Supplementary Text: Materials and Methods.

Chemicals and reagents

Dextran sulfate sodium (DSS) was purchased from MP Biomedicals (molecular weight, 36,000-50,000). Concanavalin A (diluted to a final concentration of 2.5 μg/mL) was purchased from Sigma-Aldrich.

Analysis of the Hexane Fraction of AG

With further long-term Bioassay-Guided Fractionation studies ongoing and given our current findings, we thought it was prudent to provide a preliminary analysis of the Hexane Fraction of AG for potential bioactive compounds. To provide additional hexane fraction of AG for chemical analysis by gas and liquid chromatography and ultimately further sub-fractionation and bioassay, 100 g of the AG extract was dissolved in 1 L of water and partitioned against 8 x 400 mL of hexane using a 2 L separatory funnel. The combined hexane fractions were filtered through glass fiber to remove particulates and evaporated down using a vacuum centrifuge to yield 0.54 g of a free flowing, light yellow colored oil.

Fatty acid analysis by gas chromatography (GC)-mass spectrometry (MS) and flame ionization detector (FID)

For fatty acid analysis, two aliquots, nominally 50 mg, of the Hexane Fraction of AG were transferred to 10 mL reaction vials, and exact weights recorded. An accurately weighed spike of 23:0 methyl ester solution was added to each vial as an internal standard prior to the reaction and the contents thoroughly mixed by brief sonication. The spiked hexane extracts were directly transesterified using 3 mL of 2% H2SO4 in methanol for 30 min at 90oC in a

thermostated heating block (Pierce, Rockford, IL). After cooling and addition of 1 mL water, the fatty acid methyl esters were recovered by partitioning into hexane (2 x 2 mL) and brought to a final volume of 5 mL in a volumetric flask.

Fatty acid methyl esters were analyzed on a Varian Saturn 2200 GC-MS system (Varian Inc., Palo Alto. CA). The GC was a model CP3800 equipped with both a CTC Analytics CombiPal autosampler (Zwingen, CH) and a flame ionization detector (FID). Samples (1 uL) were injected at a temperature of 250oC with split ratios of 1/25 (FID) or 1/100 (MS) on a 30 m x 0.25 mm ID x 0.25μm film thickness Famewax column (Restek Corp., Bellefonte, PA). The temperature program was as follows: initial temperature 195oC, ramped at 5oC/min to 240oC and held for 9 min for a total run time of 18 min. Ultra high purity helium was used as a carrier gas at a flow rate of 1.1 mL/min.

Liquid Chromatography (LC)-UV analysis

The Hexane Fraction of AG was also quantitatively analyzed by LC-MS specifically to look for the presence of ginsenosides and their aglycones (protopanaxdiol and protopanaxtriol) using single ion monitoring in negative ion mode using authentic in-house materials as standards. Two replicate samples of the hexane extract, accurately weighed, were prepared in MeOH at a nominal concentration of 0.5 mg/mL. Ginsenoside analysis was done on a 2.1 x 100 mm Waters Symmetry Shield C-18 column using gradient elution from 20 to 40% ACN in water in 40 min at a flow rate of 0.3 mL/min and an injection volume of 2 μL and aglycone analysis was done on a 2.1 x 50 mm Waters Symmetry Shield C-8 column using gradient elution from 40 to 75% ACN in water in 15 min at a flow rate of 0.4 mL/min and an injection volume of 2 μL.

Cell culture and treatment

ANA-1 murine macrophage cells were received as a kind gift from Michael Espey (National Cancer Institute, Bethesda, MD) and maintained in Dulbecco's modified Eagle's media (Hyclone, Logan, UT) supplemented with 10% New Born Calf serum (NBCS) (Biofluids, Rockville, MD), 2 mM glutamine (Biofluids), penicillin (10 U/ml) and streptomycin (10 μg/ml, Biofluids) in growing suspension culture at 37°C in a humidified 5% CO_2 atmosphere. Experiments with AG extract/fractions were carried out by pre-incubating cells with indicated concentrations of AG extract/fractions for indicated times. All AG extracts/fractions were dissolved in DMEM medium (0.1% NBCS). Following a wash, cells were activated by exposure to 100 U/ml interferon (IFN)-γ (R&D Systems, Minneapolis, MN).

TK6 lymphoblastoid cells were a kind gift from Curtis C. Harris (National Cancer Institute, Bethesda, MD), originally derived from Dr. William Thilly's and Howard Liber's labs. TK6 cells are a lymphoblastoid cell line derived from the spleen more than 30 y ago (26). TK6 cells were maintained in exponentially growing suspension culture at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L l-glutamine. No authentication of the ANA-1 or TK6 cell lines was done by the authors.

$CD4^+/CD25^-$ cells from C57BL/6 mice were purified from the spleens using nylon wool columns (Polysciences) followed by depletion of B cells and macrophages. The purity of T cells was 90% as determined by flow cytometry (Cytomics FC 500, Beckman Coulter). $CD4^+/CD25^-$ T cells were then isolated using a MACS mini separator and CD4 and CD25 microbeads according to the manufacturer's instructions (Miltenyi Biotec) by depletion of $CD4^-CD25^+$ T cells (negative selection). $CD4^+/CD25^-$ effector T cells (1×10^6) were cultured in six-well plates

overnight followed by experimentation as indicated. All cells were maintained in exponentially growing suspension culture at 37°C in a humidified, 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

DSS mouse model of colitis

We followed our previous protocol for our DSS (MP Biomedicals, Solon, OH: 36 000-50 000 mw) mouse model of colitis (11). Briefly, 8-10 week old C57BL/6 mice received either water *ad libitum* or 1% DSS. All mice were on an AIN93M diet as described previously (27). 11.9 mg/kg of whole AG extract or the Hexane Fraction of AG were dissolved in 100 µl 1x PBS per mouse and administered daily by oral gavage (per os, PO). 11.9 mg/kg daily, which is the human equivalent dose of 58 mg daily (28). Of note, currently the use of ginseng in human clinical trials can range anywhere from 200 mg to 9 g daily (29, 30). The control group of mice was given 100 µl of maltodextrin dissolved in 1x PBS by oral gavage. All procedures performed were in accordance with the Guide for care and Use of laboratory animals (National Research Council, Washington, DC) and approved by the Animal Resource Facility, University of South Carolina, Institutional Animal Care and Use Committee. To determine whether the Hexane Fraction of AG can reverse/treat colitis, mice were fed 1% DSS for 1.5 cycles (7 days DSS, 7 days water and 7 days DSS) and then given control, the whole AG extract, or the Hexane Fraction of AG daily by oral gavage throughout the course of the experiment. Mice were euthanized at 0 cycles, 1.5 cycles, 3.5 cycles (another 2 cycles of DSS and water) and 5.5 cycles (another 4 cycles of DSS and water) (Supplementary Figure 1). For pathology and immunohistochemistry, colon tissue samples were washed with phosphate-buffered saline (PBS;

Mediatech, Herndon, VA), cut longitudinally, swiss-rolled, then formalin fixed overnight, and paraffin embedded.

Disease activity index (DAI)

The DAI, which monitors weight loss, stool consistency, and blood in the stool as a measure of disease severity, was scored for each animal every third day throughout the experiment. The DAI was calculated for each animal as done previously (13). With this scoring system, the DAI is calculated by scoring each animal for weight loss, stool consistency, and blood in the stool and then dividing the total score by 3. For example, an animal that lost 12% of its body weight (score of 3) with evidence of loose stool (score of 2) plus gross rectal bleeding (score of 4) would have a calculated DAI of 3.

Quantification of inflammation to examine effects on colitis

Paraffin embedded tissues were serially sectioned, and one section from each mouse was stained with H&E. Sections were microscopically examined for histopathologic changes using the following scoring system. Histology score was determined by multiplying the percent involvement for each of the three following histologic features by the percent area of involvement (13) : inflammation severity (0, none; 1, minimal; 2, moderate; 3, severe), inflammation extent (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), and crypt damage (0, none; 1, one third of crypt damaged; 2, two thirds of crypt damaged; 3, crypts lost, surface epithelium intact; 4, crypts lost, surface epithelium lost). Percent area involvement was defined as: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%. Therefore, the minimal score is

0 and the maximal score is 40. The intensity of the staining was evaluated independently by two blinded investigators (D.P and A.C).

AOM/DSS-induced colon cancer model

Supplementary figure 2 outlines the time line of the protocol. We followed a modified protocol outlined recently by the Wirtz et al (31) to chemically induce colon cancer in the C57BL/6 mice. Briefly 8- to 12- week-old male C57BL/6 mice were weighed and given a single intraperitoneal (IP) injection of AOM (10 mg/kg) or vehicle (1x PBS) on experimental day 0. One week later, animals received 1% DSS in their drinking water. Throughout the course of the experiment the animals were fed the AIN 93M chow. Colitis associated colon cancer was induced with cyclical DSS treatment (following the initial single IP injection of AOM), which consisted of 7 days of 1% DSS and 14 days of normal drinking water (a total of 21 days). The cycle here consisted of 14 days normal drinking water for recovery, because in our experience, mice were too distressed with a shorter recovery when given both AOM and 1% DSS for the colon cancer model. 11.9 mg/kg of the Hexane fraction of AG, whole AG extract and vehicle groups (1x PBS), were given to the mice at day 14 (after AOM and first week of DSS) by oral gavage and continued daily throughout the course of the experiment. The mice were euthanized at day 35 (1 ½ cycles), and day 50 (2 cycles. For pathology and immunohistochemistry, colon tissue samples were washed with 1x PBS (Mediatech, Herndon, VA), cut longitudinally, swiss-rolled, then formalin fixed overnight, and paraffin embedded.

Definition of terms to quantify the effects of treatment on pre-cancerous and cancerous lesions in the AOM/DSS mouse model.

All lesions were examined blindly by a trained pathologist, specializing in mouse tissues. Inflammatory lesions were characterized by increased intensity of lamina propria cellular infiltrate with alterations of the composition and changes in distribution. Based on the area of the inflammatory stretch and its extension into the submucosa, the inflammatory lesions were categorized into severe and mild. For ulceration, the infiltrate was identified as extensive and extends diffusely towards deeper areas (transmucosal). The presence of neutrophils, indicating a change in the composition of inflammatory infiltrate was a feature of Ulcerative colitis (UC) and this activity could be recognized by the presence of neutrophils infiltrating the walls of some crypts. Polyps were defined as well demarcated circumscribed lumps of epithelial dysplasia, with uncontrolled crypt cell division. Dysplasia in non-invasive adenomas was identified where part of the epithelium was replaced by cells showing varying degree of atypia, with changes in architecture and aberrant differentiation. Nuclear changes include hyperchromatism and enlargement with nuclear crowding and frequent overlapping with nuclei typically stratified near the base of the crypts and cytoplasm shows increased basophilia. Low Grade Dysplasia was defined as mild and moderate dysplasia in which architectural changes are modest and nuclear stratification was confined to the lower half of the cell. High Grade Dysplasia was defined by more profound architectural alterations and stratification of nuclei into the upper half of the cells. Invasive Adenocarcinoma is recognized by neoplastic cells that invades or infiltrates through the muscularis mucosa, into or beyond the submucosa.

Immunohistochemical staining

For immunohistochemical staining, serial sections of mouse colon tissues (processed as described above) were incubated with antibodies against p53 (Rabbit polyclonal, cat# 31333,

diluted 1 in 1000; Abcam, Cambridge, MA), cyclooxygenase-2 (Cox-2) (Rabbit polyclonal, cat# 160126; diluted 1 in 5000; Cayman Chemical, Ann Arbor, MI) or iNOS (Rabbit Polyclonal, Cat# 160862, diluted 1 in 3500; Cayman Chemical, Ann Arbor, MI). To ensure even staining and reproducible results, sections were incubated by slow rocking overnight in primary antibodies (4°C) using the Antibody Amplifier™ (ProHisto, LLC, Columbia, SC). Following incubation with primary antibody, sections were processed with EnVision+ System-HRP kits (DakoCytomation, Carpinteria, CA) according to the kit protocol. The chromogen was diaminobenzidene and sections were counter stained with 1% methyl green. The positive control tissue was colon cancer sections. The negative control was devoid of primary antibody incubation. Immunohistochemistry was quantified as we described previously (27), with a slight modification. The intensity of the staining was evaluated independently by two blinded investigators (D.P and A.C). For each tissue section, the percentage of positive cells was scored on a scale of 0 to 5 for the percentage of tissue stained: 0 (0% positive cells), 1 (<10%), 2 (11% to 25%), 3 (26% to 50%), 4 (51% to 80%), or 5 (> 80%). Staining intensity was scored on a scale of 0 to 3: 0-negative staining, 1-weak staining, 2-moderate staining, or 3-strong staining. The two scores were multiplied resulting in an immunoreactivity score (IRS) value ranging from 0 to 15.

Western blot analysis and antibodies

Western blots were carried out as described previously (32). Antibodies used include: iNOS (Rabbit polyclonal, diluted 1 in 500, cat#160862; Caymen Chemicals, Ann Arbor, MI), Cox-2 (Rabbit polyclonal, diluted 1 in 500, cat#160106; Caymen Chemicals, Ann Arbor, MI), PARP (Full length) (Rabbit polyclonal, diluted 1 in 500, cat#9542; Cell Signaling Technology,

Danvers, MA), Cleaved PARP (Rabbit polyclonal, diluted 1 in 500, cat# 9544; Cell Signaling Technology, Danvers, MA), p53 (Mouse monoclonal, DO-1, diluted 1 in 500, cat# OP43T; Calbiochem, Gibbstown, NJ), Phospho-p53-Serine 15 (Rabbit polyclonal, diluted 1 in 500, cat#9284; Cell Signaling Technology, Danvers, MA), PUMA (Rabbit polyclonal, diluted 1 in 500, cat#4976; Cell Signaling Technology, Danvers, MA), Wip1 (Rabbit polyclonal, diluted 1 in 500, cat#AP8437b, Abgent, Inc., San Diego, CA), and Actin (Mouse monoclonal, diluted 1 in 1000, cat#A5316m Calbiochem, St. Louis, MO). For all the blots, a standard protein (BenchMark Prestained Protein Ladder; Invitrogen, Carlsbad CA) was run to ensure the correct molecular weight of each bands observed. Where possible, purified protein was also run as a positive control. For iNOS, Cox-2, WIP1, p53, phosphor-p53-serine 15, and Actin there was only one clear band shown at 140 kDa, 72 kDa, 61 kDa, 53 kDa, 53 kDa, and 42 kDa, respectively. For PARP, the cleaved and full length antibodies incubated concurrently, and 3 bands were observed [116 kDA (Full Length), 89 kDa (Cleaved PARP), and 50 kDa (presumabely non-specific binding)]. For PUMA, 3 bands were observed, and the band at 23 kDa was chosen because this band corresponds to the molecular weight of PUMA. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ). Both secondary antibodies were diluted at 1:2000. All antibodies were diluted in 5% milk/PBST (1% Tween 20 in 1× PBS). Western blot signal was detected by Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) and developed onto Hyperfilm.

Real-Time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, CA). One μg of total RNA served as template for single strand cDNA synthesis in a reaction using oligo(dT) primers and AMV reverse transcriptase (Promega Corp, WI) under conditions indicated by the manufacturer. PCR of cDNA samples was performed with samples amplified for 30 cycles of denaturation at 94^{o}C for 30 s, annealing at 50^{o}C for 30 sec, and extension at 72^{o}C for 30 sec with final extension at 72^{o}C for 10 min. The sequences for PCR primers used were: COX-2 Forward 5'-CCC CCA CAG TCA AAG ACA CT-3', COX-2 Reverse 5'-CTC ATC ACC CCA CTC AGG AT-3'; iNOS Forward 5'-CAC CTT GGA GTT CAC CCA GT-3', iNOS Reverse 5'-ACC ACT CGT ACT TGG GAT GC-3'; GAPDH Forward 5'-ACC CAG AAG ACT GTG GAT GG-3', GAPDH Reverse 5'-CAC ATT GGG GGT AGG AAC AC-3' (Integrated DNA Technologies, Inc). Real-time PCR (qPCR) was performed using the 7300 Real-Time PCR Assay System (Applied Biosystems, CA) with Power SYBR green PCR master mix (Applied Biosystems, CA) and primers for iNOS, COX-2 and GAPDH according to the vendor's protocol. Both iNOS and COX-2 gene expression was normalized by GAPDH gene expression.

Annexin V assay

$CD4^{+}/CD25^{-}$ effector T cells were seeded at 1×10^{6} per well into six-well dishes for 24 h. Following this, fresh medium or medium containing concanavalin A (Con-A, 2.5 μg/mL) was added to cells and cultured for 12 h. Con-A was then washed off, and fresh medium or fresh medium containing freshly dissolved indicated concentrations of AG or the Hexane Fraction of AG (0 - 300 μg/mL) was added for 24 h, as indicated. TK6 cells were seeded at 1×10^{6} per well into six-well dishes for 24 h and treated with AG or the Hexane Fraction of AG (0 - 1000 μg/mL). Cells were then harvested for Annexin V according to instructions provided by the kit

manufacturer (BD Biosciences). Annexin V/propidium iodide (PI) staining was examined using a Beckman Coulter Cytomics FC500 flow cytometer.

TUNEL assays

A TUNEL (TdT-mediated dUTP Nick-End Labeling) assay was carried out to assess apoptosis. For *in vivo* detection, we used the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI), according to the manufacturer's directions. Briefly, this is a modified TUNEL Assay designed to provide simple, accurate and rapid detection of apoptotic cells in situ at the single-cell level. The system measures nuclear DNA fragmentation, an important biochemical indicator of apoptosis. Biotinylated nucleotides are incorporated at the 3′-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). Horseradish-peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown. The counterstain was CAT Hematoxylin (Biocare Medical, Concord, CA). Labeling was carried out on serial sections to that we used to score inflammatory index. TUNEL labeling in 10 separate sections from 10 individual mice was quantified in both the epithelial areas, and the mesenteric lymph nodes (MLNs). For the epithelial areas, 10 random fields were evaluated per slide. Because there are relatively fewer MLNs, we evaluated each MLN for TUNEL labeling. Labeled tissues were examined for intensity of staining using a method similar to that previously described (33). Briefly, intensity of staining was evaluated independently by blinded investigators. For each tissue section, the percentage of positive cells in either the epithelium, or in the MLNs, was scored as described for immunohistochemical staining.

To measure apoptosis in cell culture, we used the TUNEL assay involving end-labeling of DNA with fluorescein-dUTP, followed by analysis using flow cytometry. For exposure to the Hexane Fraction of AG, cells were incubated in 1% New-Born Calf Serum (NBCS)-supplemented DMEM media for 24 hrs. The media was changed and the cells were either treated with Hexane Fraction of AG (260 μg/ml) or non-treated (1% NBCS supplemented media) for 0, 6, and 8 hrs. Cells were harvested and TUNEL assay was performed as described by vendor (Roche Diagnostics, IN). Briefly, 1 X 10^6 cells were fixed using a 100 μl of fixation solution (2% paraformaldehyde) and permeabilized using a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). Cells were washed and incubated with TUNEL reaction mixture (Label Solution and Enzyme Solution) (Roche Diagnostics, IN). Apoptosis in the samples was analyzed by flow-cytometry (Beckman Coulter, CA). The fluorescence was evaluated using the excitation wavelength of 488nm and detected in the range of 515 -565 nm (green, FL-1 channel). The dot plot of FS Vs FL-1 and histogram plot of (Number of Event) Vs (FL-1 Channel) were plotted to obtain a percentage increase in the apoptosis of the Hexane fraction of AG treated cells. The positive control for apoptosis was fixed and permeabilized cells with DNase I recombinant (3 U/ml in 50 mM Tris-HCL, pH 7.5, 1 mg/ml Bovine Serum Albumin) (Invitrogen, CA) to induce DNA strand breaks prior to labeling. The negative control for apoptosis was non-treated, healthy cells. Isogenic enzyme control is the fixed and permeabilized cells with the labeling solution but without the terminal transferase enzyme.