The Microenvironment of Injured Murine Gut Elicits a Local Pro-
restitutive Microbiota

Authors:

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1. Supplementary Information Figures (1 to 8).
2. Relative abundance of bacterial genus.
Supplementary information Figure 1

a. Endoscope and biopsy forceps used to inflict mucosal wounds in murine distal colon.

b. Hallmarks of mucosal wound restitution during day 2 post injury:
1. Wound adjacent crypts are marked by increased proliferation and rapid migration of epithelial cells at day 2 post injury.
2. Wound beds are populated with neutrophils.

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1. Wound adjacent crypts are marked by increased proliferation and rapid migration of epithelial cells at day 2 post injury.
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D. Elucidation of spatiotemporal changes in microflora and microenvironment in mucosal wound bed:
1. High throughput sequencing of bacterial 16s rRNA genes (V4 region)
2. Determination of anaerobic microenvironment and gene expression
Supplementary Information Figure 1 | Generation of transmucosal wounds in the distal mouse colon by miniaturized endoscopy and biopsy forceps. **a**, Miniaturized endoscope and biopsy forceps used to make mucosal wounds in mouse colon⁹. **b**, *En face* image of the colonic wounds (*n* = 4 per group) at day 0 and day 2 post injury. Highlighted areas were collected to extract bacterial DNA and for subsequent high throughput sequencing analysis. **c**, Hematoxylin and eosin staining of a biopsy wound bed from wild-type (WT) mice 1, 2, 4 and 6 days post injury. **d**, Diagram showing scheme of sample collections for characterization of the spatiotemporal shifts of mucosa-associated microbiota as well as changes in wound microenvironment.
Supplementary Information Figure 2

(a) Alcian blue staining of intact injured colonic mucosa from WT mice (\(n=6\)).
(b) FISH analysis showing localization of microbiota using a pan-bacterial 16S rRNA gene probe (red). (\(n=6\)). Yellow arrow indicates presence of microbiota. Scale bars, 50 µm.
(c) Wheat germ agglutinin (WGA) staining of intact colonic mucosa.
(d) Adjacent crypts and wound.
(e) Immunofluorescence staining of MUC2 mucin in colonic mucosa. \(n=5\) per group.
(f) MUC2 DNA staining in intact and wounded colonic mucosa.
(g) Antibacterial activity test results: A. muciniphila, E. coli, Prevotella copri, Salmonella, L. plantarum, L. rhamnosus, Lysenibacillus.
Supplementary information Figure 2  | Absence of secreted mucin layer in wound bed results in intimate contact of epithelial cells and wound associated microbiota.  

a,b, Alcian blue staining of intact injured colonic mucosa from WT mice (n = 6).  
c,d, FISH analysis showing localization of microbiota using a pan-bacterial 16S rRNA gene probe (red). (n = 6). Yellow arrow indicates presence of microbiota.  

Scale bars, 50 µm.  

e, f, Immunofluorescence staining of MUC2 (green) mucin in colonic mucosa (n = 5 per group).  

Akkermansia specific FISH probe was tested against pure cultures of Akkermansia and a number of enteric bacteria, including E. coli, P. copri, Salmonella, L. plantarum, L. rhamnosus GG (LGG), and Lysenibacillus.
Supplementary Information Figure 3

(a) Relative abundance (%) of Lactobacillus over time.

(b) PCoA plot of microbiota of mouse colon.

(c) Mucosal infiltrating Ly6G+ cells (neutrophils) / 200 µm² fields in mucosal wound bed.

(d) MPO activity (U/g of tissue).

(e) Mucin 3 Actin.

(f) Mucin 3 Actin.

(g) Mucin 3 Actin.
Supplementary information Figure 3 | Restitution of wound mucosa is accompanied by both changes in wound microenvironment and microbiota. Relative abundance of *Lactobacillus* in intact and wounds mucosa determined by HTS. The data represent mean of relative abundance of microbiota of individual wounds (*n* = 5 per group). b, PCoA plot of microbiota of mouse colon. (*n* = 5 per group). c, Quantitative representation of immunofluorescence analysis (by ImageJ software; expressed in units of fluorescence) of Pimonidazole HCl adduct staining (hypoxia) in wound beds (*n* = 10 per group). **, *P* < 0.01. d, Mucosal infiltrating Ly6G+ cells (neutrophils) / 200 µm² fields in mucosal wound bed (mean ± s.e.m., *n* = 5) detected by IF staining. *, *P* < 0.05. n.s. means not significant. e, Myeloperoxidase (MPO) activity (mean ± s.e.m.) assay (*n* = 10). **, *P* < 0.01. f, Immunofluorescence staining of MUC3 mucin in restitutive wounds (*n* = 5 per group). g, Left panel, real-time qPCR analysis (mean ± s.e.m.) of *muc3* expression in wounds. Right panel, real-time qPCR analysis of fold change in specific relative abundance of *Akkermansia* in wounds to compare against intact mucosa of WT mouse (mean ± s.e.m.). Wounds were harvested from mice administered with a potent HIF-1α inhibitor (BAY 87-2243; 4 mg/kg) or a MUC3 peptide (Asp2236~Val2356), and were used for qPCR analysis of *glut1* (data not shown), *muc3* and *Akkermansia*. *n* = 6 per group.
Supplementary Information Figure 4

A. muciniphila

a

WT, control

WT

FPR1<sup>−/−</sup>

Day 2

Day 6

b

% wound closure

WT

WT

Fpr<sup>−/−</sup>

AM

WT, control

WT, A. muciniphila

DSS (3%)

AM

DSS (3%)

AM

WT, control

WT, A. muciniphila

WT, control

WT, A. muciniphila

WT, control

WT, A. muciniphila

% wound closure

Control

A. muciniphila

A. muciniphila + NAC

NAC

A. muciniphila + BOC2

BOC2

* P < 0.05; AM denotes A. muciniphila.

# P < 0.01. **P < 0.001.

Supplementary information Figure 4

Akkermansia muciniphila enhances FPR1-dependent wound restitution. a, Endoscopic images of bioptic wounds (n = 12 per group) in mouse colon. Mice were treated as above for 6 days with Hank’s balanced salt solution (HBSS; Control) or A. muciniphila. Dotted lines outline the depressed area margin of the lesion at each time point. b, Graph shows A. muciniphila-enhanced percent of wound closure (mean ± s.e.m; n = 12 per group) on day 6. *, P < 0.05; AM denotes A. muciniphila. c, Percent body weight loss of mice subjected to dextran sodium sulfate (DSS) colitis for 6 days followed by recovery from colitis for 6 days. n = 10 per group, mean ± s.e.m; P < 0.05. d, Representative photomicrographs of hematoxylin and eosin–stained histological sections. e, Percentage of ulceration in whole colon samples. P < 0.01. f, SK-CO15 monolayers were subjected to scratch wound assay in the presence of A. muciniphila. Wound widths were determined at 0 and 6 hours. SK-CO15 cell monolayers were also incubated with Akkermansia and NAC or FPR inhibitor BOC2. # P < 0.01. **P < 0.001.
Supplementary information Figure 4 | *Akkermansia muciniphila* enhances FPR1 dependent wound restitution. 

a, Endoscopic images of bioptic wounds (n = 12 per group) in mouse colon. Mice were treated as above for 6 days with Hank’s balanced salt solution (HBSS; Control) or *A. muciniphila*. Dotted lines outline the depressed area margin of the lesion at each time point. 

b, Graph shows *A. muciniphila*-enhanced percent of wound closure (mean ± s.e.m; n = 12 per group) on day 6. *, P < 0.05; AM denotes *A. muciniphila*. 

c, Percent body weight loss of mice subjected to dextran sodium sulfate (DSS) colitis for 6 days followed by recovery from colitis for 6 days. n = 10 per group, mean ± s.e.m; P < 0.05. 

d, Representative photomicrographs of hematoxylin and eosin–stained histological sections. 

e, Percentage of ulceration in whole colon samples. P < 0.01. 

f, SK-CO15 monolayers were subjected to scratch wound assay in the presence of *A. muciniphila*. Wound widths were determined at 0 and 6 hours. SK-CO15 cell monolayers were also incubated with *Akkermansia* and NAC or FPR inhibitor BOC2. # P < 0.01. **P < 0.001.
**Supplementary Information Figure 5**

**Supplementary information Figure 5 | Akkermansia muciniphila induces cellular ROS generation.** Mice were loaded with hydrocyanine 3 followed by biopsy wounding (BW) of the epithelium, as described previously\(^9\) and in methods. Wound mucosa (\(n = 10\) per group) was luminally treated for 15 min with Hank’s balanced salt solution (HBSS; Control) or different suspensions of *A. muciniphila* (2.5 \(\times\) \(10^7\) to 2.5\(\times\)\(10^9\) CFU/ml). Fluorescence was determined from *en face* wound bed by confocal laser scanning microscopy (Zeiss). The graph shows a quantitative representation of ROS production (mean ± s.e.m.) in the top panel. *, \(P < 0.05\). Fluorescence intensity was measured by the ImageJ software and expressed in units of fluorescence.

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<table>
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<th>2.5(\times)(10^8) cfu/ml</th>
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<th>2.5(\times)(10^9) cfu/ml</th>
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<td>40</td>
<td>50</td>
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\(A. muciniphila\)
Supplementary Information Figure 5

**a** *In vivo* EdU incorporation (proliferation) assay:

Biopsy injury

I.P. injection of EdU 2hr prior to wound bed collection & EdU staining to detect proliferation

Day 0

Day 2

Intrarectal administration of control buffer or *A. muciniphila* suspension for 2 days

**b** P-ERK

Control  fMLF  AM  AM + Boc2

**c** EdU DNA

Control  fMLF  AM

AM + Boc2  AM + NAC

Graph showing EdU + cells per field
Supplementary information Figure 6 | *A. muciniphila* stimulates ERK activation and cellular proliferation in cultured colonic epithelial cells. 

**a,** Experimental scheme to determine proliferating cells. 

**b,** Immunofluorescence analysis for phospho-ERK in cultured SK-CO15 cells treated with FPR inhibitor BOC2 30 min prior to stimulation with *A. muciniphila* (6 x 10⁷ CFU/ml) or fMLF (500 nM) for 15 min. AM denotes *A. muciniphila.* 

**c,** EdU incorporation into cultured SK-CO15 cells treated with BOC2 and the antioxidant NAC prior to incubation for 4 h with *A. muciniphila* (6 x 10⁷ CFU/ml) or fMLF (500 nM). AM denotes *A. muciniphila.* 

EdU-stained sections of a WT mouse treated with control buffer at days 1, 2 and 4 post biopsy injury showing Adjacent crypts. EdU (Chase) and β-catenin (to visualize intestinal epithelial cells).
Supplementary Information Figure 7

**In vivo EdU chase assay:**

<table>
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<tr>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 4</th>
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<tr>
<td>Biopsy injury &amp; EdU injection (I.P.)</td>
<td>Wound bed harvesting &amp; staining for EdU</td>
<td>Control buffer or <em>A. muciniphila</em></td>
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**b** *β*-catenin EdU (Chase) DNA

<table>
<thead>
<tr>
<th>WT, Day 1 wound</th>
<th>WT, Day 2 wound</th>
<th>WT, Day 4 wound</th>
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<tbody>
<tr>
<td>Control buffer</td>
<td>Control buffer</td>
<td>Control buffer</td>
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</table>

**Supplementary information Figure 7 | A. muciniphila-stimulated enterocyte migration in murine colon.**

*a*, Schematic diagram of EdU (5-ethynyl-2'-deoxyuridine) chase assay showing time of biopsy infliction and EdU injection (intraperitoneally; I.P.) as described in Figure 4F. In order to label cells at the time of injury, and then chase them for several days, Edu injection was performed right after the infliction of biopsy injury. Mice were treated intrarectally with *A. muciniphila* (6.0x10⁸ CFU/ml) suspension or control buffer (Hank’s balanced salt solution, HBSS; Control) for 0 - 4 days. 

**b**, Wound beds (*n*=12) were harvested on specified days and serial sections were stained for EdU-positive cells and *β*-catenin (to visualize intestinal epithelial cells). EdU-stained sections of a WT mouse treated with control buffer at days 1, 2 and 4 post biopsy injury showing
(EdU positive; red) epithelial cells emanating from a crypt at day 1, and readily present in
the thin layer of epithelial layer covering the wound bed on day 4. Most of the wounds re-
epithelialized within 4 days in the control group as described in reference\textsuperscript{5}. To chase
EdU-labeled cells for several days, mice were injected with EdU (I.P.) immediately after
endoscopic injury as described in Figure 4F and in Extended Data Figure 7a. White lines
show crypts adjacent to the wound.
Supplementary Information Figure 8

A model showing rapid, reversible and local alterations in the mucosal microbiota that occur in the wound microenvironment. These ecological changes are dependent on FPR1/NOX2-mediated local tissue hypoxia and expression of muc3 mucin. While non-microbial environmental signals clearly have major effects on wound healing, characterization of a dominant member of this wound associated consortia, Akkermansia, an anaerobic, mucinophilic commensal bacterium, revealed a FPR1/NOX1 dependent pro-restitutive function.
Relative abundance of the bacterial genus determined by high throughput sequencing (see Methods for the protocol of microbiota sequencing and analysis KASK).

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<th>WT, Day 6</th>
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**Methods** (WT, Intact WT, Day 2 WT, Day 4 WT, Day 6WT). DOI: www.nature.com/naturemicrobiology
**SUPPLEMENTARY INFORMATION**

**Methods**

KASK

DOI: 10.1038/NMICROBIOL.2015.21
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