thermo Fisher Scientific) supplemented with 1% GlutaMax (Thermo Fisher Scientific). Indentation measurements were performed by compressing the non-adherent cells between the wedged cantilever and the plastic bottom petri-dish as illustrated in *Fig. 6D*. Forward and retraction velocities were set to 5 µm/s. Force-distance curves were recorded on cells at 2 nN loading force and were analyzed using JPK data processing software (Bruker JPK). The Young's modulus (E) of non-adherent cells was determined using the Hertz model corrected for spherical indenters (Sneddon) using the following mathematical equation: $F = \frac{E}{1-\nu^2} \times 2 \left(\frac{r^2 + R^2}{2} \ln \frac{R+r}{R-r} - Rr \right)$ and $\delta = \frac{r}{2} \ln \left(\frac{R+r}{R-r} \right)$ where F is the indentation force, ν is the assumed Poisson's ratio of the sample ($\nu = 0.5$, Sneddon, 1965), δ is the indentation depth, r is the cell average radius (~ 1.5 µm for mouse and ~ 2.5 µm for human) and $R = \sqrt{\delta \cdot r}$ is the radius of the circular contact area. The E values were obtained fitting the curves up to an indentation depth of 500 nm, and the fit was then corrected for the stress on the cells coming from the dish bottom causing additional deformation of the spherical shape [11,12].

Fluorescence recovery after photobleaching (FRAP)

Il3r^{-/-} and *Il3r^{+/+}* thymocytes were seeded in a 6 well plate and treated with BacMam2.0 (60 µl per 10⁶ cells, Thermo Fisher) for at least two days. 8 well glass bottom plates (Ibidi) were coated with CellTak (Thermo Fisher Scientific) and BacMam-treated cells

were placed inside the wells. After 10 minutes at room temperature, cells were immobilized on the CellTak treated surface. FRAP experiments were performed using a Zeiss LSM880 microscope. Actin monomers were bleached with a 405nm laser. The set up for the FRAP measurements was following the protocol of Fritzsche et al. [13]. Analysis of FRAP data was performed using a homebuilt Python3 script and using the suggested model of Fritzsche et al. [13].

Scanning electron microscopy (SEM)

SEM imaging of the actin network in *I13r^{-/-}* and *I13r^{+/+}* thymocytes was performed according to the protocol described by Schu et al. [14] with minor modifications. Shortly, cells were immobilized on CellTak (Thermo Fisher Scientific)-treated cover glass. The cell membrane was extracted using 0.5 % Triton X-100 (Merck) in buffer M supplemented with 10 μ M Phalloidin (Merck). The cells were incubated with membrane extraction solution between 30 sec and 5 min to obtain optimal membrane extraction. Afterwards, cells were rinsed with buffer M three times before fixing them in 2 % Glutaraldehyd (Merck) and 2 % PFA (Science Service) diluted in 0.2 M sodium cacodylat buffer for 1 hour. Cells were dehydrated according to Schu et al. [14] and sputter coated with 4 nm platinum. All SEM images were obtained using a SEM Quanta 400 FEG (FEI) with vacuum mode activated. For mesh hole size analysis the homebuilt software FiNTA was used [15].

Microarray

The raw data of the open available dataset GSE97012 was downloaded from the Gene Expression Omnibus [16]. All microarray duplicates with the same probe name,

accession number and gene name were aggregated and averaged. Quantile normalization was conducted and subsequently, all probes with intensity values lower than the 5th quantile were removed. Differential expression statistics were calculated with Kolmogorov-Smirnov test and adjusted for multiple testing with the Holm-Šídák method using the Python packages Scipy v1.6.2 [17] and Statsmodels v0.12.2 [18], respectively.

RNA-sequencing of mouse thymocytes

RNA from unchallenged mouse *I13r^{-/-}* and *I13r^{+/+}* thymocytes was isolated with the NucleoSpin RNA Kit for RNA isolation (Machery Nagel). RNA-Analysis and sequencing was performed by the Core unit next generation sequencing of the University Hospital Erlangen. The quality of isolated RNA was determined using an Agilent 2100 bioanalyzer with RNA 6000 Nano kit and related software (Agilent). Sequencing libraries were generated from RNA samples using the TruSeq Stranded mRNA Kit (Illumina). Libraries were sequenced on a HiSeq 2500 platform (Illumina). The reads were aligned with Star v2.5.4b [19] against the GRCm38 reference genome. Reads were counted with featureCounts of the Rsubread package v2.8.1 [20], using the GRCm38 version 96 GTF file. Differential expression analysis was conducted using DeSeq2 v1.34.0 [21].

Significantly differentially expressed genes were uploaded onto the DAVID Bioinformatics database (2021 update) [22]. Functional annotation and pathway enrichment analysis was performed with classification stringency at lowest. In Annotation Cluster 2, 90 genes were grouped under the keyword 'cytoskeleton'. For heatmap depiction, the expression values of the 90 cytoskeleton-associated genes

identified in the DAVID analysis were normalized to the mean of the expression values of *Il3r^{+/+}* controls.

Kinome activation analysis

Kinome activation profiling microarray of thymocytes from *Il3r^{-/-}* and *Il3r^{+/+}* mice ($n = 6$ per group) was performed by Pamgene International BV, 's-Hertogenbosch, Netherlands, as previously described [23]. Briefly, 1×10^7 freshly isolated thymocytes per mouse were washed, spun down and snaped-frozen in liquid nitrogen prior to shipment as detailed in Pamgene protocol 1161.

Protein isolation and serine/threonine (STK) and protein-tyrosine (PTK) kinases activity profiling assays based on measuring peptide phosphorylation by protein kinases were performed by the service provider (PamGene International BV). Briefly, protein of frozen cell pellets was isolated using M-PER mammalian protein extraction reagent supplemented with PhosphoSTOP (Roche) and cComplete Tablets (Roche). 1 μ g protein per sample was loaded on PamChip®4 arrays containing 144 (STK) or 196 (PTK) peptide sequences (13 amino acids long and correlating with one or multiple upstream kinases) immobilized on a porous aluminum oxide membrane. Fluorescently labelled anti-phospho antibodies were used to detect phosphorylation activity of kinases present in the sample, imaged and controlled by the EVOLVE v2.0 software and quantified using BioNavigator v6.3 (BN6; PamGene International BV). Log₂-transformed integration (linear regression, S100) of signal intensities at multiple exposure times was normalized using a Combat correction model for batch correction with scaling parameters (mean/sd) estimated using an empirical Bayes approach (effective noise reduction associated with applying the correction) [24,25]. Unpaired t-

tests or the upstream kinase analysis (UKA) tool (BN6; PamGene international BV) were used to compare the normalized values.

Significantly differentially activated kinases lists of STK and PTK were uploaded to DAVID Bioinformatics database (2021 update) [22]. Functional annotation clustering was performed with standard settings. In these clusters, keywords related to cytoskeleton were identified and kinases belonging to these keywords were extracted (11 of 74 for STK and 27 of 75 for PTK). Coral Kinome Tree plot was prepared combining STK and PTK data and plotted using CORAL software as described by Metz et al. [26]. For volcano plots, mean specificity scores and mean kinase statistics of all kinases were plotted and the 11 (STK) or 27 (PTK) significant differentially activated kinases from DAVID analysis were highlighted in red. For heatmap plots, mean kinase statistic of these kinases was plotted.

Graphs and statistics

Graphs were plotted and statistical analyses were performed using Prism v8.3.0 software (GraphPad). Outliers identified by the outliers plugin (rout Q=1%) were removed. Following normality analysis ($\alpha=0.05$), according to data distribution and data pairing, significance tests and correlations were performed as mentioned in the figure legends. $P < 0.05$ was considered statistically significant in all tests with asterisks indicating the following levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The effect size (Cohen's d for Mann-Whitney and Wilcoxon tests, Cohen's f for one- or 2-way ANOVA) for RT-DC measurements was calculated using G*Power v3.1.9.7 software [27] with $\alpha = 0.05$ and a power ($1-\beta$) of 0.95.

Patient and public involvement

This study was driven by the patients' need for optimized therapies for IBD based on further insights into the IBD pathogenesis, but patients or the public were not involved in the design and conduct of the study.

Data availability

RNAseq data will be deposited on a publicly available database. All other data that support the findings of this study are available from the corresponding author upon reasonable request.

References

- 1 Wirtz S, Popp V, Kindermann M, *et al.* Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc* 2017;**12**:1295–309. doi:10.1038/nprot.2017.044
- 2 McHedlidze T, Waldner M, Zopf S, *et al.* Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity* 2013;**39**:357–71. doi:10.1016/j.immuni.2013.07.018
- 3 Zundler S, Schillinger D, Fischer A, *et al.* Blockade of α E β 7 integrin suppresses accumulation of CD8+ and Th9 lymphocytes from patients with IBD in the inflamed gut in vivo. *Gut* 2017;**66**:1936–48. doi:10.1136/gutjnl-2016-312439
- 4 Collison LW, Vignali DAA. In vitro Treg suppression assays. *Methods Mol Biol* 2011;**707**:21–37. doi:10.1007/978-1-61737-979-6_2
- 5 Becker E, Dedden M, Gall C, *et al.* Residual homing of α 4 β 7-expressing β 1+PI16+ regulatory T cells with potent suppressive activity correlates with exposure-efficacy of vedolizumab. *Gut* 2021;;gutjnl-2021-324868. doi:10.1136/gutjnl-2021-324868
- 6 Erben U, Loddenkemper C, Doerfel K, *et al.* A guide to histomorphological evaluation of intestinal inflammation in mouse models. *Int J Clin Exp Pathol* 2014;**7**:4557–76.
- 7 Toepfner N, Herold C, Otto O, *et al.* Detection of human disease conditions by single-cell morpho-rheological phenotyping of blood. *Elife* 2018;**7**. doi:10.7554/eLife.29213
- 8 Mokbel M, Mokbel D, Mietke A, *et al.* Numerical Simulation of Real-Time Deformability Cytometry To Extract Cell Mechanical Properties. *ACS Biomater Sci Eng* 2017;**3**:2962–73. doi:10.1021/acsbomaterials.6b00558
- 9 Mietke A, Otto O, Girardo S, *et al.* Extracting Cell Stiffness from Real-Time Deformability Cytometry: Theory and Experiment. *Biophys J* 2015;**109**:2023–36. doi:10.1016/j.bpj.2015.09.006
- 10 Stewart MP, Hodel AW, Spielhofer A, *et al.* Wedged AFM-cantilevers for parallel plate cell mechanics. *Methods* 2013;**60**:186–94. doi:10.1016/j.ymeth.2013.02.015
- 11 Dokukin ME, Guz NV, Sokolov I. Quantitative Study of the Elastic Modulus of Loosely Attached Cells in AFM Indentation Experiments. *Biophys J* 2013;**104**:2123–31. doi:10.1016/j.bpj.2013.04.019
- 12 Glaubitz M, Medvedev N, Pussak D, *et al.* A novel contact model for AFM indentation experiments on soft spherical cell-like particles. *Soft Matter* 2014;**10**:6732–41. doi:10.1039/C4SM00788C

- 13 Fritzsche M, Charras G. Dissecting protein reaction dynamics in living cells by fluorescence recovery after photobleaching. *Nat Protoc* 2015;**10**:660–80. doi:10.1038/nprot.2015.042
- 14 Schu M, Terriac E, Koch M, *et al.* Scanning electron microscopy preparation of the cellular actin cortex: A quantitative comparison between critical point drying and hexamethyldisilazane drying. *PLOS ONE* 2021;**16**:e0254165. doi:10.1371/journal.pone.0254165
- 15 Flormann DAD, Schu M, Terriac E, *et al.* A novel universal algorithm for filament network tracing and cytoskeleton analysis. *The FASEB Journal* 2021;**35**:e21582. doi:10.1096/fj.202100048R
- 16 Tang MS, Bowcutt R, Leung JM, *et al.* Integrated Analysis of Biopsies from Inflammatory Bowel Disease Patients Identifies SAA1 as a Link Between Mucosal Microbes with TH17 and TH22 Cells. *Inflamm Bowel Dis* 2017;**23**:1544–54. doi:10.1097/MIB.0000000000001208
- 17 Virtanen P, Gommers R, Oliphant TE, *et al.* SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat Methods* 2020;**17**:261–72. doi:10.1038/s41592-019-0686-2
- 18 Seabold S, Perktold J. Statsmodels: Econometric and Statistical Modeling with Python. *Proceedings of the 9th Python in Science Conference* 2010;:92–6. doi:10.25080/Majora-92bf1922-011
- 19 Dobin A, Davis CA, Schlesinger F, *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013;**29**:15–21. doi:10.1093/bioinformatics/bts635
- 20 Liao Y, Smyth GK, Shi W. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Research* 2019;**47**:e47. doi:10.1093/nar/gkz114
- 21 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 2014;**15**:550. doi:10.1186/s13059-014-0550-8
- 22 Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009;**4**:44–57. doi:10.1038/nprot.2008.211
- 23 Müller K, Honcharova-Biletska H, Koppe C, *et al.* JNK signaling prevents biliary cyst formation through a CASPASE-8-dependent function of RIPK1 during aging. *Proc Natl Acad Sci U S A* 2021;**118**:e2007194118. doi:10.1073/pnas.2007194118
- 24 Chen C, Grennan K, Badner J, *et al.* Removing batch effects in analysis of expression microarray data: an evaluation of six batch adjustment methods. *PLoS One* 2011;**6**:e17238. doi:10.1371/journal.pone.0017238
- 25 Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 2007;**8**:118–27. doi:10.1093/biostatistics/kxj037

- 26 Metz KS, Deoudes EM, Berginski ME, *et al.* Coral: Clear and Customizable Visualization of Human Kinome Data. *Cell Syst* 2018;**7**:347-350.e1. doi:10.1016/j.cels.2018.07.001
- 27 Faul F, Erdfelder E, Buchner A, *et al.* Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. *Behavior Research Methods* 2009;**41**:1149–60. doi:10.3758/BRM.41.4.1149