Mutualism by a microbial symbiosis factor prevents intestinal inflammatory disease

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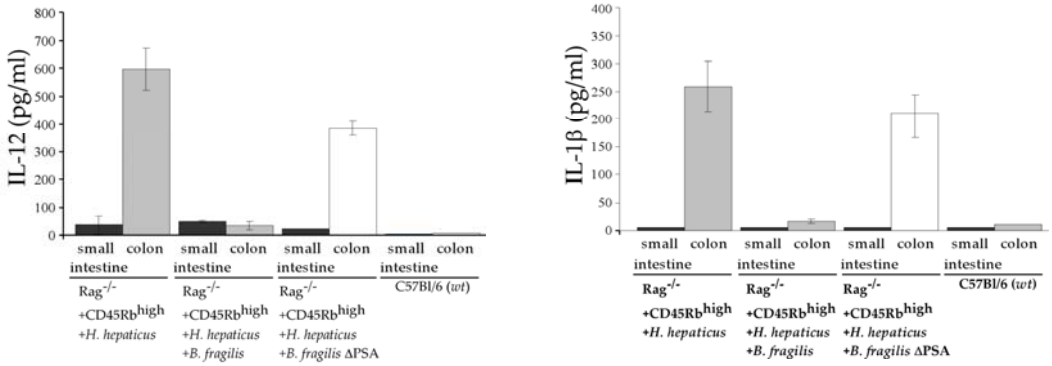
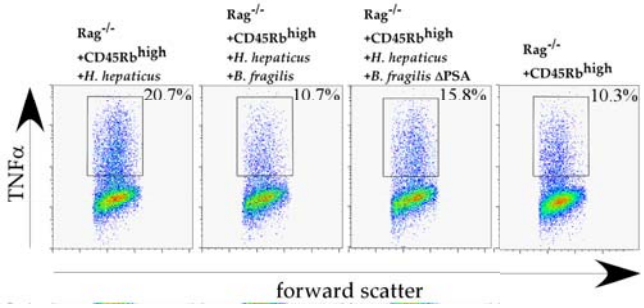
Supplemental Information

Supplemental Fig. 1



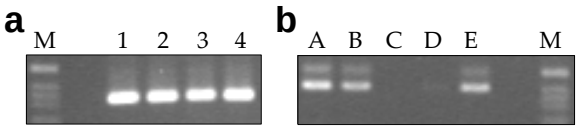
S. Fig. 1| Cultures of colons from various groups of animals demonstrate the specific increase in pro-inflammatory cytokines in diseased colons (but not in small intestines). This increase is significantly reduced in animals co-colonized with PSA-producing *B. fragilis*. Conversely, animals colonized with *B. fragilis* ΔPSA express greatly increased pro-inflammatory cytokine levels over those in control animals (C57BL/6). ELISA results for the pro-inflammatory cytokines IL-12p40 (left) and IL-1β (right) are shown. Results are from one trial of 2 independent experiments. Error bars indicate SD values from studies of colons recovered from 4 animals per group.

Supplemental Fig. 2



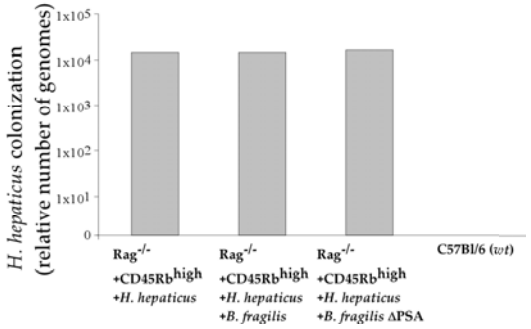
S. Fig. 2| Expression of the TNFα by CD4$^+$ T cells is reduced by wild-type *B. fragilis* colonization during experimental colitis. CD4$^+$ cells were purified from pooled splenocytes from each group (4 mice per group) and restimulated *in vitro* with PMA and ionomycin in the presence of brefeldin A for 4 hours. Cells were stained for intracellular TNFα. Cells within the lymphocyte gate were included in the analysis, and numbers indicate the percentage of cells producing TNFα. Purified cells were > 90% CD4$^+$. Animals colonized with PSA-producing *B. fragilis* during protection displayed lower TNFα levels than diseased animals.

Supplemental Fig. 3



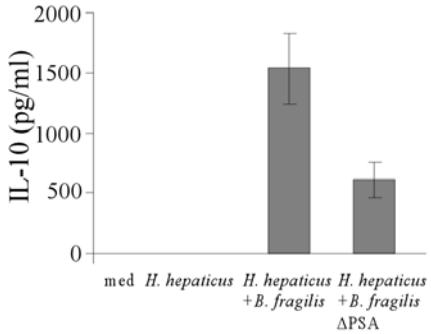
S. Fig. 3| Experimental animals remain colonized with *H. hepaticus* and *B. fragilis* throughout the course of disease. (**a**) Ethidium bromide–stained gel electrophoresis of *H. hepaticus*–specific Q-PCR shows that co-colonization with *B. fragilis* does not induce clearance of bacteria after 8 weeks. M: Marker. 1: $Rag2^{-/-}$ animals with $CD4^{+}CD45Rb^{high}$ T cell transfer colonized with *H. hepaticus* alone. 2: $Rag2^{-/-}$ animals with $CD4^{+}CD45Rb^{high}$ T cell transfer colonized with *H. hepaticus* and *B. fragilis* 9343 (wt). 3: $Rag2^{-/-}$ animals with $CD4^{+}CD45Rb^{high}$ T cell transfer colonized with *H. hepaticus* and *B. fragilis* ΔPSA. 4: C57BL/6 mice colonized with *H. hepaticus* alone. Note: *H. hepaticus* readily colonized animals but did not induce disease (Fig. 1). Primers for *H. hepaticus* 16S rDNA: (HB-15) 5'-GAAACTGTTACTCTG-3' and (HB-17) 5'-TCAAGCTCCCCGAAGGG-3'. (**b**) Ethidium bromide–stained gel electrophoresis of *B. fragilis*–specific Q-PCR showing stable bacterial colonization after 8 weeks. A: $Rag2^{-/-}$ animals with $CD4^{+}CD45Rb^{high}$ T cell transfer colonized with *H. hepaticus* and *B. fragilis* 9343 (wt). B: $Rag2^{-/-}$ animals with $CD4^{+}CD45Rb^{high}$ T cell transfer colonized with *H. hepaticus* and *B. fragilis* ΔPSA. C: $Rag2^{-/-}$ animals with $CD4^{+}CD45Rb^{high}$ T cell transfer colonized with *H. hepaticus* alone. D: C57BL/6 mice colonized with *H. hepaticus* alone. E: *B. fragilis* genomic DNA (positive control). M: Marker. Primers for *B. fragilis ssr3* (*finB*) gene: (ssr3-F) 5'-TATTTGCGAGAAGGTGAT-3' and (ssr3-r) 5'-TAAACGCTTTGCTGCTAT-3'.

Supplemental Fig. 4



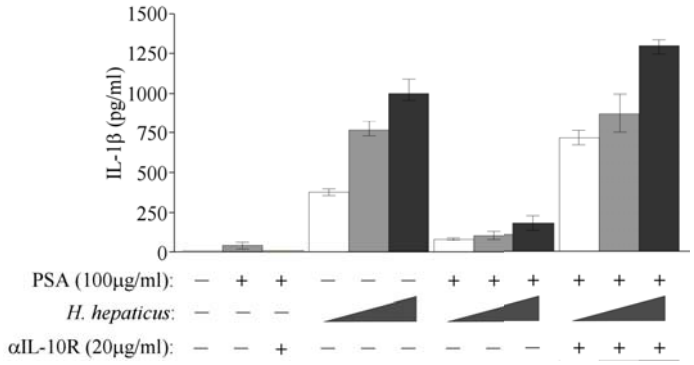
S. Fig. 4| Quantitation of *H. hepaticus* colonization demonstrates that the organism is present in equal numbers regardless of PSA-mediated protection. Fecal samples were collected from each experimental group, and total DNA was extracted (Qiagen DNAeasy tissue kit). Equal amounts of DNA (50 ng) were used in Q-PCR (Bio-rad) with *H. hepaticus*–specific primers. Q-PCR for *H. hepaticus* colonization was assessed according to *Young et al*., 2004 [1] as $\log^{10}$ number of copies of a known gene (cytolethal distending toxin). Animals contained equivalent levels of *H. hepaticus* at the end of the experiment.

Supplemental Fig. 5



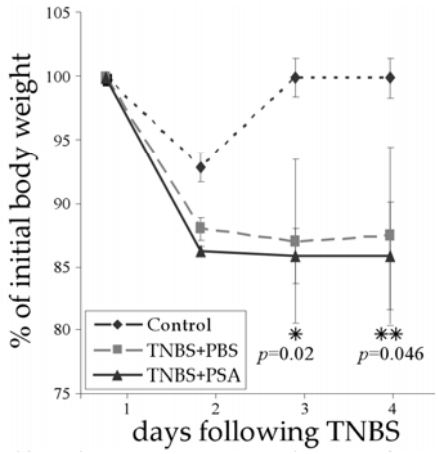
S. Fig. 5| PSA from *B. fragilis* induces expression of IL-10 in vitro. Results are shown for IL-10 ELISA of supernatants of primary BMDC–T cell co-cultures incubated for 48 hours with *H. hepaticus* alone or with *H. hepaticus* and*B. fragilis* (wild-type or ΔPSA) at a multiplicity of infection of 5.. Error bars show SD values for samples run in duplicate and represent 3 independent experiments.

Supplemental Fig. 6



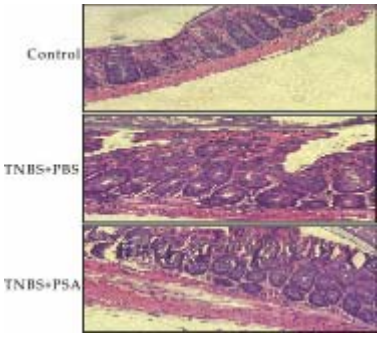
S. Fig. 6| PSA reduces expression of IL-1β in an IL-10-dependent manner. Infection of BMDC–T cell co-cultures with increasing concentrations of live *H. hepaticus* (multiplicity of infection: 0.1, 1.0, and 10, as depicted by triangles) results in release of the cytokine IL-1β. Treatment of infected cells with PSA reduces IL-1β levels, as shown in the middle three bars. Neutralization of IL-10 signaling by addition of an IL-10 receptor antibody (αIL-10R) alleviates suppression of *in vitro* inflammatory responses, resulting in increased levels of IL-1β (left three bars). Error bars show SD values for experiments run in triplicate.

Supplemental Fig. 7



S. Fig. 7| PSA does not prevent weight loss in TNBS-treated IL-10$^{-/-}$ animals. Groups of 4 C57BL/6 mice were treated with PSA (or PBS) and then subjected to rectal administration of TNBS or vehicle (control). Mean body weights (shown as percentages of initial weight) are shown for each group; SD values indicate that, in the absence of IL-10, PSA cannot restore TNBS-induced weight loss. ANOVA demonstrates that weight loss in both TNBS-treated groups is statistically different from that in control animals.

Supplemental Fig. 8



S. Fig. 8| PSA does not prevent pathology in TNBS-treated IL-10$^{-/-}$ animals. Groups of 4 C57BL/6 mice were treated with PSA (or PBS) and then subjected to rectal administration of TNBS or vehicle (control). Histologic analysis of H&E-stained sections from a representative animal from each group is shown. Thickening of the colon and epithelial hyperplasia are noted in both TNBS-treated groups of IL-10$^{-/-}$ animals, regardless of PSA treatment. Thus, in the absence of IL-10, PSA does not reduce intestinal injury in TNBS-treated IL-10$^{-/-}$ mice. Results represent 2 independent experiments.

METHODS

Bacterial strains and animals. *B. fragilis* NCTC9343 and *H. hepaticus* ATCC51149 were obtained from the American Type Culture Collection. Conventionally reared SPF mice of strains C57BL/6NTac, C57BL/6NTac IL-10$^{-/-}$, and B6.129S6-Rag2^{tm1Fwa} N12 (Rag2$^{-/-}$) were purchased from Taconic Farms (Germantown, NY) and screened negative for *B. fragilis* and *H. hepaticus*. Swiss-Webster germ-free (SWGF) mice were purchased from Taconic Farms. Upon delivery in sterile shipping containers, the mice were transferred to sterile isolators (Class Biologically Clean, Madison, WI) in our animal facility. Animals were screened weekly for bacterial, viral, and fungal contamination as previously described[2]. All animals were cared for under established protocols and the IACUC guidelines of Harvard Medical School and the California Institute of Technology.

Flow cytometry, fluorescence-activated cell sorting (FACS), and staining. Lymphocytes were isolated from mouse spleens that were mechanically disrupted into single-cell preparations. Red blood cells were lysed, and splenocytes ($1x10^6$) were incubated with various combinations of antibodies (BD Pharmingen, San Diego, CA) at 2 μg/mL for 30 min at 4°C. Cells were then washed and either fixed or used directly. For intracellular cytokine flow cytometry, samples were analyzed on a model FC500 cytometer (Beckman Coulter, Fullerton, CA) or a FacsCalibur (Becton Dickson), and data were analyzed with RXP Analysis Software (Beckman Coulter) or FlowJO. FACS was performed on a BD FACSAria, and cell purity was always >99%.

In vitro cytokine assays. For colon organ cultures, procedures were followed as previously reported[3]. For co-culture, CD4$^+$ T cells were purified from splenic lymphocytes (prepared as described above) with a CD4$^+$ T Cell Subset Kit (R&D Systems, Minneapolis, MN) used as instructed by the manufacturer. Cell purity was always >95%. BMDCs were purified from femurs of mice after extraction and washing in PBS. Cells were cultured for 8 days in C-RPMI-10 in the presence of GM-CSF (20 ng/mL; Biosource, Camarillo, CA). Medium was replaced after 4 days, and adherent cells were cultured for an additional 4 days, at which point nonadherent cells were recovered, washed, and used directly. Cells were >95% CD11c$^+$ at the time of use. Purified CD4$^+$ T cells ($1x10^6$) were mixed with purified CD11c$^+$ BMDCs ($1x10^6$) in a 48-well plate and were incubated at 37°C in an atmosphere containing 5% CO_2. Various stimuli were used, as described in Results. ELISA was performed with pre-coated plate kits (BD Pharmingen) according to the manufacturer's guidelines. In some assays, *H. hepaticus*, with or without wild-type *B. fragilis* or *B. fragilis* ΔPSA, was added at various concentrations.

Induction of experimental colitis. As assessed by PCR, $Rag2^{-/-}$ and control C57Bl/6 mice were negative for *H. hepaticus* colonization at the time of delivery. Splenic lymphocytes were harvested from wild-type donor mice, and $CD4^{+}CD45Rb^{high}$ cells were purified from lymphocyte populations by FACS as described above. Cells were washed with PBS, and $3x10^{5}$ cells were injected intraperitoneally in a volume of 0.2 mL into recipient *H. hepaticus*–colonized $Rag2^{-/-}$ animals. For colonization experiments, both *H. hepaticus* ($1x10^{8}$ organisms) and *B. fragilis* ($1x10^{8}$ organisms) were introduced at the time of cell transfer. Throughout PSA treatment studies, animals received 50 μg of PSA by gavage 3 times per week. Animals were weighed throughout the experiment until sacrifice at 8 weeks.

Induction of intestinal inflammation-TNBS colitis. The backs of wild-type (C57BL/6) male mice were shaved, and pre-sensitization solution (150 μL; acetone with olive oil in a 4:1 ratio mixed with 5% TNBS in a 4:1 ratio) was slowly applied. Seven days after sensitization, mice were anesthetized with isofluorene and TNBS solution (100 μL; 1:1 5% TNBS with absolute ethanol) administered rectally through a 3.5F catheter (Instech Solomon; SIL-C35). Mice were analyzed 4-6 days after TNBS administration.

Histologic tissue analysis. Mouse tissues in Bouin's fixative (VWR, West Chester, PA) were embedded in paraffin, sectioned (6-μm slices), mounted onto slides, and stained with hematoxylin and eosin. Sections were evaluated in blinded fashion by a single pathologist (Dr. R. T. Bronson, Harvard Medical School).

Quantitative real-time PCR. RNA was extracted with Trizol per the manufacturer's instructions (Invitrogen). RNA (1 μg) was reverse transcribed into cDNA with an iScript cDNA synthesis kit (Bio-Rad). cDNA was diluted by addition of 60 μL of water, and a 2-μL volume of this solution was used for Q-PCR. Q-PCR was performed using IQ SYBR Green supermix (Bio-Rad) and primers were used at 0.2 μm. Q-PCR was performed on a Bio-Rad iCycler IQ5. Sequences of Q-PCR primers were as follows 5'-3': *IL-23 (p19)* F: AGC TAT GAA TCT ACT AAG AGA GGG ACA R: GTC CTA GTA GGG AGG TGT GAA GTT G. *IL-17A F*: TTA AGG TTC TCT CCT CTG AA R: TAG GGA GCT AAA TTA TCC AA. *TNFα* F: ACG GCA TGG ATC TCA AAG AC R: GTG GGT GAG GAG CAC GTA GT. *IL-10* F: CTG GAC AAC ATA CTG CTA ACC G R: GGG CAT CAC TTC TAC CAG GTA A *RORγT* F: CCG CTG AGA GGG CTT CAC R: TGC AGG AGT AGG CCA CAT TAC A *IL-21* F: ATC CTG AAC TTC TAT CAG CTC CAC

R: GCA TTT AGC TAT GTG CTT CTG TTT C *IL-27* F:CTG TTG CTG CTA CCC TTG CTT

R: CAC TCC TGG CAA TCG AGA TTC.

1. Young, V. B. et al. In vitro and in vivo characterization of Helicobacter hepaticus cytolethal distending toxin mutants. Infect Immun 72, 2521-7 (2004).
2. Mazmanian, S. K., Liu, C. H., Tzianabos, A. O. & Kasper, D. L. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 122, 107-18 (2005).
3. Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S. & Medzhitov, R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118, 229-41 (2004).