uicc global cancer control

IJC
International Journal of Cancer

Parabacteroides distasonis attenuates toll-like receptor 4 signaling and Akt activation and blocks colon tumor formation in high-fat diet-fed azoxymethane-treated mice

Gar Yee Koh [1], Anne Kane[2], Kyongbum Lee[3], Qiaobing Xu[3,4,5], Xian Wu[1], Jatin Roper[5], Joel B. Mason[1,5,6] and Jimmy W. Crott[1]

[1] Jean Mayer USDA Human Nutrition Research on Aging at Tufts University, Boston, MA
[2] Phoenix Laboratory, Tufts Medical Center, Boston, MA
[3] Department of Chemical and Biological Engineering, Tufts University, Medford, MA
[4] Department of Biomedical Engineering, Tufts University, Medford, MA
[5] Tufts University School of Medicine, Boston, MA
[6] Friedman School of Nutrition Science and Policy, Boston, MA

Gut dysbiosis may play an etiological role in colorectal tumorigenesis. We previously observed that the abundance of *Parabacteroides distasonis* (Pd) in stool was inversely associated with intestinal tumor burden and IL-1β concentrations in mice. Here, we assessed the anti-inflammatory capacity of Pd membrane fraction (PdMb) in colon cancer cell lines. In addition, we tested whether Pd could suppress colon tumorigenesis in mice. Six-week-old male A/J mice were fed a low-fat (LF) diet, high-fat (HF) diet or HF+ whole freeze-dried Pd (HF + Pd, 0.04% wt/wt) for 24 weeks. After 1 week on diet, mice received 4 weekly injections of azoxymethane. PdMb robustly suppressed the production of pro-inflammatory cytokines and lowered the abundance of MyD88 and pAkt (ser473) induced by *E. coli* lipopolysaccharide in colon cancer cell lines. Moreover, PdMb induced apoptosis in colon cancer cell lines and blocked TLR4 activation in a reporter line. Colon tumors were observed in 0% of LF (0 of 19), 25% of HF (5 of 20) and 0% of HF + Pd mice (0 of 20) ($p = 0.005$). The latter group also displayed a lower abundance of MyD88 and pAkt (ser473) in colonic mucosa than HF mice. Taken together, these data suggest that Pd has anti-inflammatory and anti-cancer properties that are likely mediated by the suppression of TLR4 and Akt signaling, as well as promotion of apoptosis. Further work is needed to confirm these findings in additional models and fully elaborate the mechanism of action.

Colorectal cancer (CRC) is the 3rd most common cancer in the US and the lifetime risk for this disease is estimated to be ~4.5%. There are 135,000 new cases diagnosed and approximately 50,000 deaths from this disease annually in the US alone.[1]

Findings that the gut microbiome differs between individuals with CRC and healthy controls,[2,3] and that alterations are already present in those with precancerous adenomas,[4–7] suggest that dysbiosis of the gut microbiome could play an etiologic role in CRC. A causal role for gut microbiota in colorectal tumorigenesis is further supported by observations that gut microbiota from tumor-bearing mice[8] or specific human gut bacterial communities[9] promote colon tumorigenesis when inoculated into germ-free mice. Moreover, individual species including *Fusobacterium nucleatum,*[10] enterotoxigenic *Bacteroides fragilis*[11] and *Escherichia coli NC101*[12] can also promote tumor formation in mice. Further supporting a role for the gut microbiota in CRC are observations that antibiotic treatment[8] and germ-free conditions[13] suppress tumor formation in mice. One mechanism by which bacterial dysbiosis might promote CRC is by inciting inflammation. Inflammation affects most of the major processes that govern the evolution of a tumor including mutation, proliferation, apoptosis, vascularization, invasion and immune evasion.[14] For this reason, inflammation is widely considered a promising target for cancer prevention.[15]

In addition to tumor-promoting gut bacteria, there is a great interest in identifying bacteria with anti-inflammatory and anti-cancer properties. In our previous study, we observed the abundance of *Parabacteroides distasonis* (Pd), a Gram-negative anaerobe, to be inversely associated with colonic interleukin (IL)-1β as well as intestinal tumor burden in Apc^{1638N} mice. Others report that Pd was more frequently

Key words: *Parabacteroides distasonis*, colorectal cancer, toll-like receptor 4, protein kinase B, inflammation
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: U.S. Department of Agriculture; **Grant number:** 58-1950-4-003; **Grant sponsor:** Office of the Vice Provost for Research, Tufts University
DOI: 10.1002/ijc.31559
History: Received 7 Feb 2018; Accepted 12 Apr 2018; Online 26 Apr 2018
Correspondence to: Jimmy W. Crott, Ph.D., Vitamins and Carcinogenesis Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, Massachusetts 02111, USA, Tel: [+1-617-556-3117], E-mail: jimmy.crott@tufts.edu

What's new?

Inflammation constitutes a major mechanistic link between obesity and tumor risk. Gut microbial dysbiosis may also play an etiological role in colorectal tumorigenesis. The abundance of commensal Gram-negative anaerobe *Parabacteroides distasonis* (Pd) in stool was previously observed to be inversely associated with intestinal tumor burden and IL-1β in mice. Here, in an obesity-driven model of colorectal cancer, Pd was shown to have anti-inflammatory and anti-cancer properties that are likely mediated by the suppression of TLR4 and Akt signaling, and promotion of apoptosis. The findings suggest that administering specific protective bacterial species could have utility in the prevention of colorectal tumorigenesis in at-risk populations.

absent in colon biopsies from Crohn's disease patients than controls, especially in those with active compared to quiescent disease.[16] Moreover, Kverka *et al.*[17] demonstrated that feeding various fractions of Pd to mice could effectively suppress chemically induced colitis in mice and inhibit the secretion of pro-inflammatory cytokines in macrophages *in vitro*.

Consumption of high-fat diet has been shown to alter the composition of gut microbiota that leads to metabolic endotoxemia,[18] a phenomenon characterized by increased circulating level of bacterial lipopolysaccharides (LPS). The recognition of bacterial LPS by toll-like receptor 4 (TLR4) is known to trigger a cascade of cellular signaling to induce inflammation. In fact, it is demonstrated that overexpression of TLR4 promotes systemic inflammation and intestinal neoplasia in mice.[19,20] Compelling evidence indicates that elevated inflammation constitutes a major mechanistic link between obesity and increased tumor risk.[21,22] Further, our laboratory has demonstrated a distinct colonic transcriptome in obese mice that converge on Akt signaling pathway,[23] a critical mediator of carcinogenesis.[24–26] Hence, based on these observations, we hypothesized that Pd may possess some utility in the suppression of CRC by inhibiting colonic inflammation and the pro-tumorigenic TLR4 and Akt signaling pathways. We sought to evaluate this hypothesis in an obesity-driven model of CRC because obesity is a major risk factor for CRC.[27,28] For the first time, we observed Pd to completely block high-fat (HF) and azoxymethane (AOM)-driven colon tumorigenesis concomitant with reduced activation of Akt and TLR4 signaling.

Methods

Bacterial culture and preparation

Pd 8503 was purchased from ATCC (Manassas, VA), reconstituted in brain–heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD), streaked out on chocolate agar (Remel, Lanexa, KS), and incubated in a Gas-Pak EZ anaerobic containment system (Becton, Dickinson) at 37°C. Stocks were prepared by freezing aliquots from the plate at −80°C in 10% glycerol/BHI. Working cultures were started by streaking for isolation from the glycerol stock on a chocolate agar plate (Remel, Lanexa, KS). After 24 hr incubation, additional plates were sub-cultured from the starter plate to inoculate the volume of culture required: 1 plate per 600 mL bottle of final culture. After 24 hr, a generous swab from each plate was re-suspended in 12 mL BHI, which was then introduced into 500 mL BHI broth in a glass bottle. The bottles were then filled to the top with BHI (final volume ~600 mL), the caps were secured tightly, and they were incubated for 24–48 hr. Cultures were harvested at 10,000*g* for 10 min at 4°C and washed twice in sterile ice-cold 10 mM phosphate buffered saline (PBS) at pH 7.5 with protease inhibitor. To prepare lyophilized whole cells, the washed pellet was re-suspended in PBS and aliquots into 50 mL tubes and frozen at −80°C prior to overnight lyophilization. Total viable plate count was then performed and expressed as colony forming units per mL (cfu/mL) to confirm viability of lyophilized whole cells. To isolate the membrane fraction of *P. distasonis* (PdMb), cell pellets were passed twice through a French Pressure Cell Press (SLM Aminco/Thermo Electron Corporation, Waltham, MA) on ice at 1,500 psi. The lysate was centrifuged at 8,600*g* for 30 min at 4°C. The pellet was frozen at −80°C, followed by freeze drying. Lyophilized Pd and its membrane fractions were kept at −20°C until use.

Cell culture studies

The colorectal adenocarcinoma cell lines, HT-29 and SW480 (ATCC, Manassas, VA), were cultured in dulbecco modified eagle medium (DMEM) (Corning Inc., Corning, NY) supplemented with 10% fetal bovine serum (Gibco/Life Technologies, Grand Island, NY), 2 mmol/L of L-glutamine (Gibco/Life Technologies), and 1% penicillin–streptomycin (Gibco/Life Technologies). All cells were maintained at 37°C with 5% CO_2 and were used between passages 3 and 20.

To study pro-inflammatory signaling and cytokine production, cells were allowed to attach overnight and then switched to serum-free DMEM. Twenty-four hours later, cells were pre-treated with vehicle (media) or PdMb (25–400 μg/mL) for 2 hr and then challenged with *E. coli* LPS (1 μg/mL, variant O11:B4, Sigma Aldrich, St. Louis, MO) or vehicle.

To study IL-8 production, cells were seeded at 2×10^4 cells/well in a 96-well plate and supernatant collected 24 h after LPS challenge and stored at −80°C. Cells were then washed twice with PBS and incubated with serum-free DMEM to measure viability using the XTT assay (Thermo Fisher, Waltham, MA). IL-8 in supernatant was measured by ELISA (R&D Systems, Minneapolis, MN). For additional cytokines, cells were seeded at 5×10^4 cells/well in a 48-well plate and supernatant collected 24 hr after LPS challenge. IL-

1β, IL-6, IL-8 and tumor necrosis factor (TNFα) were measured in supernatant using a multiplex electrochemiluminescent array according to manufacturer's protocols (Meso Scale Discovery, Rockville, MD). For intracellular protein analyses, cells were seeded at 8×10^5 cells/well in a 6-well plate. One hour after LPS challenge cells were lysed with RIPA buffer (Alfa Aesar, Ward Hill, MA) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN) and lysates were stored at −80°C until immunoblot analyses.

To assess the effect of PdMb on cell proliferation, cells were seeded at a lower density on a 96-well plate (1×10^4 cells/well) and allowed to attach overnight. The cells were then treated with PdMb (25–400 μg/mL) or vehicle for 48 hr and cell viability was measured with the XTT assay. To study apoptosis, cells were seeded at 5×10^5 cells/well on a 6-well plate and allowed to attach overnight. Cells were then switched to serum-free media for 6 hr and then treated with PdMb (100 μg/mL) or vehicle. Forty eight hours later cells were lysed and stored frozen (–80°C) until the measurement of caspase-3 activity using a dye cleavage assay according to manufacturer's protocol (Biovision Inc., Milpitas, CA).

TLR4 signaling was assessed using HEK-Blue™ hTLR4 cells (InvivoGen, San Diego, CA). These are HEK 293 cells stably-transfected with human TLR4, MD2/CD14 co-receptor genes and a secreted embryonic alkaline phosphatase (SEAP) plasmid containing NF-kB response elements. Stimulation of TLR4 activates NF-kB, which subsequently induces the production of SEAP. The cells were maintained and sub-cultured according to the manufacturer's instructions and only cells between passages 3 and 6 were used. HEK-Blue hTLR4 cells were seeded on a 96-well plate at 2×10^4 cells/well. PdMb at various concentrations were added to each well and incubated for 18 hr in HEK-Blue™ Detection media containing 100 ng/mL of *E. coli* LPS. Production of SEAP was measured at 630 nm on a microplate reader (BioTek, Winooski, VT). Activation of TLR4 was expressed as a ratio to the negative (blank) control.

Mouse study

All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee of Tufts University. Mice were maintained on a 12-hr light/dark cycle, were individually housed and provided *ad libitum* access to food and water.

Six-week old male A/J mice (Jackson Lab, Bar Harbor, ME) were randomized into three groups ($n = 21$/group) receiving either a low-fat (LF) diet (10% calories from fat), HF diet (60% calories from fat) or a HF diet supplemented with 0.04% wt/wt freeze-dried Pd (HF + Pd). Diets (D12450J and D12492) were purchased from Research Diets Inc. (New Brunswick, NJ). After 1 week on diet, all mice received a weekly intraperitoneal injection of AOM (Sigma Aldrich, St. Louis, MO) at 10 mg/kg for 4 weeks. The mice were maintained on respective diets throughout the study for a total of 24 weeks. Body weight was recorded weekly and food intake was recorded every other week. Lean and fat mass was analyzed *via* MRI (EchoMRI, Houston, TX) on the final day of treatment. After 24 weeks on diet (20 weeks after final AOM injection) mice were euthanized by isoflurane inhalation, cervical dislocation, and exsanguination by cardiac puncture. The abdomen was then opened and gonadal fat removed and weighed. The colon was then removed, opened longitudinally and washed with ice-cold PBS with protease inhibitors (Roche, Indianapolis, IN). The colon was inspected under a dissecting microscope for the presence of tumors by a blinded observer. Tumor size and location were noted and tumors were photographed before being excised and fixed in formalin for later histological determination. Colonic mucosa was collected by gently scraping the remaining normal-appearing colon with glass microscope slides, frozen in liquid N_2 and stored at −80°C.

For colonic cytokine measures, mucosal scrapings were lysed in ice-cold RIPA buffer (Alfa Aesar, Ward Hill, MA) containing protease inhibitors (Roche, Indianapolis, IN) and protein concentrations determined *via* bicinchoninic acid assay (Thermo Fisher, Waltham, MA). Colonic IL-1β, IL-6 and TNFα were measured by ELISA according to manufacturer's instructions (R&D Systems, Minneapolis, MN) and normalized by protein concentrations.

TLR4, MyD88 and Akt were quantified by Western blotting. Briefly, protein samples (40 μg) were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were probed with primary TLR4 (Novus Biologicals, Littleton, CO), MyD88 (Cell Signaling, Danvers, MA), pAkt (Cell Signaling, Danvers, MA), Akt (Cell Signaling, Danvers, MA) or GAPDH (Abcam, Cambridge, MA) antibodies and then incubated with anti-rabbit (Cell Signaling, Danvers, MA) or anti-mouse (Santa Cruz, Dallas, TX) secondary antibody (1:2,000 dilutions). Bands were visualized with enhanced chemiluminescence reagent and X-ray film (Thermo Fisher, Waltham, MA). Bands were quantified using a Gel-Doc image analysis system (Quantity One Software, Bio-Rad) and proteins of interest normalized to GAPDH.

The composition of the gut microbiome was profiled and differences were evaluated as previously described.[29]

Statistical analyses

All data were analyzed with the Statistical Analysis System (SAS 9.3). Analysis of variance (ANOVA) with repeated measures was performed on body weight and food intake. All other end-points were evaluated for difference between groups using Student's t test (two groups) or one-way ANOVA followed by Tukey's *post hoc* test (multiple comparison). Comparison of tumor incidence among groups was performed by Fisher's exact test. All data are expressed as mean ± standard error unless otherwise stated. Significance was accepted at $p < 0.05$.

Results

Cell culture studies

The anti-inflammatory effect of PdMb was assessed *in vitro* using human colorectal adenocarcinoma cells as well as a reporter line. First, pre-treating SW480 and HT-29 cells with PdMb (25–400 μg/mL) caused a dose-dependent inhibition of IL-8 release induced by 1 μg/mL *E. coli* LPS (Supporting Information Figs. S1A and S1B). Compared to LPS control, pre-treatment with PdMb caused a 55% and 29% ($p < 0.05$) reduction in media IL-8 concentrations in HT-29 and SW40 cells, respectively. Importantly, PdMb treatment, even at the highest dosage, did not affect cell viability. Thus, the reductions in cytokine concentrations observed are due to reduced cytokine production from viable cells (Supporting Information Figs. S1C and S1D). In the subsequent experiments, we utilized 100 μg/mL of PdMb. In SW480 cells, this dose significantly attenuated LPS-induced production of IL-1β, IL-6, IL-8 and TNFα by 45%, 62%, 46% and 24%, respectively (Figs. 1*a*–1*d*). Similarly, in HT29 cells, except for IL-6, LPS induced a significant elevation in IL-1β, IL-8 and TNFα production that was reduced by 92%, 76% and 38% with PdMb pre-treatment (Figs. 1*e*–1*h*). Heat treatment (80°C, 30 min) caused a significant reduction in the ability of PdMb to suppress LPS-induced cytokine production suggesting that the active component(s) are likely protein(s) (Supporting Information Fig. S2).

In addition to the attenuation of cytokine production in LPS-stimulated cells, pre-treatment with PdMb downregulated the LPS-induced expression of TLR4 and MyD88 in SW480 cells by 28% ($p = 0.06$) and 44% ($p = 0.02$), respectively (Figs. 2*a*–2*c*). Similarly, the LPS-induced elevation of pAkt tended to be reduced by PdMb (Fig. 3*dp* = 0.14). No changes in TLR4 or MyD88 protein levels in HT29 cells due to LPS and PdMb treatment were detected (Figs. 2*f* and 2*g*). However, the LPS-induced elevation of pAkt in HT29 cells was completely blocked by PdMb (Fig. 2*h*).

Apoptosis and proliferation were also evaluated because they are relevant to tumorigenesis and both modulated by Akt signaling. Although PdMb did not affect cell proliferation (Supporting Information Fig. S3), it caused a 43% ($p = 0.05$) and 52% ($p = 0.02$) increase in caspase-3 activity in HT29 and SW480 cells, respectively (Fig. 3), suggesting an induction of apoptosis *in vitro*.

In order to directly assess an effect of PdMb on TLR4 signaling, we utilized a TLR4 reporter cell line in which the read-out of activation is SEAP expression. Treatment of cells with PdMb alone (25–100 μg/mL) did not affect the production of SEAP (Fig. 4), indicating that PdMb by itself does not activate TLR4. In contrast, treating cells with LPS induced a robust activation of TLR4 that was attenuated by PdMb in a dose-dependent fashion. Specifically, PdMb at 25, 50, 100 and 200 μg/mL attenuated LPS-induced TLR4 activities by 17%, 20%, 53% and 85%, compared to LPS-treated cells, respectively (Fig. 4).

Mouse study

Twenty-one mice were assigned to each group at the beginning of the study. One mouse per group died shortly after the first AOM injection. One mouse from LF group was found dead on Week 18 without apparent cause and did not display any tumors. After an initial decline in body weight during AOM administration all mice recovered and gained weight rapidly (Supporting Information Fig. S4). Although weights for the two HF-fed groups were consistently numerically higher than those on the LF diet throughout the study, those differences did not reach significance ($p = 0.48$). At the end of the study, HF mice tended to have a higher fat mass and lower lean mass compared to the LF but differences were also not significant. In contrast, gonadal fat weight was 32% higher in HF than LF mice ($p < 0.001$, Supporting Information Table S1). Pd administration did not affect any index of adiposity. Caloric intake of HF mice was significantly higher than in LF mice, but no difference was detected between HF and HF + Pd mice ($p = 0.27$, Supporting Information Fig. S5).

Colon tumors, histologically confirmed to be neoplastic, were observed in 0% (0 of 19), 25% (5 of 20) and 0% (0 of 20) of LF, HF and HF + Pd mice, respectively (Fisher's exact test = 0.005) (Table 1). Concentrations of IL-6 in the colonic mucosa, but not TNF-α and IL-1β, were elevated in HF, compared to LF mice; however Pd addition did not significantly affect any of these cytokines (Table 1). We next measured the expression of TLR4 and MyD88 and, although TLR4 did not differ between groups (Figs. 5*a* and 5*c*), MyD88 was significantly lowered by Pd consumption (Figs. 5*a* and 5*d*, $p = 0.004$). Consistent with cell culture data, Pd consumption reduced pAkt (ser473) in HF-fed mice by 47% (Figs. 5*b* and 5*e*), however no differences were observed between LF and HF mice.

The fecal microbiome was profiled by 16S rRNA gene sequencing. An average of 61,385 reads were retrieved per sample (range = 26,364–109,326). The number of sequences was normalized to 26,100. There was no effect of diet or Pd addition on chao1, observed species, Shannon, PD whole tree or equitability measured of diversity (GLM, $p > 0.05$). Similarly, the abundance of each phyla or the ratio of firmicutes to bacteroidetes was not affected by diet or Pd addition (GLM, $p > 0.05$). Using Multivariate Association with Linear Models (MAsLin),[30] we identified 20 taxa that were significantly associated with diet, and one taxa (genus *Anaerotruncus*) was associated with Pd supplementation (Supporting Information Table S2).

Discussion

Our previous studies found the abundance of Pd in stool to be inversely associated with intestinal tumor burden and IL-1β concentrations in obese Apc^{1638N} mice.[29] This finding, together with reports that Pd administration significantly attenuated chemically induced colitis in mice,[17] led us to hypothesize that Pd would be effective in suppressing CRC by

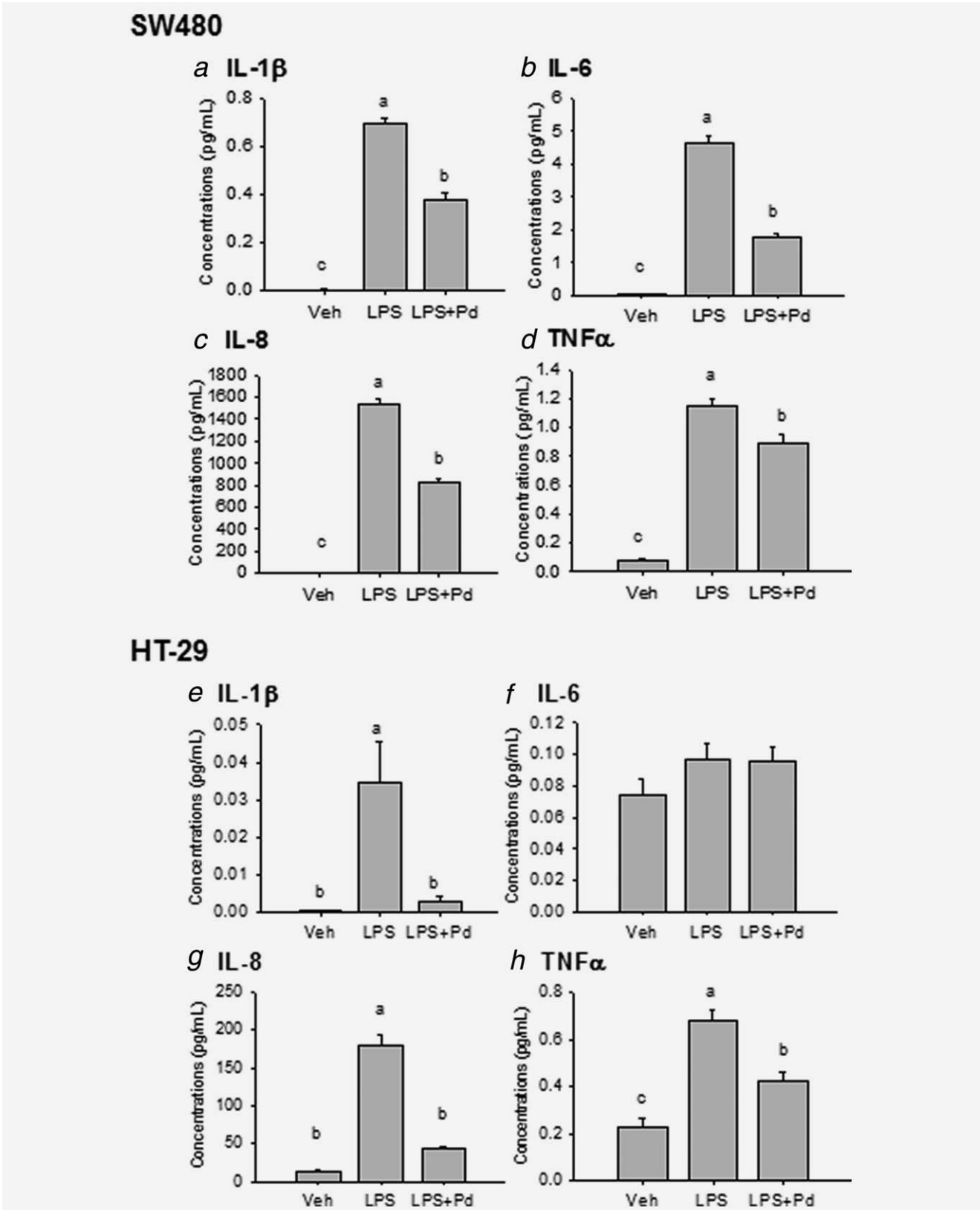

Figure 1. Pre-treatment with PdMb attenuated cytokine production in LPS-stimulated SW480 cells (*a–d*) and HT29 cells (*e–h*). Veh, vehicle-treated cells; LPS, vehicle-treated cells stimulated with 1 μg/mL of LPS; LPS + Pd, Pd-treated cells stimulated with 1 μg/mL of LPS. Data are shown as mean ± SEM ($n = 3$) and representative of three independent experiments. Groups with different letters are significantly different ($p < 0.05$).

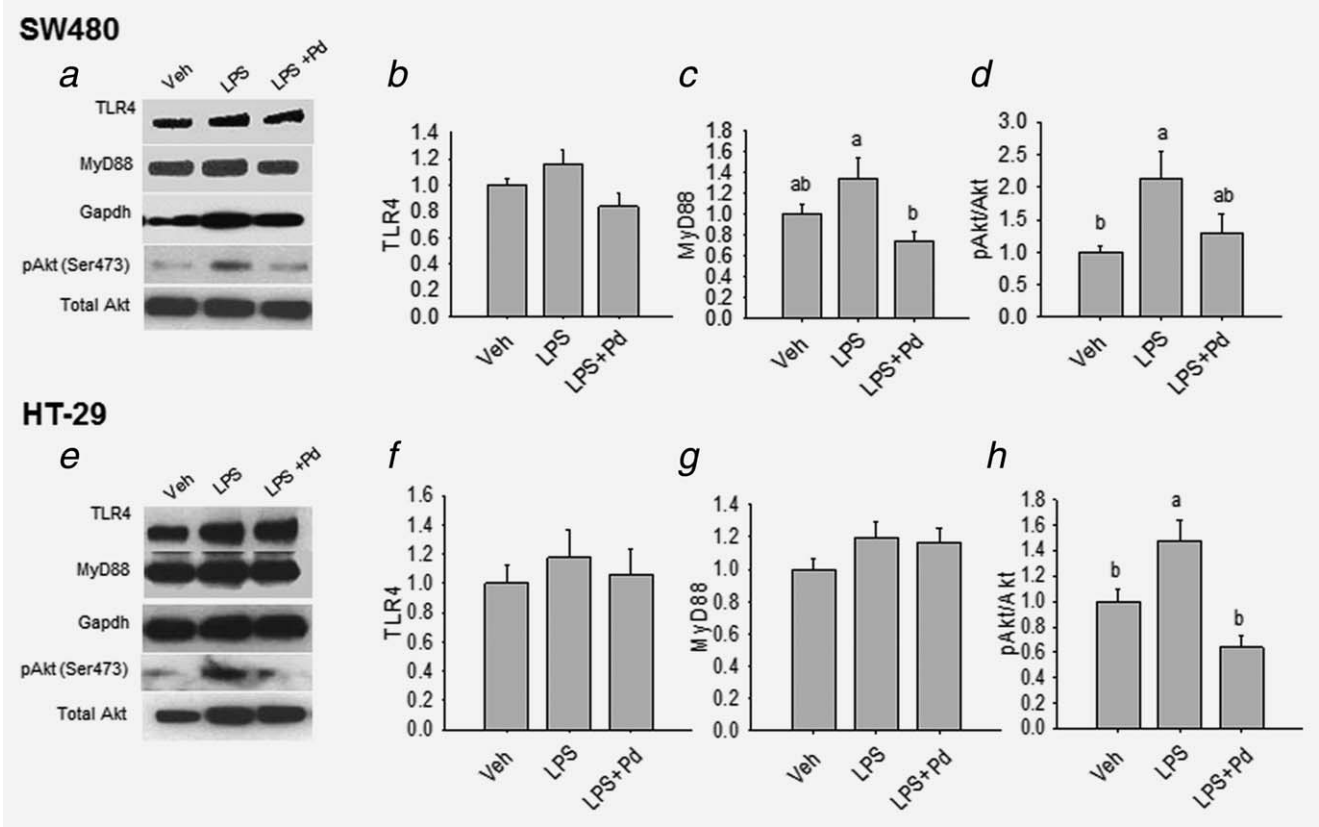

Figure 2. Effects of PdMb on innate immune signaling and phosphorylated Akt expression in LPS-stimulated SW480 (*a–d*) and HT29 (*e–h*) cells. Veh, vehicle-treated cells; LPS, vehicle-treated cells stimulated with 1 μg/mL of LPS; LPS + Pd, Pd-treated cells stimulated with 1 μg/mL of LPS. Data are shown as mean ± SEM ($n = 3$) and representative of two independent experiments. Groups with different letters are significantly different ($p < 0.05$).

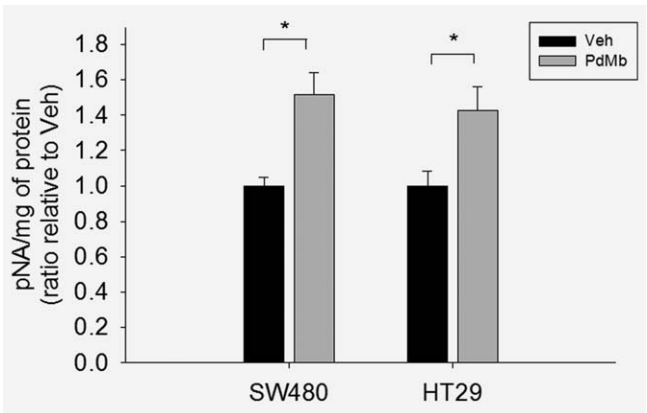

Figure 3. PdMb suppressed caspase-3 activity in SW480 and HT-29 cells. Veh, vehicle-treated cells; PdMb, Pd-treated cells at 100 μg/mL. Data are shown as mean ± SEM ($n = 3$). *$p < 0.05$.

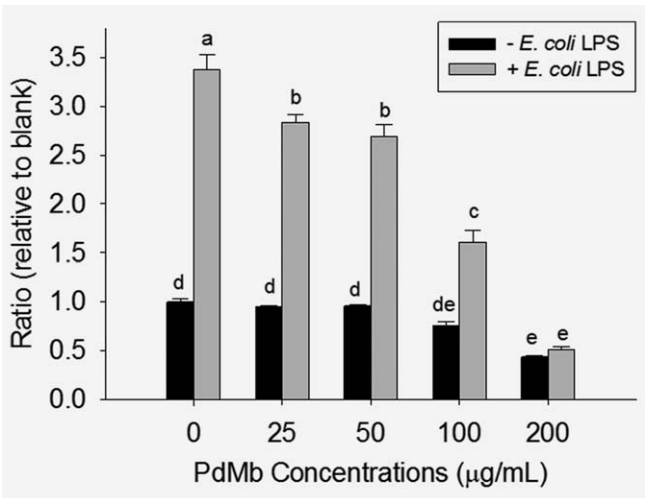

Figure 4. PdMb inhibited LPS-induced TLR4 activation in a dose-dependent manner. Data are shown as mean ± SEM ($n = 3$) and representative of three independent experiments. Groups with different letters are significantly different ($p < 0.05$).

blocking inflammation. Using CRC cell lines, we found that a crude Pd membrane extract robustly suppressed the release of inflammatory cytokines elicited by *E. coli* LPS. This data is in good agreement with reports that PdMb suppressed LPS-induced TNF-α production by RAW264.7 macrophages.[17] Taken together, these data strongly indicate that Pd has a direct anti-inflammatory effect on multiple cell types.

To test the anti-cancer potential of Pd, we employed a model of CRC driven by AOM and HF feeding. Unlike our cell culture studies which utilized a crude membrane extract, whole lyophilized bacteria were used for this study. As

Table 1. Tumor incidence and colonic cytokine concentrations of AOM-treated mice by group

	LF	HF	HF + Pd
No. mice with tumors (%)	0 of 19 (0[b])	5 of 20 (25[a])	0 of 20 (0[b])
Colonic IL-6 (pg/mg of protein)	5.41 ± 0.44[a]	7.46 ± 0.62[b]	6.58 ± 0.66[ab]
Colonic IL-1β (pg/mg of protein)	3.41 ± 0.37	4.23 ± 0.37	4.90 ± 0.43
Colonic TNF-α (pg/mg of protein)	2.81 ± 0.31	3.70 ± 0.41	2.61 ± 0.31

Data are expressed as mean ± SEM ($n = 15–20$). Mean values across the row with different letters are differ, $p \leq 0.05$.

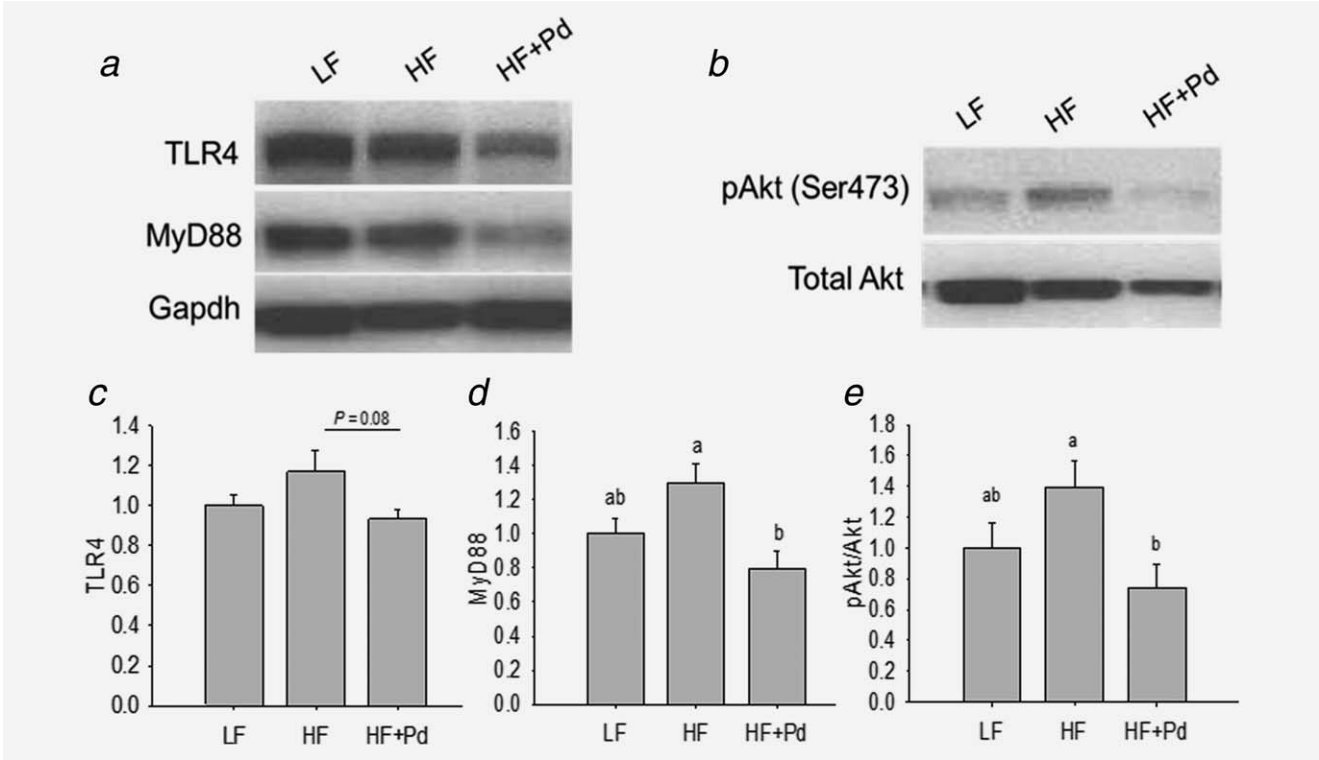

Figure 5. Pd attenuated the colonic expression of TLR4, MyD88 and pAkt in AOM-treated high-fat fed mice. (a) Representative Western blot analysis of TLR4 and MyD88. (b) Representative Western blot analysis of pAkt. (c) Quantification of TLR4 (n = 10), (d) MyD88 (n = 10), and (e) pAkt (n = 10) expression in LF, HF and HF + Pd mice. GAPDH was used as a loading control and protein expression was calculated as fold-changes relative to LF. Groups with different letters are significantly different ($p < 0.05$). Data are representative of two independent experiments.

expected, colon tumor incidence in HF mice was significantly higher than in LF control mice (25% *vs.* 0%). Addition of Pd to the HF diet completely blocked tumor formation (0% incidence), demonstrating that Pd has cancer preventive capabilities. Because the viability of this lyophilized Pd preparation is very low (2×10^4 cfu/mg of lyophilized Pd), we predict that its action is mainly due to a factor present on the cell surface rather than the secretion of a factor that requires an active process and viable cells. Indeed, using 16S rRNA gene sequencing we were unable to detect Pd in the stool of mice—indicating that no live Pd was able to colonize these mice. Only one taxa, Anaerotruncus, was associated with Pd supplementation (Supporting Information Table S2); however, there are no reports of this taxa affecting colorectal tumorigenesis.

To evaluate possible mechanisms for the apparent anti-inflammatory and anti-tumor activities of Pd, we scrutinized members of the TLR4 and Akt signaling pathways. TLR4 incites inflammation by interacting with the adaptor protein MyD88 then activating Ikkβ and finally NF-κB to activate the expression of inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8.[14] Observations that TLR4 is up-regulated in human CRCs[31,32] and that TLR4 overexpression promotes,[20] while knockout attenuates[33] tumorigenesis in mice, implicate this pathway in colorectal tumorigenesis. Further, constitutive activation of Ikkβ and NF-κB has also been shown to promote colonic inflammation and tumorigenesis in mice.[34,35] Serving as the ligand for TLR4, *E. coli* LPS caused an elevation of MyD88 in CRC cell lines *in vitro*. Consistent with an inhibition of TLR4 signaling, PdMb pre-

treatment suppressed the LPS-induced MyD88 increase in these cells. Similarly, Pd also lowered the abundance of MyD88 protein in the colon of HF fed mice. To directly evaluate an effect of Pd on this pathway, we employed a TLR4 reporter cell line and demonstrated that PdMb caused a dose-dependent inhibition of LPS-induced TLR4 signaling. Together, these observations indicate that Pd can suppress TLR4 activation, however it remains to be determined where in the pathway this inhibition occurs (*i.e.*, upstream at the receptor or somewhere downstream), and whether this inhibition is causally involved in the observed suppression of cytokine release and tumor formation or merely associated.

TLR4 activation can lead to the activation of Akt through PI3K, which also contributes to activation of NF-κB.[36,37] We previously found that both diet and genetically induced obesity induced robust changes in gene expression consistent with an elevation of Akt signaling.[23] Akt signaling regulates several cellular pathways relevant to carcinogenesis, including proliferation and apoptosis.[38] Aberrations in the Akt pathway are a frequent event in several human cancers.[39] In the current study, we assessed Akt activation by probing for pAkt (ser473) and found that Pd reduced this active form of Akt in CRC cell lines and in the colon of HF fed mice. These data demonstrate that Pd can suppress the activation of TLR4 and Akt and provide a plausible explanation of how Pd suppresses tumorigenesis on a molecular level. In an effort to understand how these molecular changes might alter tumorigenesis, we evaluated apoptosis and proliferation—processes that are both modulated by Akt signaling. In cell culture, we found no evidence of an effect of Pd on cellular proliferation; however we did observe a robust induction (43%–52% increase) of apoptosis in cells treated with Pd. Thus, we speculate that Pd might suppress tumor formation in mice by promoting apoptosis.

Our findings that PdMb suppressed cytokine release and TLR4 signaling *in vitro*, and that whole lyophilized Pd blocked high fat-driven colon tumor formation, is in good agreement with data by Kverka *et al.*[17] who showed that PdMb suppressed cytokine release from RAW264.7 macrophages *in vitro* and attenuated acute and chronic dextran sodium sulfate (DSS)-induced colitis in mice. Interestingly, these findings contrast with those of Dziarski *et al.*[40] who reported that gavaging with Pd (2×10^8 cfu), either once in antibiotic-treated mice or five times in mice not treated with antibiotics, significantly increased the severity of DSS-induced colitis as well as mortality. It is difficult to reconcile these contrasting outcomes; however, key differences in experimental design are apparent. Of note, the latter group employed a very harsh 5% DSS regimen which resulted in >60% mortality in the control group after 14 days. Importantly, established protocols recommend using 2% DSS.[41,42] Kverka *et al.*[17] used 3% DSS and did not report any mortality with PdMb even after 4 one-week DSS cycles. Another key difference between these studies is that Dziarski *et al.*[40] utilized live Pd while Kverka *et al.*[17] utilized PdMb and other fractions. However, others report that live Pd did not cause any adverse effect upon inoculation into germ-free mice. Rather, mice mono-associated with Pd had a higher abundance of anti-inflammatory regulatory T-cells in their colonic mucosa as well as higher concentrations of the short chain fatty acids butyrate and acetate than germ-free mice.[43] In our own studies, we did not observe any adverse effects of gavaging obese mice with live Pd (1×10^9 cfu) every other day for 2 weeks and actually saw a trend for reduced colonic and plasma inflammatory cytokines (data not shown).

Two factors should be considered when interpreting the current mouse data. First, we utilized A/J mice which, although sensitive to AOM-induced tumorigenesis,[44] are relatively resistant to diet-induced obesity.[45] Thus, the degree of obesity achieved was exceedingly mild and only manifested to a significant degree as an increase in visceral adiposity (*i.e.*, increased gonadal fat mass). Consistent with this mild degree of obesity, only a very mild degree of colonic inflammation was achieved as evidence by a modest 38% increase in IL-6 and insignificant changes in TNF-α and IL-1β. Given this mild degree of inflammation, we speculate that inflammation is not the only driver of tumorigenesis in the HF mice, nor its attenuation the mechanism by which Pd blocked tumor formation in this setting. Thus, we speculate that the robust degree of chemoprevention afforded by Pd consumption is likely due to the promotion of apoptosis. Second, in an effort to elicit a modest colon tumor burden (which might be more amenable to modulation *via* Pd), we administered only four AOM injections rather than the commonly used six injections which is reported to cause an average of eight tumors per mouse.[44] As a result of this milder carcinogen regimen, we did not observe any tumors in the LF control mice and only a 25% penetrance in the HF-fed mice. It remains to be determined how effective Pd might be in suppressing tumorigenesis in models with a higher tumor burden. Furthermore, it remains to be determined whether Pd might also protect against tumor formation in mice consuming regular chow or other low fat diets.

In conclusion, we have demonstrated that a crude membrane fraction of the Gram-negative anaerobe *P. distasonis* can dose-dependently suppress the release of inflammatory cytokines from colon cancer cell lines as well as TLR4 signaling in a reporter line. Moreover, Pd membrane fraction suppressed activation of Akt signaling as evidenced by a reduced phosphorylated Akt. Consistent with these observations, feeding whole freeze-dried Pd reduced the level of TLR4 and Akt activation in HF-fed mice. Finally, the reduced activity of these pro-tumorigenic pathways and induction of apoptosis constitutes a highly plausible mechanism underlying the blockade of tumor formation observed in these mice. Based on these data we speculate that administering components of Pd or interventions to expand resident Pd communities could have utility in suppressing colonic inflammation and tumorigenesis in at-risk individuals such as the obese.

Acknowledgements

Any opinions, findings, conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture. The authors thank Dr. Donald Smith and the staff of Comparative Biology Unit at the HNRCA for assistance with animal care, Shahin Sarkarati-Smith of the Nutrition Evaluation Laboratory for assistance with cytokine analyses and Dr. Roderick T. Bronson (Dana-Farber/Harvard Cancer Center Rodent Histopathology Core) for his expertise on tumor classification. Special thanks for Dr. Michael Malamy for helpful comments and suggestions.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016;66:7–30.
2. Wang T, Cai G, Qiu Y, et al. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J* 2012;6: 320–9.
3. Ahn J, Sinha R, Pei Z, et al. Human gut microbiome and risk for colorectal cancer. *J Natl Cancer Inst* 2013;105:1907–11.
4. McCoy AN, Araujo-Perez F, Azcarate-Peril A, et al. Fusobacterium is associated with colorectal adenomas. *PLoS One* 2013;8:e53653.
5. Shen XJ, Rawls JF, Randall T, et al. Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. *Gut Microb* 2010;1:138–47.
6. Peters BA, Dominianni C, Shapiro JA, et al. The gut microbiota in conventional and serrated precursors of colorectal cancer. *Microbiome* 2016;4: 69.
7. Hale VL, Chen J, Johnson S, et al. Shifts in the fecal microbiota associated with adenomatous polyps. *Cancer Epidemiol Biomarkers Prev* 2017; 26:85–94.
8. Zackular JP, Baxter NT, Iverson KD, et al. The gut microbiome modulates colon tumorigenesis. *mBio* 2013;4:e00692-13.
9. Baxter NT, Zackular JP, Chen GY, et al. Structure of the gut microbiome following colonization with human feces determines colonic tumor burden. *Microbiome* 2014;2:20.
10. Kostic AD, Chun E, Robertson L, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 2013;14: 207–15.
11. Wu S, Rhee KJ, Albesiano E, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med* 2009;15:1016–22.
12. Arthur JC, Perez-Chanona E, Muhlbauer M, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* 2012;338:120–3.
13. Uronis JM, Muhlbauer M, Herfarth HH, et al. Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. *PloS One* 2009;4:e6026.
14. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006;441:431–6.
15. Terzic J, Grivennikov S, Karin E, et al. Inflammation and colon cancer. *Gastroenterology* 2010;138: 2101 e5–2114 e5.
16. Zitomersky NL, Atkinson BJ, Franklin SW, et al. Characterization of adherent bacteroidales from intestinal biopsies of children and young adults with inflammatory bowel disease. *PloS One* 2013; 8:e63686.
17. Kverka M, Zakostelska Z, Klimesova K, et al. Oral administration of *Parabacteroides distasonis* antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. *Clin Exp Immunol* 2011;163:250–9.
18. Cani PD, Amar J, Iglesias MA, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007;56:1761–72.
19. Wang L, Chen Q, Qi H, et al. Doxorubicin-induced systemic inflammation is driven by upregulation of toll-like receptor TLR4 and endotoxin leakage. *Cancer Res* 2016;76:6631–42.
20. Santaolalla R, Sussman DA, Ruiz JR, et al. TLR4 activates the beta-catenin pathway to cause intestinal neoplasia. *PloS One* 2013;8:e63298.
21. Aleman JO, Eusebi LH, Ricciardiello L, et al. Mechanisms of obesity-induced gastrointestinal neoplasia. *Gastroenterology* 2014;146:357–73.
22. Yehuda-Shnaidman E, Schwartz B. Mechanisms linking obesity, inflammation and altered metabolism to colon carcinogenesis. *Obes Rev* 2012;13: 1083–95.
23. Pfalzer AC, Kamanu FK, Parnell LD, et al. Interactions between the colonic transcriptome, metabolome, and microbiome in mouse models of obesity-induced intestinal cancer. *Physiol Genomics* 2016;48:545–53.
24. Nakatani K, Thompson DA, Barthel A, et al. Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J Biol Chem* 1999;274:21528–32.
25. Rychahou PG, Kang J, Gulhati P, et al. Akt2 overexpression plays a critical role in the establishment of colorectal cancer metastasis. *Proc Natl Acad Sci USA* 2008;105:20315–20.
26. Khan MW, Keshavarzian A, Gounaris E, et al. PI3K/AKT signaling is essential for communication between tissue-infiltrating mast cells, macrophages, and epithelial cells in colitis-induced cancer. *Clin Cancer Res* 2013;19:2342–54.
27. Bardou M, Barkun AN, Martel M. Obesity and colorectal cancer. *Gut* 2013;62:933–47.
28. Larsson SC, Wolk A. Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies. *Am J Clin Nutr* 2007;86:556–65.
29. Pfalzer AC, Nesbeth PD, Parnell LD, et al. Diet- and genetically-induced obesity differentially affect the fecal microbiome and metabolome in Apc1638N mice. *PloS One* 2015;10:e0135758.
30. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;12:R60.
31. Doan HQ, Bowen KA, Jackson LA, et al. Toll-like receptor 4 activation increases Akt phosphorylation in colon cancer cells. *Anticancer Res* 2009;29: 2473–8.
32. Lu CC, Kuo HC, Wang FS, et al. Upregulation of TLRs and IL-6 as a marker in human colorectal cancer. *IJMS* 2014;16:159–77.
33. Fukata M, Chen A, Vamadevan AS, et al. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. *Gastroenterology* 2007;133:1869–81.
34. Vlantis K, Wullaert A, Sasaki Y, et al. Constitutive IKK2 activation in intestinal epithelial cells induces intestinal tumors in mice. *J Clin Invest* 2011;121:2781–93.
35. Shaked H, Hofseth LJ, Chumanevich A, et al. Chronic epithelial NF-kappaB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation. *Proc Natl Acad Sci USA* 2012;109:14007–12.
36. Bauerfeld CP, Rastogi R, Pirockinaite G, et al. TLR4-mediated AKT activation is MyD88/TRIF dependent and critical for induction of oxidative phosphorylation and mitochondrial transcription factor A in murine macrophages. *J Immunol* 2012;188:2847–57.
37. Laird MH, Rhee SH, Perkins DJ, et al. TLR4/MyD88/PI3K interactions regulate TLR4 signaling. *J Leukoc Biol* 2009;85:966–77.
38. Chan CH, Jo U, Kohrman A, et al. Posttranslational regulation of Akt in human cancer. *Cell Biosci* 2014;4:59.
39. Garcia-Echeverria C, Sellers WR. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. *Oncogene* 2008;27:5511–26.
40. Dziarski R, Park SY, Kashyap DR, et al. Pglyrp-regulated gut microflora *Prevotella falsenii*, *Parabacteroides distasonis* and *Bacteroides eggerthii* enhance and *Alistipes finegoldii* attenuates colitis in mice. *PloS One* 2016;11:e0146162.
41. Wirtz S, Popp V, Kindermann M, et al. Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc* 2017;12:1295–309.
42. Wirtz S, Neufert C, Weigmann B, et al. Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2007;2:541–6.
43. Faith JJ, Ahern PP, Ridaura VK, et al. Identifying gut microbe-host phenotype relationships using combinatorial communities in gnotobiotic mice. *Sci Transl Med* 2014;6:220ra11.
44. Bissahoyo A, Pearsall RS, Hanlon K, et al. Azoxymethane is a genetic background-dependent colorectal tumor initiator and promoter in mice: effects of dose, route, and diet. *Toxicol Sci* 2005; 88:340–5.
45. Bullen JW, Jr., Ziotopoulou M, Ungsunan L, et al. Short-term resistance to diet-induced obesity in A/J mice is not associated with regulation of hypothalamic neuropeptides. *Am J Physiol Endocrinol Metab* 2004;287:E662–E670.