

1 **A purified membrane protein from *Akkermansia muciniphila* or the pasteurized**
2 **bacterium blunts colitis associated tumourigenesis by modulation of CD8⁺ T cells**
3 **in mice**

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9 **Supplementary data**

10 **MATERIALS AND METHODS**

11 **Western blotting**

12 Amuc_1100 was a protein of 32 kDa, which was one of the most abundant outer
13 membrane proteins encoded by a specific Type IV pili gene cluster in *A.muciniphila*.
14 To test this protein, we added a His tag to the c-terminal of Amuc_1100. The purified
15 Amuc_1100 with the His tag could be detected with a His-tag antibody. An equal
16 amount of protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel
17 electrophoresis and transferred onto polyvinylidene difluoride membranes. The
18 membranes were stained with a primary antibody toward the His tag (A100186-100,
19 GenScript) and then incubated with secondary antibody. Protein bands were visualized
20 by a gel imaging system (Bio-Rad).

21 **Figure legends**

22 Supplementary Figure 1. Expression and purification of Amuc_1100 *in vitro*. (A)
23 Representative Gram-staining of *A. muciniphila*. (B) The coding sequence of
24 Amuc_1100 amplified by PCR. (C) His-tagged Amuc_1100 was cloned into the pET-
25 26b(+) vector using restriction endonuclease sites (NdeI/XhoI). (D) The recombinant
26 plasmids were transformed into BL21 *E. coli*. Plasmid digested by MluI and XhoI were
27 analysed by 1% agarose gel electrophoresis. (E) The purified Amuc_1100 protein was
28 stained by Coomassie brilliant blue. His-tagged Amuc_1100 was eluted with different
29 concentrations of imidazole. (F) The purified Amuc_1100 was detected by Western
30 blotting.

31

32 Supplementary Figure 2. Effects of pasteurized *A. muciniphila* or Amuc_1100 on the
33 faecal microbiota in colitis mice. (A) Schematic diagram of the experimental study
34 design. (B) PLS-DA plot of the faecal microbiota colitis of mice treated with *A.*
35 *muciniphila* or Amuc_1100. The Shannon (C), Chao (D), Ace (E) and Sobs (F)
36 indices on the OUT level were analysed. (G) Relative abundance of bacterial species.
37 (H) Ternary plots of the relative abundances of species in colitis mice. (I) Pie chart of
38 the faecal microbiota composition on the species level. Data are presented as the
39 means± SEM and were analyzed ordinary one-way ANOVA with Tukey's multiple
40 comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with respective control.

41

42 Supplementary Figure 3. Supplementation with pasteurized *A. muciniphila* or

43 Amuc_1100 suppressed carcinogenesis in mice. (A) Schematic diagram of colitis
44 associated colon cancer (CAC) model induced by AOM/DSS in mice. (B) The colon
45 length at the 8th, 12th and 23rd week. (C) Tumour incidence.

46

47 Supplementary Figure 4. The microbial composition in control, ulcerative colitis
48 (UC), adenoma (Ad) and CRC patients. (A) Partial least squares discriminant analysis
49 (PLS-DA) plot of the faecal microbiota. (B) Rarefaction curves were used to evaluate
50 the microbial richness of human faeces. (C) Shannon, (D) Chao, (E) ACE and (F)
51 Sobs indices were analysed on OUT level in human. Sobs, Chao and Ace indices
52 reflect community richness, and the Shannon index represents community diversity.
53 Data are presented as the means± SEM and analyzed by ordinary one-way ANOVA
54 with Tukey's multiple comparisons. *** $P < 0.001$, compared with control; # $P < 0.05$,
55 ## $P < 0.01$, ### $P < 0.001$, compared with UC.

56

57 Supplementary Figure 5. The microbial composition of mice with CAC. (A) PLS-DA
58 plot of the faecal microbiota. (B) Rarefaction curves of the microbial richness in mice.
59 (C) Shannon, (D) Sobs, (E) Chao and (F) ACE indices on the OUT level were
60 analyzed. Sobs, Chao and Ace indices reflect community richness, and the Shannon
61 index represents community diversity. Data are presented as the means± SEM and
62 were analysed by ordinary one-way ANOVA Tukey's multiple comparisons. ** $P <$
63 0.01, *** $P < 0.001$, compared with 0 w.

64

65 Supplementary Figure 6. FCM gating strategies. (A) Gating strategy for the analysis
66 of the percentage of CD8⁺ T cells in CD3⁺ T cells and PD-1⁺ or TNF- α ⁺ cells in CD8⁺
67 T cells. (B) Gating strategy for the analysis of the percentage of CD11b⁺ F4/80⁺ cells
68 in lymphocytes, CD16/32⁺ cells in CD11b⁺ F4/80⁺ cells and PD-1⁺ cells in CD16/32⁺
69 CD11b⁺ F4/80⁺ cells.

70

71 Supplementary Figure 7. Effects of *A. muciniphila* or Amuc_1100 on T cells in colitis
72 mice. (A) The percentage of CD4⁺ T cells in CD3⁺ T cells in the spleen. (B) The
73 fraction of CD8⁺ T cells in CD3⁺ T cells and (C) the expression of PD-1 in CD8⁺ T
74 cells in the mesenteric lymph node (MLN). Data are presented as the means \pm SEM
75 and were analysed by ordinary one-way ANOVA with Tukey's multiple comparisons.
76 ** $P < 0.01$, *** $P < 0.001$, compared with respective control.

77

78 Supplementary Figure 8. *A. muciniphila* or Amuc_1100 enhanced CTLs activation in
79 CAC mice. FCM analysis of CTLs, PD-1⁺ CTLs and TNF- α CTLs in the MLN in the
80 8th (A) and 23rd week (B).

81

82 Supplementary Figure 9. Effects of pasteurized *A. muciniphila* or Amuc_1100 on
83 CD8⁺T cells in the spleens of CAC mice. (A) The percentage of CD8⁺ T cells at 8th
84 and 23rd week. (B) The fraction of TNF- α ⁺ cells in CD8⁺T cells at 8th and 23rd week.
85 (C) The fraction of PD-1⁺ cells in CD8⁺T cells at 8th and 23rd week. Data are
86 presented as the means \pm SEM and were analysed with ordinary one-way ANOVA

87 with Tukey's multiple comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared
88 with control; # $P < 0.05$, ## $P < 0.01$, compared with AOM+DSS group.
89
90 Supplementary Figure 10. Effects of pasteurized *A. muciniphila* or Amuc_1100 on
91 macrophages in the MLN of CAC mice. (A) The percentage of macrophage
92 (CD11b⁺F4/80⁺) cells in the 8th, 12th and 23rd week. (B) The expression of CD16/32 in
93 macrophages. (C) The expression of PD-1 in macrophages. Data are presented as the
94 means ± SEM and were analyzed with ordinary one-way ANOVA with Tukey's
95 multiple comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control;
96 # $P < 0.05$, ### $P < 0.001$, compared with AOM+DSS group.

Supplementary Table 1. Patients Characteristic

	Control (n=72)	ulcerative colitis (n=58)	adenomatous polyps (n=18)	colorectal cancer (n=22)
Gender, male/female	36/36	31/27	13/5	17/5
Age (year)	44.75±17.7	42.8±14.9	62.6±9.1	64.3±10.1
Smoking	13.9%	5.2%	22.2%	40.9%
Glucose (mmol/L) ^a	4.95±0.72	4.32±0.65	4.95±0.72	5.37±1.33
TG (mmol/L) ^a	1.58±1.21	1.22±0.69	1.95±1.03	1.38±0.21

^a, Mean±SEM

Supplementary Table 2. Scoring system for disease activity index

score	Body weight loss	stool consistency	bleeding
0	none	none	none
1	0-5%	---	trace
2	5-10%	loose stool	mild hemocult
3	10-20%	---	obvious hemocult
4	>20%	diarrhea	gross bleeding

Supplementary Table 3. Specific primers sequences

Gene Name	Primer sequence	
	Forward	Reverse
<i>A. muciniphila</i>	AGAGGTCTCAAGCGTTGTTTCGGAA	TTTCGCTCCCCTGGCCTTCGTGC
Amuc_1100	GGGTACCATATGATCGTCAATTCCAAACGC	CCTTGGCTCGAGATCTTCAGACGGTTCCTG
EUB	AGAGTTTGATCCTGGCTC	TGCTGCCTCCCGTAGGAGT
16S rRNA	GTGCCAGCMGCCGCGGTAA	GGACTACHVGGGTWTCTAAT
Mus-TNF- α	CCCTCACACTCACAAACCAC	ACAAGGTACAACCCATCGGC
Mus-IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTTCATACA
Mus-IL-18	GTTTACAAGCATCCAGGCACAGC	GGTTTGAGGCGGCTTTCTTTG
Mus-IFN- γ	GCGCCAAGCATTCAATGAGC	ATCTCTTCCCCACCCCGAAT
Mus-IL-1 β	GCCACCTTTTGACAGTGATGAG	ATGTGCTGCTGCGAGATTTG
Mus-IL-33	AAGTACAGCATTCAAGACCAGC	TTATTTTGCAAGGCGGGACC
Mus-Caspase-3	TCTGACTGGAAAGCCGAAACT	AGGGACTGGATGAACCACGAC
Mus-Bcl-2	ATGATAACCGGGAGATCGTG	G TTCAGGTACTCAGTCACC

Mus-GAPDH

AGGTCGGTGTGAACGGATTTG

TGTAGACCATGTAGTTGAGGTCA

Supplementary Table 4. Antibodies

Antibodies	Use	Source	Identifier
CD3 PerCP-eFluor 710	Flow Cytometry	eBioscience	46-0032-80
CD16/32-PerCP-Cy5.5	Flow Cytometry	eBioscience	45-0161-80
FcR Block	Flow Cytometry	eBioscience	14-0161-86
Fixable Viability Dye eFlour™ 780	Flow Cytometry	eBioscience	65-0865
CD279 (PD-1)-BV421	Flow Cytometry	BD Biosciences	562584
CD4-FITC	Flow Cytometry	BD Biosciences	553046
CD8a-FITC	Flow Cytometry	BD Biosciences	553030
CD11b-FITC	Flow Cytometry	BD Biosciences	557396
F4/80-PE	Flow Cytometry	BD Biosciences	565410
TNF-PE	Flow Cytometry	BD Biosciences	561063
Annexin V/PI	Flow Cytometry	BD Biosciences	556547
CD8 (IHC)	IHC	Abcam	ab108343
F4/80 (IHC)	IHC	Santa Cruz Biotechnology	sc-377009

Cleaved-caspase 3	IHC	Cell Signaling Technology	9664
γ H2AX	IHC	Cell Signaling Technology	7631
Ki67	IHC	Cell Signaling Technology	9449
His-tag	WB	GenScript	A100186-100
