Original research

Neutrophils prevent rectal bleeding in ulcerative colitis by peptidyl-arginine deiminase-4-dependent immunothrombosis

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ABSTRACT

Objective Bleeding ulcers and erosions are hallmarks of active ulcerative colitis (UC). However, the mechanisms controlling bleeding and mucosal haemostasis remain elusive.

Design We used high-resolution endoscopy and colon tissue samples of active UC (n = 36) as well as experimental models of physical and chemical mucosal damage in mice deficient for peptidyl-arginine deiminase-4 (PAD4), gnotobiotic mice and controls. We employed endoscopy, histochemistry, live-cell microscopy and flow cytometry to study eroded mucosal surfaces during mucosal haemostasis.

Results Erosions and ulcerations in UC were covered by fresh blood, haematin or fibrin visible by endoscopy. Fibrin layers rather than fresh blood or haematin on erosions were inversely correlated with rectal bleeding in UC. Fibrin layers contained ample amounts of neutrophils coaggregated with neutrophil extracellular traps (NETs) with detectable activity of PAD. Transcriptome analyses showed significantly elevated PAD4 expression in active UC. In experimentally inflicted wounds, we found that neutrophils underwent NET formation in a PAD4-dependent manner hours after formation of primary blood clots, and remodelled clots to immunothrombi containing citrullinated histones, even in the absence of microbiota. PAD4-deficient mice experienced an exacerbated course of dextrane sodium sulfate-induced colitis with markedly increased rectal bleeding (96% vs 10%) as compared with controls. PAD4-deficient mice failed to remodel blood clots on mucosal wounds eliciting impaired healing. Thus, NET-associated immunothrombi are protective in acute colitis, while insufficient immunothrombosis is associated with rectal bleeding.

Conclusion Our findings uncover that neutrophils induce secondary immunothrombosis by PAD4-dependent mechanisms. Insufficient immunothrombosis may favour rectal bleeding in UC.

INTRODUCTION

Ever since the initial therapeutic studies of Truelove and Witts, rectal bleeding has been considered as an important component of the clinical features of patients suffering from severe ulcerative colitis (UC). Increased rectal bleeding may require hospitalisation and rarely emergency surgical interventions. In UC, the epithelial lining is breached and emergency barriers are immediately required before epithelial restitution can be achieved. Emergency barriers need to provide provisional control of microbial invasion, avoid loss of blood and mucosal tissue fluids and support a timely restitution of mucosal epithelial integrity. Colitis can

Summary box

What is already known about the subject?

⇒ Rectal bleeding is a significant and worrisome feature of active ulcerative colitis (UC) and is present in many cases. Here we study the role of neutrophil-mediated immunothrombosis and extracellular trap formation in damaged intestinal mucosae and describe their beneficial haemostatic function during flares of UC.

What are the new findings?

⇒ Activated neutrophils migrate to sites denuded of intestinal epithelium, aggregate and extrude decondensed chromatin as neutrophil extracellular traps (NETs) in a peptidyl-arginine deiminase-4 (PAD4)-dependent manner.

⇒ PAD4-dependent NET formation is closely linked to the remodelling of blood clots to secondary immunothrombi. PAD4-dependent immunothrombosis achieves successful mucosal haemostasis and limits rectal bleeding in UC and in experimental models of disease.

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

⇒ The concept of a beneficial role of immunothrombosis and extracellular trap formation at mucosal surfaces challenges the prevailing view of harmful NETs. Therapeutic approaches in active UC need to carefully balance rather than abrogate the neutrophil response.
be caused by various infectious microorganisms. Additionally, non-infectious causes of mucosal damage exist, ranging from drug-induced to diet-induced mucosal stress. Failure to clear instigating factors or repeated challenges in a structurally vulnerable microenvironment favour sustained chronic inflammation in UC and the development of flares of acute inflammation. Flares often feature rectal bleeding and the presence of large numbers of polymorphonuclear granulocytes.

Neutrophil granulocytes may trespass epithelial layers, phagocyte and serve as a first line of defense. Neutrophil granulocytes may further extrude decondensed chromatin decorated with granular and nuclear constituents termed neutrophil extracellular traps (NETs). Peptidyl arginine deiminase-4 (PAD4) contributes to chromatin decondensation in the course of NET formation in response to select triggers. NETs tend to aggregate, contribute to host defense and are increasingly appreciated as a prothrombotic element. We observed that granulocytes and NETs represent a dominant element of the mucosal surface in UC, especially in areas of erosions and ulcerations. In experimental models, we noticed NET formation in direct proximity of blood clots and hypothesised a role of neutrophils and NETs in the remodelling of blood clots, mucosal haemostasis and control of rectal bleeding. In this study, we, thus, closely characterised surface remodelling and NET formation on mucosal erosions in both UC and experimental models, determined its functional role in mucosal wound healing and acute colitis and its dependence on PAD4.

** MATERIALS AND METHODS **

**Patient and public involvement**

Patients suffering from acute UC (n = 36) were recruited from the academic IBD centre of the University Hospital Erlangen, Germany. Partial Mayo scoring of rectal bleeding was performed on a clinical visit. Routine colonoscopy was performed in accordance to standard clinical practice and colon tissue biopsies were collected after informed consent in agreement to the approval granted by the Ethics Committee of the Friedrich-Alexander-University Erlangen-Nürnberg. Further clinical information on localisation and extent of disease, severity, age, sex and past or current therapies are included in the online supplemental table S1 and S2. Patients or the public were not involved in the design of this study. No additional burden was inflicted to study patients as interventions were planned for diagnostic and therapeutic reasons.

**Endoscopic grading**

A total of at least five images per patient derived from different areas of the colon were assessed by an experienced endoscopist in a blinded fashion for a graded assessment of the frequency of mucosal erosions and ulcerations inspired by the Blackstone score: 0: no visible erosions, 1: less than 10 erosions (< 5 mm in size) per 10 cm section, 2: more than 10 erosions (< 5 mm in size) per 10 cm section to 3: more than 10 erosions (< 5 mm in size) and ulcerations (> 5 mm in size) per 10 cm section.

**Human tissue samples**

Colonoscopy was performed according to clinical guidelines after informed consent of the patient. Paraffin-embedded tissues (10 colon ulcers (including UC and diverticulitis) and 22 samples of eroded surface in active UC) were subjected to staining by immunofluorescence. All samples were derived from routine clinical practice after informed consent in Mainz, Bayreuth and Erlangen and a positive ethical review of the local authorities.

**Transcriptomic analyses**

The Predicting Response to Standardized Pediatric Colitis Therapy (PROTECT) study was a multicentre inception cohort study based at 29 centres in the USA and Canada providing RNAseq analyses of 206 patients with UC. Additionally, RNAseq analyses of an independent cohort of the RISK study encompassing 55 non-IBD controls, 43 patients with UC and 92 patients with Crohn’s disease (CD) was analysed based on publicly available datasets (PROTECT (GSE109142), RISK (GSE117993)). Additionally, RNA sequencing was performed in-house on murine colon tissue wounds as described before.

In short, a total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer’s recommendations and index codes were added to attribute sequences to each sample. polymerase chain reaction (PCR) was performed with Phusion High-Fidelity DNA polymerase (New England Biolabs), Universal PCR primers and Index [X] Primer. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated followed by data analyses as described.

The newly developed dataset is publicly available at the European Bioinformatics Institute ArrayExpress website (https://www.ebi.ac.uk/arrayexpress) under the accession number (E-MTAB-10824).

Genes related to each topic (clot remodelling, neutrophils, myeloid cells, lymphocytes, fibroblasts) were individually selected inspired by published studies and gene ontology terms. For cell-related genes, specificity of expression was analysed using data assembled by the ImmGen consortium.

**Mice**

PAD4−/− mice were kindly provided by K Mowen, Scripps Institute, La Jolla, California, USA and have been described previously. All mice used were on the C57BL/6 background. For each individual experiment, age-matched and sex-matched mice were used. Mice aged 6−14 weeks were used for experimental procedures. In experiments comparing PAD4-deficient mice to wild-type, PAD4-proficient littermates were used as controls. All mice were kept under specific pathogen-free (SPF) conditions at the animal facility of the University of Erlangen. C57BL/6 mice raised under gnotobiotic conditions were provided by A Bleich and kept in isolators for the course of the experiment performed under sterile conditions with control C57BL/6 mice kept under SPF conditions in separate cages. Experimental procedures were
Experimental models of disease
Acute colitis was induced by the administration of 3–4 % dextran sodium sulfate (DSS, 36-50 kD) (MP Biotech, Santa Ana, California, USA) to the drinking water of mice. Weight and clinical features were documented throughout the experiment. If scheduled by the experiment, deoxyribonuclease I (DNase I) (5 U/g body weight) (Sigma-Aldrich) was injected intravenously each day. Endoscopic analysis of the colonic mucosa was performed on day 7 of DSS administration. Mice were euthanised if a weight loss of 20 % occurred during the course of the disease or sacrificed at day 9 for further examinations. COLOVIEW high-resolution mouse video endoscopic system (Karl Storz, Tuttlingen, Germany) was used for mouse colonoscopy. Colonic wounds were inflicted using an endoscopic forceps during mouse colonoscopy (size: 3 Fr.) as described previously. Colonuscopy was performed on a daily basis in order to kinetically evaluate the development of the wound area. Wound area calculation was performed on screen shots as described. 25 Mice were sacrificed at the indicated time points and colon tissue was dissected and wounds were recovered using a punch biopsy. Samples were subjected to further analysis using RNA and protein isolation techniques as well as analyses by histochemistry.

Cell isolation procedures
Murine lamina propria mononuclear cells were isolated as previously described. In brief, colonic tissue was mechanically dissected, and intestinal epithelial cells were removed by incubation in ethylenediaminetetraacetic acid (EDTA). Remaining tissue was digested in collagenase D (Roche Diagnostics, Mannheim, Germany), DNase I (Sigma-Aldrich, Munich, Germany), and dispase II (Roche Diagnostics). Digested tissue was passed through a 100 µm cell strainer, and the remaining cellular content was prepared for flow cytometry using fluorescently labelled antibodies directed against CD45, Siglec F, Ly6C and Ly6G (BioLegend). Human peripheral blood neutrophils were isolated from healthy donors after informed written consent in agreement with local ethical regulations and separated using PanColl (PanBiotech, Germany) density gradient centrifugation. Granulocytes were enriched from the erythrocyte pellet by consequent dextrane sedimentation (60 min, 1 %, centrifugation. Granulocytes were isolated from snap frozen samples using Mammalian Protein Extraction Reagent (MEPER) complete buffer (Thermo Scientific) and mechanical disruption using a ball mill. Protein quantification was performed using Bradford reagent (Carl Roth). For dot blots, protein lysates were directly administered to nitrocellulose membranes. For further analysis, Western blots were performed after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using ready-made gels (Bio-Rad).

Calculation of ulcerated area
Tissue sections stained with H&E were used for a blinded morphometric analysis calculating the area affected by ulceration relative to the total sectional mucosal surface.

Assessment of fecal bacterial load
Stool samples of gnotobiotic and SPF mice were collected before and 18 hours after wound infliction. Stool samples were suspended in a weight-normalised amount of sterile phosphate-buffered saline (PBS). Filtrates were plated on sterile LB agar plates without antibiotics, incubated at 37 °C for 24 hours and bacterial colonies were counted.

PAD4-dependent plasmin activity assay
Various concentrations of human α2-antiplasmin (Merck Millipore, Darmstadt, Germany) were preincubated with or without active PAD4 enzyme (Cayman Chem, Ann Arbor) for 2 hours at 37 °C in a buffer containing 100 mM Tris-HCl, 160 mM lysine and 10 mM CaCl2. Afterwards, human active plasmin enzyme was added and incubated for 30 min at 37 °C. The fluorescent plasmin substrate N-Succinyl-Ala-Phy-Lys-AMC (Merck Millipore) was then added to each well and the fluorescence intensity measured in a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) with ex/em 345/465 nm at 37 °C over the Roche LightCycler system (Roche, Penzberg, Germany). Expression was calculated relative to the housekeeping gene Hprt using the deltadelta threshold cycle (ΔΔCt) algorithm. Fold difference to control treated animals or unstimulated control, respectively, was calculated as a ratio to the respective control mean.

Immunofluorescence and blotting techniques
Histological staining was performed on paraffin-embedded sections with the classical haematoxylin-eosin (H&E) staining procedure. Immunofluorescence of cryosections or paraffin-embedded slides was performed as described below and recorded on either a confocal laser scanning-microscope or a standard fluorescence microscope (Leica, Germany) using overnight hybridisation with primary Abs specific for α-smooth muscle actin (Abcam, Cambridge, UK, 1:500), Beta-Catenin (Cell Signalling, 1:500), E-Cadherin (BD, 1:100), EpCAM (BioLegend, 1:100), citrullinated histone H3 (Abcam, 1:200), MPO (Abcam, 1:200), C3d (R&D Systems, 1:100). Detection was performed using either biotinylated secondary Abs (goat anti-rabbit or anti-rat, Abcam, 1:1000) and TSA Fluorescein/Cy3 kits (PerkinElmer, Waltham, Massachusetts, USA) or directly labelled Alexa 488 or Alexa 555-conjugated goat anti-rat antibodies (Abcam, 1:200–1:1000). Before examination, the nuclei were counterstained with either Hoechst 33342, propidium iodide or SYTOX Green (Invitrogen Molecular Probes, Karlsruhe, Germany; BD, Heidelberg, Germany). Autofluorescence of blood clots in paraffin-fixed sections was determined at excitation 488 nm and emission 525 nm in the absence of Alexa 488/fluorescein isothiocyanate (FITC)-based immunofluorescence. Tissue-derived proteins were isolated from snap frozen samples using Mammalian Protein Extraction Reagent (MEPER) complete buffer (Thermo Scientific) and mechanical disruption using a ball mill. Protein quantification was performed using Bradford reagent (Carl Roth). For dot blots, protein lysates were directly administered to nitrocellulose membranes. For further analysis, Western blots were performed after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using ready-made gels (Bio-Rad).

Real-time quantitative PCR
Tissue RNA was isolated by directly freezing tissue samples in liquid nitrogen in lysis buffer of thepeqGOLD Total RNA Kit (Peqlab, Erlangen, Germany). RNA quantification was performed using Nanodrop technology (Thermo Scientific, Wilmington, Delaware, USA). Reverse transcription into complementary DNA (cDNA) was performed using the BioRad iScript cDNA synthesis Kit (Bio-Rad Laboratories, Munich, Germany). Real-time quantitative PCR (qPCR) was performed using QuantiTect Primer Assays for Actb, Aqp9, Csf2, Csf3, Cxcl5, Cxcr2, Hprt, Il1b, Il6, Nos2, Tnfa, Pad2, Pad4, S100a9 (Qiagen, Hilden, Germany) and QuantiTect SYBR Green qPCR Kit (Qiagen) on the Roche LightCycler system (Roche, Penzberg, Germany). Expression was calculated relative to the housekeeping gene Hprt using the deltadelta threshold cycle (ΔΔCt) algorithm. Fold difference to control treated animals or unstimulated control, respectively, was calculated as a ratio to the respective control mean.

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a period of 60 min. Results were analysed using Microsoft Excel (Microsoft, Redmond, Washington, USA).

Assessment of transglutaminase activity in colon wounds
The transglutaminase substrate 5-(Biotinamido)pentylamine (Merck Millipore) was dissolved in sterile PBS and injected intraperitoneally at a concentration of 100 mg/kg body weight in wild-type and PAD4−/− mice immediately before colon wound infliction.27 After 6 hours, the tissue was harvested, frozen and cut. Wound sections were fixed and stained with a fluorescent streptavidin conjugate (Dylight 488, Thermo Fisher). After counterstaining with Hoechst, imaging was performed using a standard fluorescence microscope (Leica, Germany). Relative fluorescence intensity in the wound clot area was assessed by digital image analyses. D-dimer concentration in colon wound homogenates was measured using the Abbexa D-dimer ELISA kit (Cambridge, UK) according to manufacturer’s instructions.

Flow cytometric analysis of the cellular composition of colon wounds
Three to four colonic wounds per mouse were inflicted using an endoscopic forceps during mouse colonoscopy and colon tissue was isolated after 18 hours. The wound area was punched out using a 3 mm biopsy punch and wounds and the residual non-wounded colon tissue were retained in separate tubes. Lamina propria leukocytes (LPL) and intraepithelial leukocytes (IEL) were isolated separately from both tissue fractions using the Lamina Propria Dissociation Kit for mouse (Miltenyi Biotec, order no. 130-097-410). Briefly, the tissue was cut into small pieces, incubated with predigestion solution at 37 °C for 2×20 min with vigorous shaking and then filtered through a 100 μm cell strainer. The filtrate containing the IEL fraction was kept on ice while the remaining tissue pieces were incubated with HBSS without Ca2+ and Mg2+ + 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 37 °C for 20 min with vigorous shaking. After repeated filtration, the tissue pieces were enzymatically digested for 30 min at 37 °C and subsequently shredded using the gentleMACS Dissociator (Miltenyi Biotec). The remaining LPLs were transferred into a fresh tube and centrifuged in parallel with the IEL filtrate to pelletise the cells. The pelleted LPL and IEL fractions were purified using a Percoll Cytiwa density gradient and washed with flow cytometry buffer in a final step. After staining with fluorophore-coupled antibodies, flow cytometric analysis was performed using a LSRFortessa Cell Analyzer and FlowJo software (BD Biosciences, Franklin Lakes, New Jersey, USA).

DNase-mediated digestion of wound clots in vitro
Colon wounds were inflicted on wild-type mice as described above. After 18 hours, mice were sacrificed and wound clots were picked and put on glass slides followed by immunostaining as presented above. Wounded areas were excised from the colon of sacrificed mice using a punch biopsy. The samples were mounted as a whole on top of glass slides, fixed and subjected to immunostaining as presented above. Stained samples were subjected to confocal laser-scanning microscopy (Leica SP5) and z-stacks were acquired. Further image processing was achieved by Leica and Image J software.

Confocal laser-scanning microscopic analysis of colon tissues and mucosal ulcers
Immunothrombi formed on top of mucosal wounds were manually picked and put on glass slides followed by immunostaining as presented above. Wounded areas were excised from the colon of sacrificed mice using a punch biopsy. The samples were mounted as a whole on top of glass slides, fixed and subjected to immunostaining as presented above. Stained samples were subjected to confocal laser-scanning microscopy (Leica SP5) and z-stacks were acquired. Further image processing was achieved by Leica and Image J software.

Statistical analysis
Data were analysed as indicated in the figure legends using the unpaired Student t-test using Microsoft Excel (Microsoft, Redmond, Washington, USA) or an analysis of variance with post-hoc Tukey honestly significant difference (HSD) tests, as well as Fisher’s exact test for 2×2 contingency tables and Wilcoxon rank-sum test, as indicated. Correlation of ordinal and metrically scaled parameters was performed using non-parametric Spearman rank-order correlation test.

RESULTS
Bleeding in active UC is controlled by successful formation of fibrin on mucosal erosions
Epithelial barrier dysfunction characterises UC, especially during flares of disease. In this setting, many patients experience marked rectal bleedings. We observed that patients with endoscopically active UC exhibit abundant mucosal erosions, while intensity of rectal bleeding strongly varies (figure 1A and B). We assessed both rectal bleeding as reported on clinical visits (partial Mayo score) as well as endoscopic features of disease. There was an increased frequency of mucosal erosions and ulcerations in patients suffering from rectal bleeding (figure 1B). However, the presence of erosions did not necessarily prompt rectal bleeding. Mucosal haemostasis was successfully established even in active UC, when the erosions were completely covered by fibrin (figure 1C). However, incomplete coverage by fibrin correlated

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with increased rectal bleeding. Increased occurrence of fresh blood or haematin on the surface of mucosal erosions correlated with clinical rectal bleeding (figure 1D). These findings suggested a haemostatic function of fibrin layers in active UC.

Eroded colonic mucosa features blood clots which are remodelled to a fibrin layer rich in aggregated granulocytes and NETs

As the mechanisms controlling mucosal haemostasis in UC are incompletely understood, we assessed the morphology of mucosal erosions by microscopy in subsequent studies. We studied erosions covered with blood or haematin and those covered with whitish fibrin (figure 2A and D). The surface of blood-covered erosions featured homogenous blood clots. Invasion of neutrophils from the edge of the blood clot was observed (figure 2B). MPO+ neutrophils at the edge of the autofluorescent blood clot were present in aggregates showing interspersed decondensed chromatin and featured citrullinated histone H3 (H3cit) as evidence for PAD activity (figure 2C). We speculated that infiltrating neutrophils and the associated PAD activity might be directly involved in the remodelling of the clot to a haematoxylin-affine amorphous fibrin layer. Fibrin-covered erosions also showed ample amounts of infiltrating neutrophils (figure 2D). We identified fibrin layers in active UC and on colonic ulcers to be rich in CD15+ neutrophils and myeloperoxidase (figure 2D–E, online supplemental figure 1A). Moreover, the fibrin layer on eroded surfaces was characterised by extracellular chromat in devoid of nuclear morphology displaying citrullinated histone H3 (H3cit), typical of NETs (figure 2F, online supplemental figure 1B). At the edge of blood clots on blood-covered erosions, single neutrophils were observed which also displayed H3cit. While H3cit was largely absent in MPO+ cells in the lamina propria (online supplemental figure 1A,B), strong H3cit immunopositivity and hence PAD activity was found within fibrin layers in direct proximity to aggregated granulocytes (figure 2F). Transcriptomes showed the increased presence of PAD4 in the inflamed mucosa of colonic CD, ileocolic CD and UC in two independent patient cohorts in both male and female patients alike, whereas PAD2 was reduced in active disease (figure 2F and G). Subsequent studies revealed that Padi4 expression is restricted mostly to innate immune cells of the myeloid lineage, especially neutrophil and eosinophil granulocytes (online supplemental figure 2A), while it is not detected in intestinal epithelial cells. Taken together, histone citrullination and formation of NETs is associated with increased presence of PAD4 in severe intestinal inflammation in IBD and occurs mostly in fibrin layers which cover mucosal ulcerations.

Mucosal damage leads to the formation of red blood clots subject to remodelling to neutrophil-rich fibrin layers characterised by marked PAD4-activity

Based on these findings, we hypothesised that neutrophils take part in the remodelling of red blood clots on mucosal erosions to fibrin layers. In order to dynamically model healing of mucosal ulcers in vivo, we inflicted colonic wounds in mice by endoscopy using a grasping forceps (figure 3A): directly after wounding, a red blood clot forms on the mucosal wound, which was remodelled to a whitish fibrin layer hours after wounding (figure 3A). 18 hours after wounding, a breach of the epithelial lining was still appreciable (figure 3B). At the edge of blood clots on ulcerated areas, single neutrophils were observed which also displayed H3cit. While H3cit was largely absent in MPO+ cells in the lamina propria (online supplemental figure 1A,B), strong H3cit immunopositivity and hence PAD activity was found within fibrin layers in direct proximity to aggregated granulocytes (figure 2F). Transcriptomes showed the increased presence of PAD4 in the inflamed mucosa of colonic CD, ileocolic CD and UC in two independent patient cohorts in both male and female patients alike, whereas PAD2 was reduced in active disease (figure 2F and G). Subsequent studies revealed that Padi4 expression is restricted mostly to innate immune cells of the myeloid lineage, especially neutrophil and eosinophil granulocytes (online supplemental figure 2A), while it is not detected in intestinal epithelial cells. Taken together, histone citrullination and formation of NETs is associated with increased presence of PAD4 in severe intestinal inflammation in IBD and occurs mostly in fibrin layers which cover mucosal ulcerations.
H3cit (figure 3E) and displayed the presence of C3d, indicating complement activity (figure 3F). By flow cytometry, we assessed the relative amounts of various immune cells to both wound surface and lamina propria. Both surface and lamina propria showed a strikingly increased infiltration of CD11b+ myeloid cells as compared with adjacent healthy mucosa. Both surface and lamina propria showed significant increases of CD11b+Ly6G+ neutrophils: especially the wound surface showed a striking enrichment of up to 73% of all infiltrating CD11b+ myeloid cells being neutrophils (figure 3G), while CD11b+Ly6CintLy6G-SiglecF+ eosinophils were scarce (figure 3H). The wound surface of both blood-covered and fibrin-covered erosions was additionally analysed in a top view perspective by confocal microscopy. Aggregated neutrophils and NETs were observed in direct proximity to blood clots. Ultimately, the blood clots were completely remodelled to H3cit-positive layers (figure 3I). We further quantified PAD activity in colonic wounds and detected markedly elevated H3cit in wounds (figure 3J and K). Remodeling of the blood clot on eroded surfaces thus leads to a fibrin layer.
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characterised not only by fibrin polymerisation but also by the activity of complement and a significant contribution of neutrophil granulocytes, which aggregate, form NETs and show marked PAD activity.

To underline this crucial contribution of innate immune cells to the remodelling of the wound surface, we introduced the term immunothrombus to define a primary blood clot, which has been remodelled by innate immune cells.17

An increased neutrophil-related transcriptional signature coincides with the increase of clot remodelling-associated transcripts

To further characterise the dynamic process of wound healing and wound-bed remodelling on a transcriptional level, we performed RNA sequencing of colon wounds at defined time points of 6, 24 and 48 hours after wounding as compared with healthy tissue. Specifically, we assessed dynamic changes in expression over time.
of preselected genes functionally associated to haemolysis and fibrinolysis, clot remodelling, as well as immune cell-associated signatures related to neutrophils, myeloid cells, lymphocytes and fibroblasts. Clot remodelling-associated transcripts, for example, Hp, Tgm1, Plaur, Serpine1, Serpinf2 and Hmox1 showed strongly increased abundance as soon as 6 hours after wounding (figure 4A). Interestingly, increased abundance of neutrophil-related transcripts, for example, Csf3, Clec4e, Sl100a9, Cxcr2 and Padi4 coincided with clot remodelling-associated transcripts (figure 4B). A myeloid cell-related gene set reached its maximum in abundance at 24 hours after wounding (figure 4C), whereas lymphocyte-related transcripts peaked at 48 hours (figure 4D). Fibroblast-related transcripts showed less marked alterations in abundance as compared with the aforementioned immune cell compartments (figure 4E). Taken together, abundance in clot remodelling-related genes coincides with a neutrophil-related gene signature, whereas myeloid and lymphocyte-related signatures increase at later time points after wounding (figure 4F). Findings from RNA sequencing studies were corroborated by selective qPCR analyses of colon wounds and adjacent healthy mucosa at 24 hours post wounding. Specifically, Padi4 mRNA was increased in colonic wounds, while Padi2 mRNA was not (online supplemental figure 2B).

**Deficiency of PAD4 disturbs blood clot remodelling on mucosal ulcerations and delays mucosal wound healing**

Next, we assessed the functional contribution of immunothrombi *in vivo*. We made use of PAD4-deficient mice. We observed striking morphological differences in the wound healing process in PAD4-deficient mice as compared with controls. In the absence of PAD4, H3cit was strongly reduced in the wound surface (online supplemental figure 3A) and less decondensed chromatin was present. Functionally, the remodelling of the ulcerated surface was disturbed (figure 5A, video 1): red blood clots persisted more often as analysed 6 hours after wounding and led to a continued sanguinary appearance on endoscopy (figure 5B). Following the delayed remodelling of the wound surfaces, the timely restitution of the mucosal integrity was delayed in the absence of PAD4 as compared with wild-type controls. This was evidenced by repeated endoscopy and morphometry (figure 5C and D). H&E staining of colon wound
sections evidenced the persistence of blood clots on the wound in the absence of PAD4 (figure 5E), whereas in wild-type controls remodelling to a neutrophil-rich immunothrombus occurred regularly. Neutrophil chemoattraction to the wound surface was not disturbed as evidenced by MPO staining (figure 5F). Further whole mount immunofluorescence demonstrated that neutrophil chemoattraction persisted in the absence of PAD4, while histone citrullination and chromatin decondensation was markedly disturbed (figure 5G, online supplemental figure 3A). Flow cytometric analyses corroborated the strong and equally effective chemoattraction of neutrophils to the wounds in the absence of PAD4 (figure 5H). Additionally, no significant alterations in the influx of other myeloid cells were observed (figure 5I).

**Immunothrombosis occurs on mucosal erosions even in the absence of bacteria**

Immunothrombi on colonic wounds were subject to bacterial invasion in mice: both in wild-type and PAD4-deficient mice invasion of the wound surface by bacteria was detected using 16s-rRNA eubacteria-directed fluorescent probes. Obvious tissue infiltration into deeper layers of the intestinal mucosa by bacteria was not observed (online supplemental figure 3B). We aimed to understand, whether microbial invasion was important for NET formation in immunothrombi. Gnotobiotic C57BL/6 mice were used and wounds were inflicted under sterile conditions. Bacteria could not be detected in the murine faeces of treated mice even 18 hours after the endoscopic procedure.
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We compared the course of wound healing in gnotobiotic C57BL/6 mice and mice raised under SPF conditions. We detected regular formation of blood clots and successful remodelling to immunothrombi on colonic wounds, even in the absence of microbiota (figure 6B). Neutrophils infiltrated the colonic wound surface in both gnotobiotic C57BL/6 mice and in SPF mice as evidenced by H&E staining (figure 6C) and formed immunothrombi. However, submucosal oedema and submucosal leucocyte infiltration were significantly reduced in gnotobiotic mice as compared with SPF mice (figure 6D and E). MPO⁺ cells infiltrated the wound surface in both gnotobiotic and SPF mice alike, whereas submucosal MPO⁺ cells were significantly reduced in gnotobiotic mice (figure 6F1). Both PAD (H3cit) activity (figure 6G and J) and complement (C3d) activity (figure 6H and K) persisted in immunothrombi, even under gnotobiotic conditions. Thus, neutrophil recruitment to wound surfaces, PAD activity and complement activity persist in the absence of microbiota.
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We then devised *in vitro* systems to assess the interaction of neutrophils with preformed blood clots. First, we induced a blood clot on glass cover slips (online supplemental figure 4A, B). We then applied neutrophil suspensions to a punched hole. Interestingly, neutrophils accumulated at the edge of the sterile blood clot and formed NETs as evidenced by decondensed chromatin and H3cit (online supplemental figure 4B), even in the absence of microorganisms. Over time, the blood clot destabilised and hampered further functional analyses and imaging. We asked whether soluble factors might activate this neutrophil response. Thus, an agarose gel loaded with 20% autologous serum was created in culture dishes (online supplemental figure 4C). Addition of neutrophil suspensions led to an accumulation of neutrophils at the edge of the agarose gel. At the edge, neutrophils in high cellular densities extruded decondensed chromatin (online supplemental figure 4D) in response to autologous serum (videos 2 and 3). Inhibition of PADs by BB-Cl-amidine did not hamper accumulation of neutrophils at the edge of the agarose gel (online supplemental figure 4DE, video 4). However, NET formation was abrogated, when PADs were inhibited, irrespective of the use of native or heat-inactivated serum (online supplemental figure 4FG, videos 3–6). Hence, neutrophils respond to coagulated blood and form PAD-dependent NETs at high cellular densities in response to soluble factors, even in the absence of microbiota.

**PAD4-mediated immunothrombosis controls mucosal haemostasis in acute DSS-induced colitis**

The formation of immunothrombi is not limited to physically inflicted mucosal wounds. We also observed neutrophil aggregation and NETs in the course of chemical damage to the colonic mucosa. We employed the acute DSS-induced model of colitis, which features epithelial erosions and bleeding. H&E staining of colon sections shows the marked infiltration of leucocytes to the bowel wall and an abundance of granulocytes in the lumen (figure 7A). Specifically, in the lumen in direct proximity to blood, large amounts of MPO$^+$ neutrophils can be detected (figure 7B). Immunoblots were performed, which detected the increased presence of H3cit in the inflamed colon on day 8 of acute DSS-induced colitis (figure 7C and D). In line with analyses of human tissues, we found MPO$^+$ neutrophil aggregates and H3cit preferentially on the surfaces of ulcerated mucosae (figure 7E). To assess the functional role of PAD4-mediated immunothrombosis in this model, we again made use of PAD4-deficient mice. PAD4-deficient mice featured an aggravated, accelerated course of acute DSS-induced colitis: PAD4-deficient mice suffered from an increased weight loss (figure 7F) and enhanced lethality (figure 7G). Additionally, colon length was reduced (figure 7H). Strikingly, PAD4-deficient mice displayed increased bleeding mucosal ulcerations compared with wild-type counterparts as assessed by endoscopy (figure 7I, video 7). In fact, 96% of PAD4-deficient mice displayed

**Video 2** Neutrophils were cultured in RPMI supplemented with 2% autologous serum in culture dishes previously prepared with an agarose gel in the centre prepared without autologous serum as control. The edge of the gel was imaged by live-cell microscopy (37 °C, 5% CO$_2$). This video displays high cellular densities at the gel edge, with only low amount of NET formation. Video time course 12 hours.

**Video 3** Neutrophils were cultured in RPMI supplemented with 2% autologous serum in culture dishes previously prepared with an agarose gel in the centre containing 20% autologous serum. The edge of the gel was imaged by live-cell microscopy (37 °C, 5% CO$_2$). This video displays neutrophils in high cellular densities at the edge of the serum-containing agarose gel which preferentially form NETs at the edge of the gel. Video time course 12 hours.

**Video 4** Neutrophils were cultured in RPMI supplemented with 2% autologous serum in culture dishes previously prepared with an agarose gel in the centre containing 20% autologous serum in the presence of the PAD-inhibitor BB-Cl-amidine (20 µM). The edge of the gel was imaged by live-cell microscopy (37 °C, 5% CO$_2$). This video displays neutrophils in high-cellular densities at the clot edge. BB-Cl-amidine blocks NET formation at the edge of the gel. Video time course 12 hours.

Neutrophils next to agarose gel without serum gradient

Neutrophils responding to 20% autologous serum in agarose gel

Neutrophils responding to 20% autologous serum in agarose gel in the presence of BB-Cl-amidine
rescence, we observed that wild-type mice mostly showed intact mucosal surfaces. PAD4-deficient samples, however, showed more areas denuded of epithelium as visualised by β-catenin and E-cadherin staining (online supplemental figure 5C). In acute DSS-induced colitis, we noted that wound-associated epithelia begin to cover areas with crypt loss. Interestingly, the disturbed remodelling of the ulcerated surface and disrupted immunothrombosis in PAD4-deficient mice hampered epithelial restitution by wound-associated epithelial cells (online supplemental figure 5DE). Significantly less distorted mucosa was covered by wound-associated epithelia in the absence of PAD4. Specified wound-associated epithelial cells characterised by the elevated expression of claudin-4 form at the edge of the residual epithelial lining and bridge the ulcerated surface over time. The length of the claudin-4-positive layer of wound-associated epithelia at the edge of forceps-induced colonic wounds was reduced in PAD4-deficient mice as compared with wild-type (online supplemental figure 5F, G). Additionally, we assessed the composition of myeloid cells which infiltrate the bowel wall in the course of DSS-induced colitis by flow cytometry and immunofluorescence. Here, CD11b+Ly6Chigh and CD11b+Ly6Clo myeloid cells, eosinophils and neutrophils characterise the inflammatory infiltrate. However, this approach misses neutrophils in the colon lumen (figure 7A and B, online supplemental figure 6A, B). Both PAD4-deficient and wild-type neutrophils robustly migrate to the intestinal lamina propria and are abundant in the colon lumen in the course of DSS-induced colitis. MPO immunofluorescence of cross-sections takes luminal neutrophils into account and demonstrated a significant increase of infiltrating neutrophils in PAD4−/− mice (online supplemental figure 6C) versus wild-type control. Overall, PAD4−/− mice displayed more infiltrating neutrophils as a consequence of increased mucosal ulcerations in these mice.

PAD4 affects clot remodelling by reducing serpin activity resulting in a mature immunothrombus covalently cross-linked by transglutaminases

The effect of PAD4 on enhanced stability of immunothrombi may be attributed in part to chromatin of NETs. We thus asked whether DNase I-treatment might recapitulate the observations of PAD4-deficiency in DSS-induced colitis. Daily administration of DNase I in the course of acute DSS-induced colitis did not significantly alter clinical parameters of disease severity: Weight loss (online supplemental figure 7A), colon length (online supplemental figure 7B) and the fraction of mice with rectal bleeding were unaffected by this intervention (online supplemental figure 7C). On the microscopic level, a slight protection from colonic ulceration was observed in DNase I-treated mice (online supplemental figure 7DE). We further characterised the kinetics of DNase I-mediated digestion of colonic immunothrombi. Colonic immunothrombi picked from colonic wounds and NETs generated from isolated neutrophils were subjected to DNase I treatment in vitro (online supplemental figure 6F). While NETs generated from isolated neutrophils were quickly dismantled by DNase I in the course of 90 min, DNase I-mediated digestion of colonic immunothrombosis was less efficient. After 20 hours, a reduction in DNA$^{52+}$ was noted in DNase I-treated immunothrombi. However, DNase I did not dismantle the clot and Hoehcht could still bind the immunothrombus. Thus, DNase I treatment does not phenocopy PAD4-deficient mice in the DSS-induced colitis model and only inefficiently digests colonic immunothrombi.

We further assessed PAD4-mediated functions beyond chromatin decondensation in our models. PAD4 has been shown to...
inhibit serine protease inhibitors by inactivating core P1 arginine residues in the reactive centre loop of multiple serpins with complex theoretical consequences to the coagulation and fibrinolysis cascade (online supplemental figure 8A). Indeed, in vitro studies showed the inhibition of α2-antiplasmin and increased plasmin activity in the presence of PAD4 (online supplemental figure 8B). We analysed the net effect of PAD4 downstream of coagulation and fibrinolysis by assessment of D-dimers in colonic wounds. Interestingly, the relative amount of D-dimers was significantly reduced in PAD4-deficient wounds as compared with wild-type controls (*** p < 0.001, Fisher’s two-tailed exact test). DSS, dextrane sodium sulfate; MPO, myeloperoxidase; PAD, peptidyl-arginine deiminase; WT, wild-type.
wild-type and PAD4-deficient mice and assessed transglutaminase activity in immunothrombi by streptavidin-linked fluorescence microscopy. Transglutaminase activity in immunothrombi on colon wounds was significantly reduced in the absence of PAD4 (online supplemental figure SE, F).

Taken together, neutrophils make use of PAD4 to stabilise the eroded surface in part by NETs and by the remodelling and maturation of the fibrin clot and thus favour epithelial restitution.

DISCUSSION
Rectal bleeding and bloody diarrhoea are major hallmarks of acute flares of UC. Early mucosal healing is a valuable goal to improve long-term clinical outcomes but initiation of mucosal healing in ulcerative lesions remains elusive. In this study, we uncovered a role for neutrophils and PAD4 in controlling rectal bleeding and regulating mucosal haemostasis in human IBD and experimental models of disease. Our endoscopic and microscopic studies in human UC show that successful haemostasis is associated with remodelling of blood clots to fibrin layers as an important step towards mucosal healing. Remodelling of the wound surface is guided by innate immune cells, mostly neutrophils. We thus propose the term immunothrombus to define a blood clot which has been remodelled by innate immune cells. We identified extracellular chromatin, aggregated neutrophils and PAD4 activity in immunothrombi, which cover mucosal ulcerations and erosions in UC. Erosions, ulcerations, blood, neutrophils and fibrin are positively associated with severe disease. However, we observed that blood/haematin coverage of erosions rather than fibrin coverage is associated with rectal bleeding, consistent with the role of fibrin as a protective emergency barrier. This reconciles these seemingly paradox observations: fibrin and neutrophil-rich immunothrombi occur in severe disease, yet provide protection in this setting by stabilising the damaged mucosa and successfully achieving haemostasis before epithelial restitution can occur.

We closely studied the kinetics of mucosal injury over time in mice. We detected that blood clots are remodelled in the course of hours to whitish immunothrombi by innate immune cells with highly prevalent neutrophils and PAD4-dependent NETs. In line with a protective function of PAD4-mediated NETs and immunothrombosis, PAD4-deficient mice suffered from an aggravated course of acute DSS-induced colitis with marked rectal bleeding and consecutively reduced haematocrit and haemoglobin levels. This further corroborated the role of PAD4 to support mucosal haemostasis and control rectal bleeding in colitis. Commensal bacteria are capable of infiltrating immunothrombi to a certain extent, as observed by fluorescent in-situ hybridisation. While microorganisms may stimulate neutrophils to undergo NET formation under specific settings, especially with regard to possible UC-related pathobionts, our study shows that PAD4 activity and complement activation persist in immunothrombi on colonic wounds, even in the absence of microbiota. In fact, neutrophils were strongly stimulated by coagulated blood and heat-insensitive components of serum to aggregate and form NETs. Neutrophils formed marked PAD4-dependent NETs in high cellular densities at the edges of the clots. Inhibition of PAD4 enzymes inhibited NET formation in high cellular densities at the edge of serum-containing agarose gels. These observations are in line with previous studies on the role of NETs in various diseases. While initial studies of NETs focused on their role in host defence against microorganisms, following studies highlighted the role of NETs in the course of thrombotic diseases and noninfectious inflammation. In our experiments, deficiency of PAD4 led to a failure to remodel blood clots on the mucosal surface, delayed colonic wound healing and aggravated colitis with a failure to control rectal bleeding. What is the mechanistic role of PAD4 in the remodelling of the blood clot to a stable immunothrombus? PAD4 mediates chromatin decondensation of neutrophils in the course of NET formation as observed in direct proximity to blood clots. Extracellular chromatin of NETs can occlude vessels and may serve as a scaffold for further platelet aggregation and activation of Factor XII. In our experiments, however, DNase I-treatment did not favour rectal bleeding as might have been expected if extracellular chromatin alone was responsible for clot stability. However, DNase I only inefficiently dismantled colonic immunothrombus in vitro. As large size, limited penetration of the large immunothrombus as well as DNase-insensitive sodium or calcium salts of DNA might limit DNase I efficacy in this model, an important role of extracellular chromatin in immunothrombosis cannot be excluded or suggested by these experiments. Nonetheless, we chose to look for additional effects of PAD4 beyond NET formation. On a molecular level, PAD4 mediates the modification of peptide-bound arginine to citrulline. Apart from effects on the structure of chromatin, the function of various proteases and their inhibitors including serine proteases, metalloproteases and calpains is modified by PAD4 and might be of relevance in the process of immunothrombosis. In line with findings by Tilvawala et al. we detected that serine proteases with a central P1 arginine in the reactive centre loop, for example, α2-antiplasmin, were inactivated by PAD4 in vitro. Inhibition of multiple serpins of the coagulation/fibrinolytic balance resulted in an overall reduced amount of fibrin turnover in the absence of PAD4. Reduced levels of D-dimers in colonic wounds were detected in PAD4-deficient mice. Interestingly, in the absence of PAD4 transglutaminase activity and thus covalent cross-linking of the immunothrombus by transglutaminases was reduced. This is in line with a model, in which PAD4-mediated inhibition of serpins, for example, antithrombin, is required for effective thrombin-mediated activation of the transglutaminase Factor XIIIa and subsequent stabilisation of the immunothrombus. Analyses of the colon mucosa during DSS-induced colitis demonstrate that epithelial restitution of eroded surfaces is disturbed in the absence of PAD4. Gene expression analyses did not detect PAD4 in intestinal epithelial cells arguing for an indirect effect of reduced stability of the immunothrombus and the eroded surface in the absence of PAD4 which thus hampers epithelial restitution.

Neutrophils have been observed and studied in colitis for many years: neutrophils have been implicated as drivers of...
mucosal inflammation in IBD. Specifi-cally, it has been proposed that NET formation should be therapeutically inhibited. Infection-induced or autoimmunity-induced overabundance of NET formation or defective clearance may, indeed, damage the host, especially small vessels, in particular in the presence of UC-associated pathobionts. In contrast, mixed results were reported in experimental models studying neutrophil depletion approaches in colitis in the past. Phagocyte dysfunction is implicated in the setting of monogenic IBD in childhood. Moreover, neutro-penia of diverse origin may result in the development of neutrophonic enterocolitis. In the setting of glyco-gen storage disease type Ib, chronic enteritis may develop that can be successfully treated by G-CSF infusions. This further highlights the protective potential of neutrophils in mucosal inflammation. We provide a novel perspective to this topic by describing the haemostatic function of neutrophils, NETs and immunothrombosis on ulcerations and erosions in active colitis. Our study provides an explanation for the increased bleeding as a cause of granulocyte depletion, which has been reported previously.

Taken together, we have shown that neutrophils make use of PAD4 to remodel blood clots to immunothrombi on mucosal ulcerations rich in NETs. Immunothrombi function as an emergency barrier in the setting of epithelial disruption. Our findings indicate that PAD4-dependent immunothrombi support mucosal wound healing and prevent bleeding of ulcers in severe IBD. Thus, these data suggest that the neutrophil response in active UC should be carefully balanced, rather than completely abrogated.

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REFERENCES
1 Truelove SC, Wits L. Cortisone in ulcerative colitis: a preliminary report on a therapeutic trial. Br Med J 1954;2:375–8.
2 Samuel S, Ingle SB, Dillon S, et al. Cumulative incidence and risk factors for hospitalization and surgery in a population-based cohort of ulcerative colitis. Inflamm Bowel Dis 2013;19:1858–66.
3 Robert JH, Sachar DB, Aufses AH, et al. Management of severe hemorrhage in ulcerative colitis. Am J Surg 1990;159:550–5.
4 Kasler A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annu Rev Immunol 2010;28:573–621.
5 Neurath MF, Leppkes M. Resolution of ulcerative colitis. Semin Immunopathol 2019;41:747–56.
6 Ananthakrishnan AN, Bernstein CN, Riopoulos D, et al. Environmental triggers in IBD: a review of progress and evidence. Nat Rev Gastroenterol Hepatol 2018;15:39–49.
7 Schroeder BO, Birchennough GMR, Ståhlman M, et al. Bifidobacteria or fiber protects against diet-induced Microbiota-Mediated colonic mucus deterioration. Cell Host Microbe 2018;23:27–40.
8 Fournier BM, Parkos CA. The role of neutrophils during intestinal inflammation. Mucosal Immunol 2012;5:354–66.
9 Chin AC, Lee WY, Nusrat A, et al. Neutrophil-mediated activation of epithelial protease-activated receptors-1 and -2 regulates barrier function and transepithelial migration. J Immunol 2008;181:5702–10.
10 Bernike TB, Carlse T, Ellingt E, et al. Neutrophil extracellular traps in ulcerative colitis: a proteome analysis of intestinal biopsies. Inflamm Bowel Dis 2015;21:2052–67.
11 Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303:1532–5.
12 Neel E, Khan SN, Radic M. Histone demethylation as a response to inflammatory stimuli in neutrophils. J Immunol 2008;180:1895–902.
13 Li P, Li M, Lindberg MR, et al. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med 2010;207:1853–62.
14 Wang Y, Li M, Stadler S, et al. Histone hyperacetylation mediates chromatin decondensation and neutrophil extracellular trap formation. J Cell Biol 2009;184:205–13.
15 Hemmers S, Teijaro JR, Arandjelovic S, et al. PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection. PLoS One 2011;6:e22043.
16 Knopf J, Leppkes M, Schett G, et al. Aggregated neutrophils sequester and detoxify extracellular histones. Front Immunol 2019;10:2176.
17 Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. Nat Rev Immunol 2013;13:34–45.
18 Mantooth K, Demers M, Fuchs TA, et al. Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. Proc Natl Acad Sci U S A 2013;110:8674–9.
19 Paine ER. Colonoscopic evaluation in ulcerative colitis. Gastroenterol Rep 2014;2:161–8.
20 Haberman Y, Barnet M, Oehme H, et al. Ulcerative colitis mucosal transcriptomes reveal mitochondrialopathy and personalized mechanisms underlying disease severity and treatment response. Nat Commun 2019;10:38.
21 Patankar JV, Müller TM, Kantham S, et al. E-type prostaglandin receptor 4 drives resolution of intestinal inflammation by blocking epithelial necroptosis. Nat Cell Biol 2021;23:796–807.
22 Leppkes M, Lindemann A. Temporal changes in the transcriptome of mouse colonic tissues post biopsy-induced wound generation using RNA seq. 2021. ArrayExpress. Available: http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10624
23 Heng TSP, Painter MW, Immunological Genome Project Consortium. The immunological genome Project: networks of gene expression in immune cells. Nat Immunol 2008;9:1091–4.
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24 Neurath MF, Wittekopf N, Wlodarski A, et al. Assessment of tumor development and wound healing using endoscopic techniques in mice. Gastroenterology 2010;139:1837–43.

25 Pickert G, Neufert C, Leppkes M, et al. 3D links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. J Exp Med 2009;206:1465–72.

26 Weigmann B, Tabelle J, Seidel D, et al. Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. Nat Protoc 2007;2:2307–11.

27 Jeong EM, Son YH, Choi Y, et al. Transglutaminase 2 is dispensable but required for the survival of mice in dextran sulphate sodium-induced colitis. Exp Mol Med 2016;48:e267.

28 Tilvawala R, Nguyen SH, Maurais AJ, et al. The rheumatoid arthritis-associated Cytulline. Cell Chem Biol 2018;25:691–704.

29 Lewis JD, Chuai S, Nessel L, et al. Analysis of intestinal haem-oxygenase-1 (HO-1) is associated with improved long-term clinical outcomes in ulcerative colitis. Gut et al 2011;141:1194–201.

30 Colombel JF, Rutgeerts P, Reinisch W, et al. Early mucosal healing with infliximab is associated with improved long-term clinical outcomes in ulcerative colitis. Gastroenterology 2011;141:194–201.

31 Paul G, Bataille F, Obermeier F, et al. Analysis of intestinal haem-oxygenase-1 (HO-1) in clinical and experimental colitis. Clin Exp Immunol 2005;140:547–55.

32 Dinallo V, Marafini I, Di Fusco D, et al. Neutrophil extracellular traps sustain inflammatory signals in ulcerative colitis. J Crohns Colitis 2019;13:772–84.

33 Abd El Hafez A, Mohamed AS, Shehta A, et al. Neutrophil extracellular traps-associated protein peptidyl arginine deaminase 4 immunohistochemical expression in ulcerative colitis and its association with the prognostic predictors. Pathol Res Pract 2020;216:131022.

34 Leoni G, Neumann P-A, Sumagin R, et al. Wound repair: role of immune-epithelial interactions. Mucosal Immunol 2015;8:69–88.

35 Saha P, Yeoh BS, Xiao X, et al. PAD4-dependent nets generation are indispensable for intestinal clearance of Citrobacter rodentium. Mucosal Immunol 2019;12:1761–71.

36 Schauer C, Janko C, Munoz LE, et al. Neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. Nat Med 2014;20:511–7.

37 Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A 2010;107:15880–5.

38 Leppkes M, Knopf J, Naschberger E, et al. Vascular occlusion by neutrophil extracellular traps in COVID-19. EBioMedicine 2020;58:102925.

39 Zuo Y, Yalavarthi S, Shi H, et al. Neutrophil extracellular traps in COVID-19. JCI Insight 2020;S.

40 Meng H, Yalavarthi S, Kanthi Y, et al. In vivo role of neutrophil extracellular traps in antiphospholipid antibody-mediated venous thrombosis. Arthritis Rheumatol 2017;69:655–67.

41 Bücker R, Schulz E, Günzel D, et al. α-Haemolysin of escherichia coli in IBD: a potentializer of inflammatory activity in the colon. Gut 2014;63:1893–901.

42 Kühler AA, Bäck A, Janotta M, et al. Aggravation of different types of experimental colitis by depletion or adhesion blockade of neutrophils. Gastroenterology 2007;133:1882–92.

43 Natsume M, Kawasagi A, Takizawa H, et al. Selective depletion of neutrophils by a monoclonal antibody, RP-3, suppresses dextran sulphate sodium-induced colitis in rats. J Gastroenterol Hepatol 1997;12:801–8.

44 Uhlig HH, Schwed T, Koletzko S, et al. The diagnostic approach to monogenic very early onset inflammatory bowel disease. Gastroenterology 2014;147:990–1007.

45 Gerschler M, Mey U, Strahl J, et al. Neutrophic enterocolitis in adults: systematic analysis of evidence quality. Eur J Haematol 2005;75:1–13.

46 Voiz MS, Nassir M, Treese C, et al. Inflammatory bowel disease (IBD)-like disease in a case of a 33-year-old man with glycogenosis 1b. BMC Gastroenterol 2015;15:45.