

Role of microRNAs in Resveratrol-Mediated Mitigation of Colitis-Associated Tumorigenesis in *Apc*^{Min/+} Mice[§]

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ABSTRACT

The pleiotropic effects of resveratrol include anti-inflammatory, antioxidant, and anticancer activities, and thus unique possibilities exist to explore mechanistic pathways of chemoprevention. The aim of this study was to investigate the role of microRNA (miRNA) alterations induced by resveratrol in the context of chemopreventive mechanisms against dextran sodium sulfate (DSS)-induced colitis-associated tumorigenesis in the *Apc*^{Min/+} mouse. To that end, *Apc*^{Min/+} mice were exposed to 2% DSS to enhance intestinal inflammation and polyp development. Concurrently, mice received either vehicle or resveratrol treatment via oral gavage for 5 weeks. Interestingly, treatment of DSS-exposed mice with resveratrol resulted in decreased number and size of polyps, fewer histologic signs of cell damage, and decreased proliferating epithelial cells in intestinal mucosa compared with vehicle. Resveratrol treatment dramatically reversed the effects of DSS on the numbers of specific inflammatory CD4⁺ T cells,

CD8⁺ T cells, B cells, natural killer T cells, and myeloid-derived suppressor cells in mesenteric lymph nodes. Resveratrol treatment also decreased interleukin-6 (IL-6) and tumor necrosis factor- α protein levels and reduced IL-6 and cyclooxygenase-2 mRNA expression. Microarray analysis revealed 104 miRNAs exhibiting >1.5-fold differences in expression in the intestinal tissue of resveratrol-treated mice. Among them, two miRNAs with anti-inflammatory properties, miRNA-101b and miRNA-455, were validated to be upregulated with resveratrol treatment by reverse-transcription polymerase chain reaction. Pathway analysis revealed that numerous differentially regulated miRNAs targeted mRNAs associated with inflammatory processes with known roles in intestinal tumorigenesis. These results suggest that resveratrol mediates anti-inflammatory properties and suppresses intestinal tumorigenesis through miRNA modulation.

Introduction

Bioactive dietary components offer numerous possibilities for chemoprevention, given their potential to target factors linked to the development and progression of cancer (Kim et al., 2009). Further, fewer side effects are associated with consumption of these components than prescription drugs. Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a naturally occurring stilbenoid abundant in plants and plant products such as grapes, peanuts, and red wine. Resveratrol has diverse

biologic properties including anticarcinogenic, antioxidant, anti-inflammatory, antimutagenic, proapoptotic, and immunoregulatory activities (Szekeres et al., 2011), indicating promise as a chemoprevention strategy. Recent literature supports a beneficial effect of resveratrol in various mouse models of intestinal cancer. Oral administration of resveratrol decreases polyps in the small intestine and completely suppresses polyp formation in the colon of *Apc*^{Min} (multiple intestinal neoplasia) mice (Schneider et al., 2001). Resveratrol also reduces severity of dextran sodium sulfate (DSS)-induced ulcerative colitis and attenuates chronic colonic inflammation in mice (Yao et al., 2010). Similarly, resveratrol inhibited colon carcinogenesis in an azoxymethane (AOM)/DSS murine model, reduced the multiplicity of colon neoplasms, inhibited colon cancer cell proliferation, and promoted apoptosis (Marshall and Kerkvliet, 2010). While animal studies provide promising evidence of a benefit of resveratrol on colon cancer, the mechanisms for these effects are unknown.

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ABBREVIATIONS: AOM, azoxymethane; APC, adenomatous polyposis coli; BrdU, 5-bromo-2'-deoxyuridine; COX-2, cyclooxygenase-2; DAB, 3,3'-diaminobenzidine; DSS, dextran sodium sulfate; FBS, fetal bovine serum; ICAM1, intercellular adhesion molecule 1; IL, interleukin; IL-6R, interleukin-6 receptor; IPA, ingenuity pathway analysis; MDSC, myeloid-derived suppressor cell; miRNA, microRNA; Min, multiple intestinal neoplasia; MLN, mesenteric lymph node; NF- κ B, nuclear factor- κ B; NKT, natural killer T; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; R, resveratrol; RT-PCR, reverse-transcription polymerase chain reaction; TLR, Toll-like receptor; TNF, tumor necrosis factor; V, vehicle.

Among all cancers, the evidence for a relationship between inflammation and colon cancer risk is the strongest (Fantini and Pallone, 2008). Thus, it is no surprise that anti-inflammatory strategies are known to decrease colon cancer risk. Incidentally, resveratrol has well documented anti-inflammatory mechanisms. For example, resveratrol inhibits activated immune cells and downregulates inducible nitric-oxide synthase and cyclooxygenase-2 (COX-2) via its inhibitory effects on nuclear factor- κ B (NF- κ B) (Das and Das, 2007). Although the anti-inflammatory effects of resveratrol have been characterized in a variety of disease models, including staphylococcal enterotoxin B-induced lung injury (Rieder et al., 2012), exposure to environmental toxins (Singh et al., 2011b), diabetes (Singh et al., 2011a), allergic encephalitis (Singh et al., 2007), melanoma (Guan et al., 2012), and ulcerative colitis (Singh et al., 2012a,b), there are relatively few reports of its anti-inflammatory effects in rodent models of intestinal cancer.

The $Apc^{Min/+}$ mouse model is the most widely used genetic mouse model for cancer studies that involve the gastrointestinal tract. $Apc^{Min/+}$ mice are heterozygous for a nonsense mutation at position 850 in *APC* (adenomatous polyposis coli). $Apc^{Min/+}$ mice are predisposed to developing intestinal tumors, as are humans carrying mutations in *APC*. Additionally, ~80% of human colorectal tumors harbor mutations in *APC* (Miyoshi et al., 1992). Given the rapid development of polyps, along with its similarities to familial adenomatous polyposis and inherited colon cancer syndrome in humans, the $Apc^{Min/+}$ mouse is considered a highly relevant colon cancer model (Corpet and Pierre, 2003). One drawback of this model is that tumors occur predominantly in the small intestine and not the colon, whereas humans with germline mutations in *APC* develop tumors predominantly in the colon. However, administration of DSS in the drinking water enhances colon polyp development and promotes inflammation in $Apc^{Min/+}$ mice. This mouse model is widely used in studies of anti-inflammatory agents, including both dietary supplements as well as nonsteroidal anti-inflammatory drugs (Corpet and Pierre, 2003). However, there are few studies that have examined the anti-inflammatory effects of resveratrol in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis (Hudson et al., 2013), and to our knowledge none have examined its effects in the context of DSS-induced inflammation.

MicroRNAs (miRNAs) are 20- to 25-base-pair single-stranded molecules capable of base-pairing with complementary mRNA transcripts, typically within the 3'-untranslated region. These miRNA/mRNA pairs are subsequently degraded, and translation of the targeted mRNA is thereby inhibited. Dysregulation of miRNAs is associated with development of numerous cancers including cancers of the colon (Hutchison et al., 2013). In addition to miRNAs that have cell growth and proliferation targets, new evidence suggests that dysregulation of miRNAs with inflammatory or anti-inflammatory targets also plays a mechanistic role in the pathogenesis of inflammatory bowel diseases and colon cancer development. For example, miRNAs such as miR-21 and miR-126 are differentially expressed in active versus inactive ulcerative colitis (Feng et al., 2012). Some of these altered miRNAs have targets that are associated with the downstream regulation of NF- κ B and COX-2, both relevant factors in inflammation and tumorigenesis of the colon (Strillacci et al., 2009). Importantly, the as yet poorly understood mechanisms of the pleiotropic activities of resveratrol may

act through modulation of miRNAs with targets involved in inflammation.

Thus, the purpose of this study was to examine impacts of resveratrol on inflammation and immune regulation in the $Apc^{Min/+}$ mouse model of intestinal tumorigenesis. Furthermore, a goal of this study was to explore polyp characteristics and determine the molecular mechanisms underlying the beneficial effects of resveratrol by miRNA microarray analysis. We hypothesized that resveratrol would decrease polyp number and size, decrease inflammation, suppress the immune response, and alter miRNAs known to regulate immunomodulatory pathways.

Materials and Methods

Animals. All animal care and experimental procedures were approved by the University of South Carolina's Institutional Animal Care and Use Committee and performed in accordance with the *Guide for the Care and Use of Laboratory Animals*. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council. Male $Apc^{Min/+}$ mice in a C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME) were bred with female C57BL/6 mice at the University of South Carolina's Center for Colon Cancer Research. Offspring were genotyped as heterozygotes by reverse-transcription polymerase chain reaction (RT-PCR) for *APC* by taking tail snips at weaning. The primer sequences were sense: 5'-TGAGAAAGACA-GAAGTTA-3'; and antisense: 5'-TTCCACTTTGGCATAAGGC-3'. Female $Apc^{Min/+}$ offspring were randomly assigned to either vehicle (DSS + V) or resveratrol (DSS + R) treatment groups at 4 weeks of age. An additional $Apc^{Min/+}$ group was killed at 4 weeks of age as a naïve control group. Mice were maintained on a 12-hour light/dark cycle in a low-stress environment (22°C, 50% humidity, and low noise) and provided food and water ad libitum.

Chemopreventive Effect of Resveratrol. At 4 weeks of age, $Apc^{Min/+}$ mice (DSS + V and DSS + R) were exposed to 2% DSS in their drinking water for 1 week to induce colitis and enhance polyp development. Beginning on the first day of DSS administration, mice received either resveratrol (100 mg/kg mouse body weight) or vehicle by oral gavage in a volume of 0.2 ml every other day for 5 weeks. The resveratrol was purchased from Supelco (Bellefonte, PA), resuspended in water. Mice (DSS and D + R) were killed at 9 weeks of age. An additional $Apc^{Min/+}$ group that did not receive DSS was included as a control group and was killed at 4 weeks of age.

Tissue Collection. Fifteen minutes prior to euthanasia, cells synthesizing DNA were labeled by injecting mice i.p. with 5-bromo-2'-deoxyuridine (BrdU; 120 mg/kg) (BD Biosciences, San Diego, CA), which was dissolved in normal saline solution containing 30% dimethylsulfoxide. Mice were killed via isoflurane overdose, and mesenteric lymph nodes (MLNs) were harvested and mechanically dissociated followed by lysis of red blood cells (Sigma-Aldrich, St. Louis, MO). Cell suspensions were stored on ice in media containing 2% fetal bovine serum (FBS) until analysis for inflammatory cell surface markers using flow cytometry.

The small intestine was carefully dissected distally to the stomach and proximal to the cecum. The large intestine (section 5) was removed from the distal end of the cecum to the anus. The small intestine was divided into four equal segments (sections 1–4). All intestinal sections were flushed with phosphate-buffered saline (PBS), opened longitudinally, and flattened with a cotton swab. Sections 1 and 4 of the small intestine and the large intestine (section 5) were fixed flat in 10% buffered formalin at room temperature for 24 hours for analysis of polyp counts, histopathological evaluation, and immunohistochemistry. Sections 2 and 3 were divided into two equal parts, and mucosal scrapings were performed in Iscoves medium (Invitrogen, Carlsbad, CA) [containing 5% FBS and a cocktail enzyme

inhibitor (10 mM EDTA, 5 mM benzamidine HCl, and 0.2 mM phenylmethylsulfonyl fluoride)] and QIAzol reagent (Qiagen, Valencia, CA) for protein and gene expression analysis, respectively. Samples were stored at -80°C until analysis of inflammatory mediators.

Although DSS exposure in this model allows for the promotion of more polyps in the large intestine, *Apc*^{Min/+} mice primarily develop small intestinal polyps. Previously reported findings from our group have shown that an elevation in inflammatory cytokines in the small intestine is positively correlated with the abundance of large polyps as well as overall polyp number (McClellan et al., 2012). To detect any potential anti-inflammatory effects of resveratrol in this model, we chose to perform the inflammatory outcome analysis on the small intestine.

Polyp Counts. Formalin-fixed intestinal segments from all animals were rinsed in deionized water and briefly stained in 0.1% methylene blue, and polyps were counted under a dissecting microscope. Polyps were categorized by size (>2 mm, 1–2 mm, and <1 mm).

Histologic Analyses. Tissue samples from the large intestine (section 5) were fixed in 10% formalin, dehydrated in a graded ethanol series, and Swiss rolled in paraffin-embedded sections. Samples were cut into 5- μm -thick sections using a microtome and stained with H&E. Histologic alterations including mucosal ulceration, dysplasia, and carcinoma were documented by a trained histopathologist.

Antigens were unmasked using proteinase K (Millipore, Billerica, MA), and peroxidase activity was inhibited using BLOXALL (Vector Laboratories, Burlingame, CA) for 10 minutes. For proliferating cell nuclear antigen (PCNA) staining, sections were incubated with rabbit polyclonal PCNA antibody (1:200; Abcam, Cambridge, UK) for 1 hour at room temperature. The HRP-DAB Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions. Detection was visualized by exposing sections to 3,3'-diaminobenzidine (DAB). PCNA-positive cells were visualized using the DAKO Chromavision Systems ACIS 3 system (Dako North America, Inc., Carpinteria, CA).

Flow Cytometric Analysis. Cells from the MLNs were isolated as described above for analysis of T cells (CD4 and CD8), B cells (CD19), myeloid-derived suppressor cells (MDSCs), and natural killer T (NKT) cells. In brief, for staining of cell surface antigens, cells were washed with a flow cytometry staining buffer (PBS with 2% FBS) and then stained with a fluorescein-labeled antibody [isothiocyanate, phycoerythrin, or allophycocyanin (BioLegend, San Diego, CA)] for 30 minutes at 4°C . Cells were then washed, thoroughly resuspended in flow cytometry staining buffer, and analyzed using flow cytometry (CXP FC500; Beckman Coulter, Brea, CA).

Analysis of Interleukin-6 and Tumor Necrosis Factor- α . Mucosal tissue scrapings were homogenized, and samples were centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatants were removed and analyzed for interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) using an enzyme-linked immunosorbent assay (BioLegend) according to the manufacturer's instructions. Total soluble protein cytokine levels are expressed as picograms per 100 microliters total protein.

Expression of Inflammatory Markers. RNA was reverse-transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The cDNA was amplified using forward and reverse primers. The polymerase chain reaction (PCR) products were identified using 2% agarose gel electrophoresis. The band intensity of the PCR products was quantified using the Bio-Rad Chemi Doc densitometry image analysis system.

Immunohistochemical Analysis. Intestinal tissues were fixed in 10% formalin, dehydrated through graded concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5 μm thick) were mounted on slides, cleared, deparaffinized, and rehydrated. Slides were then treated with a buffered blocking solution (3% H_2O_2 in PBS) for 10 minutes. Antigen retrieval was performed in a water bath at 89°C for 10 minutes using an antigen retrieval solution. After cooling at room temperature, sections were coincubated with primary antibodies for BrdU for 1 hour in a humidified chamber at room

temperature. Sections were washed with PBS and incubated with conjugated secondary antibody (streptavidin-horseradish peroxidase) for 30 minutes at room temperature. Thereafter, sections were washed with PBS and coincubated with a DAB solution in the dark at room temperature for 10 minutes. Sections were rinsed in PBS, counterstained with hematoxylin, and observed under a microscope. Cells that had incorporated BrdU were visualized by counting the proportion of BrdU-labeled cells in 50 typical crypts and/or villi as previously described (Mochida et al., 2003).

Microarray Analysis of miRNA. Total RNA including miRNA from intestinal scrapings was isolated using a miRNeasy kit following the manufacturer's instructions (Qiagen, Redwood City, CA). The RNA was hybridized on an Affymetrix GeneChip high-throughput miR array containing 609 murine probes (Affymetrix, Santa Clara, CA). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number INSERT. The data generated from the array were analyzed using hierarchical clustering. By use of ingenuity pathway analysis (IPA) software (Qiagen; www.ingenuity.com), the results from the miRNA microarray were analyzed to identify molecular pathways potentially altered by single or multiple miRNA target genes. In brief, this analysis compares each set of miRNAs to all available pathways in the database and assigns priority scores based on the predicted strength of the miRNA interaction with components of the target pathway. Additional analysis was performed by input of these targets into Cytoscape using the ClueGo plug-in (cytoscape.org) (Bindea et al., 2009).

Validation of Microarray Data with RT-PCR. To validate the results of the microarray analysis, two representative miRNAs (miRNA-455 and miRNA-101b), significantly changed based on microarray results and of relevance to intestinal inflammation, were subjected to RT-PCR analysis. miRNAs were reverse-transcribed (miScript II; Qiagen) to make cDNA, and RT-PCR was carried out with primers (5'-GCAGUCCACGGGCAUAUACAC-3' and 5'-UACAGUACUGUGAUAGCUGAA-3' for miRNAs 455 and 101b, respectively). Reactions were performed with SYBR Green Master Mix (Qiagen). Initiation was performed for 15 minutes at 95°C . The samples were then run for 40 cycles each at 94°C for 5 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. All samples were run in triplicate.

Statistical Analysis. All statistical analyses were conducted in Microsoft Excel and R (www.r-project.org). The two-tailed Student's *t* test was used to investigate the difference between the DSS + R and DSS + V groups, and a *P* value of <0.05 was considered statistically significant. The means \pm S.E.M. were reported. Additionally, the heat map and principle component analysis were applied to describe the correlation of miRNA expression levels among these two groups.

Results

Effect of Resveratrol on Polyp Number and Size and Histologic Changes in the Colon. In a model of colitis-associated tumorigenesis, exposure of *Apc*^{Min/+} mice to 2% DSS in drinking water for 1 week induces formation of numerous polyps in the colon. Following DSS exposure, administration of resveratrol (100 mg/kg by oral gavage for 5 weeks) reduced the overall number of polyps, although not significantly (32.6 ± 3.2 versus 42.5 ± 4.4 ; $P < 0.09$) and significantly reduced the number of large polyps (5.1 ± 1.1 versus 13.4 ± 1.5 ; $P < 0.01$) (Fig. 1A). Additionally, resveratrol treatment reduced the overall polyp number within the colon compared with mice treated with vehicle (6.67 ± 1.63 versus 16.70 ± 2.13 ; $P < 0.002$) (Fig. 1B). Although resveratrol also reduced the overall polyp burden within the small intestine, this did not reach statistical significance (data not shown). Concurrently, resveratrol treatment also reduced polyp size (representative images in Fig. 1C).

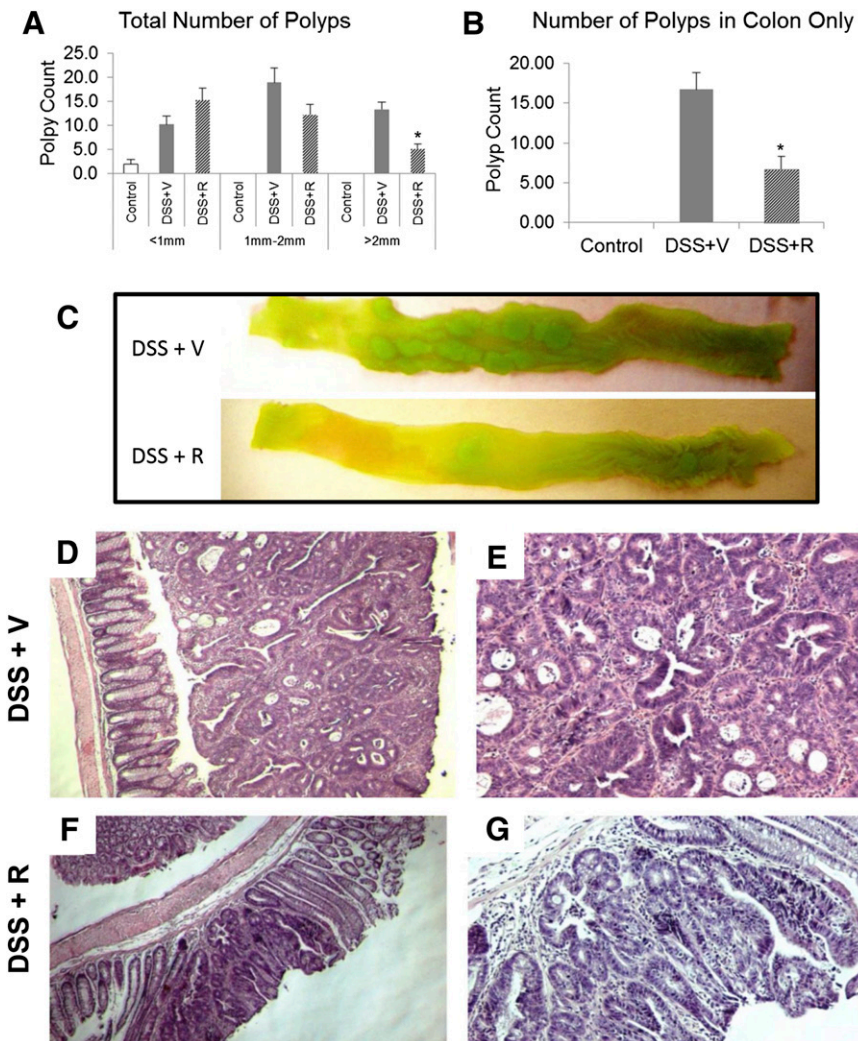


Fig. 1. Resveratrol reduces polyp number, polyp size, and histopathology in *Apc^{Min/+}* mice exposed to DSS. *Apc^{Min/+}* mice were exposed to DSS in drinking water, treated with vehicle or resveratrol, and compared with naïve control group. Resveratrol reversed the effect of DSS on number of large polyps in small and large intestine ($P < 0.01$) (A) and total polyp number in colon ($P < 0.02$) (B). No large polyps or polyps in the colon were observed in control mice. (C) Representative images of colon containing intestinal polyps from mice exposed to DSS and treated with vehicle or resveratrol. (D and E) *Apc^{Min/+}* mice given 2% DSS show H&E-stained polyps with high-grade epithelial dysplasia. (F and G) Minute foci of low-grade epithelial dysplasia comprising <1% of the surface area of the roll of colon (DSS + R group). Magnification power was 4× with a 5-mm field of view (D), 10× with a 2-mm field of view (E), 2× with a 10-mm field of view (F), and 4× with a 5-mm field of view (G).

Of note, weight loss was observed in both groups, with resveratrol-treated mice losing a maximum average of 7% body weight by day 8 and vehicle-treated mice losing up to 11% body weight by day 10. This initial weight loss was likely attributed to the DSS exposure. Weights of both groups thereafter steadily increased, with both gaining ~8% body weight by the end of the study (data not shown).

Histopathological analysis was carried out following H&E staining of paraffin-embedded, Swiss rolled colon samples from DSS-exposed *Apc^{Min/+}* mice treated with resveratrol or vehicle. In general, mice in the vehicle group presented with high-grade dysplasia and goblet cell (mucin) depletion. Glandular epithelial cells exhibited "rounding up" of cigar-shaped nuclei (Fig. 1, D and E). In contrast, resveratrol treatment dramatically reduced histologic signs of cell damage. Histopathology of samples from the resveratrol treatment group ranged from low-grade epithelial dysplasia comprising <1% of the mucosal surface area to insignificant pathologic changes (Fig. 1, F and G).

Resveratrol Treatment Decreases the Number of Proliferating Cells. We used BrdU as a marker of DNA synthesis to examine the number of proliferating cells in the colon of DSS-exposed *Apc^{Min/+}* mice treated with resveratrol or vehicle. In brief, BrdU was injected 15 minutes prior to

ethanasia, formalin-fixed colon sections were stained using a BrdU staining kit, and proliferation was determined by counting the proportion of BrdU-labeled cells in 50 typical crypts and/or villi (Mochida et al., 2003). A statistically significant (~50%) decrease in the quantification of BrdU-positive colonic epithelial cells was evident following resveratrol treatment compared with vehicle ($P < 0.01$) (Fig. 2, A–C). To support these data, PCNA staining of formalin-fixed colon sections was also performed (Fig. 2, D–F). Specimens from resveratrol-treated mice exposed to DSS showed reduced PCNA staining relative to those treated with vehicle.

Resveratrol Decreases Inflammatory Markers Associated with Colitis. The protein concentration of proinflammatory cytokines (IL-6 and TNF- α) and gene expression of inflammatory mediators (COX-2 and IL-6) in regions 2 and 3 of the small intestine of DSS-exposed *Apc^{Min/+}* mice with and without resveratrol treatment were determined. Regions 2 and 3 were chosen to represent sections of the small intestine with low and high polyp incidence, respectively. Our findings indicated that resveratrol significantly blocked increases in IL-6 and TNF- α protein concentration ($P < 0.03$ and $P < 0.01$, respectively) in DSS-exposed *Apc^{Min/+}* mice (Fig. 3, A and B).

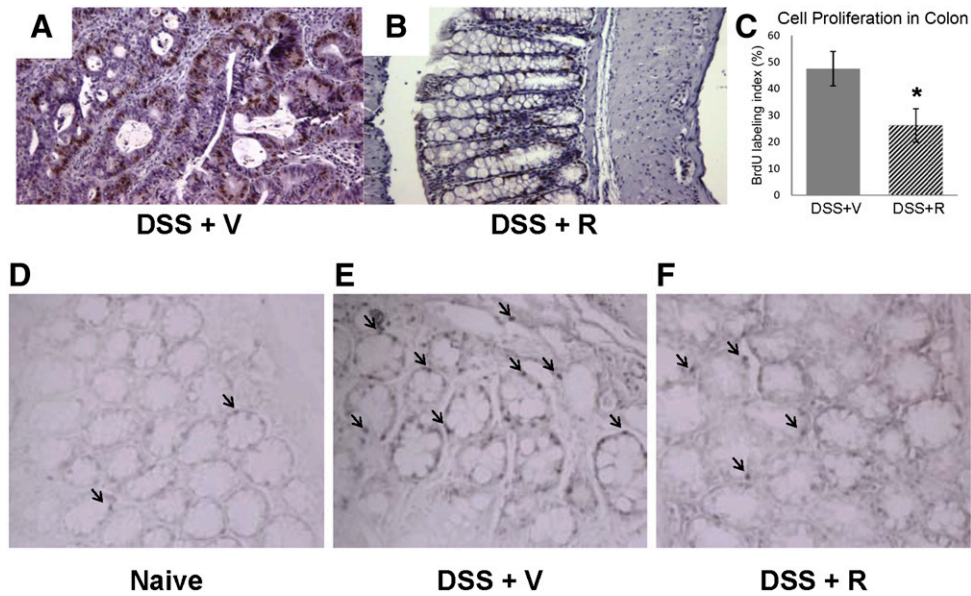


Fig. 2. BrdU-labeling index of colon crypt cells and adenomas in *Apc*^{Min/+} mice exposed to DSS. *Apc*^{Min/+} mice were exposed to DSS in drinking water, treated with vehicle or resveratrol, and compared with the naïve control group. BrdU (125 mg/kg i.p.) was administered 15 minutes before euthanasia to DSS-exposed *Apc*^{Min/+} mice treated with resveratrol or vehicle, and the labeling indices in colon crypts were assessed. BrdU staining is shown in histologic sections of intestinal mucosa from *Apc*^{Min/+} mice that received 2% DSS and were treated with vehicle (A) or resveratrol (100 mg/kg) (B). (C) The fraction of cells that are labeled with BrdU was quantified for both intestinal crypts and adenomas, and plotted. Values are averages \pm S.E.M. * $P < 0.01$ vs. DSS + V group. DSS + V ($n = 10$), DSS + R ($n = 9$), and control ($n = 2$). Magnification was 10 \times with a 2-mm field of view (A) and 4 \times with a 5-mm field of view (B). Intestinal mucosa from naïve (D), DSS + V (E), and DSS + R (F) mice were stained for PCNA, which appears as dark punctate foci on each of the images.

Likewise, resveratrol treatment offset the increase in COX-2 and IL-6 gene expression ($P < 0.02$ and $P < 0.002$, respectively) characteristic in this model (Fig. 3, C–F).

Resveratrol Modulates Immune Cell Populations. By use of flow cytometry, we examined changes in the percentage and absolute number of T cells (CD4⁺ and CD8⁺), B cells (CD19⁺), MDSCs, and NKT cells in the MLNs of DSS-exposed *Apc*^{Min/+} mice with vehicle or resveratrol treatment. While the percentages of these cells were either decreased or slightly altered, enumeration of absolute numbers of these cells revealed that resveratrol treatment reversed the DSS-induced increase in the total number of CD4⁺, CD8⁺, CD19⁺, and NKT cells and MDSCs ($P < 0.001$, $P < 0.004$, $P < 0.004$, $P < 0.01$, and $P < 0.003$, respectively) (Fig. 4).

Effect of Resveratrol on miRNA Expression Patterns by Microarray Assay, Validation by Quantitative RT-PCR, and IPA Analysis. To gain an understanding of the differences in miRNA profiles in DSS-exposed *Apc*^{Min/+} mice treated with resveratrol compared with vehicle, miRNA array analysis on intestinal tissue was performed. A total of 104 miRNAs showing a >1.5-fold change between groups were identified (Fig. 5). The heat map (Fig. 5A) of miRNA array performed on cell extracts from intestinal mucosal scrapings from two groups, DSS + V and DSS + R, showed that miRNAs are differentially expressed. Eleven miRNAs with an increase or decrease in expression by a magnitude of >2.0-fold following resveratrol treatment were plotted by principal component analysis (Fig. 5B). These altered miRNAs included 56 miRNAs that were upregulated and 48 miRNAs that were downregulated (Fig. 5C). Figure 5D depicts all assayed miRNAs within the genome and the magnitude of the fold change, with 12 miRNAs of interest labeled.

Two miRNAs (-455 and -101b) upregulated with resveratrol treatment of DSS-exposed *Apc*^{Min/+} mice by microarray analysis that have known roles in inflammatory processes were validated using quantitative RT-PCR. Consistent with the microarray results, we found a significant upregulation of miRNA-455 and miRNA-101b ($P < 0.04$ and $P < 0.02$, respectively) following resveratrol treatment compared with vehicle. In fact, quantitative estimates of the fold change from RT-PCR and microarrays were very similar (Fig. 6).

miRNAs of interest were further analyzed using IPA software. Of the miRNAs that were up- or downregulated by >1.5-fold upon resveratrol treatment, 15 had direct associations with numerous immune modulation targets (Fig. 7A). Targets that have known direct, experimentally demonstrated associations with the miRNAs of interest are schematically mapped. These miRNAs had direct affinity to mRNAs with central roles in inflammatory pathways, including IL-6, IL-6 receptor (IL-6R), TNF, TNFRSF1B (tumor necrosis factor receptor superfamily, member 1B), and CD4, among numerous targets relevant to immunomodulation. Molecules with known associations to the miRNAs of interest include transmembrane receptors [intercellular adhesion molecule 1 (ICAM1), IL-6R, IL10-RA, CD4, and numerous others], cytokines (including IL-6, IL-7, IL-25, IL-13, IL-1A, IL-10, and TNF), ligand-dependent nuclear receptors [RORC, AHR, peroxisome proliferator-activated receptor (PPAR) γ , NR5A2, and ESR2], transcriptional regulators (including SMAD3, TBX21, DDIT3, and CEBPE), and kinases (MAPK9, IKKB, and TGFBR2). The disease pathways most prevalent based on the miRNAs with greatest up- or downregulation include cancer, inflammatory disease, and gastrointestinal diseases (Fig. 7B).

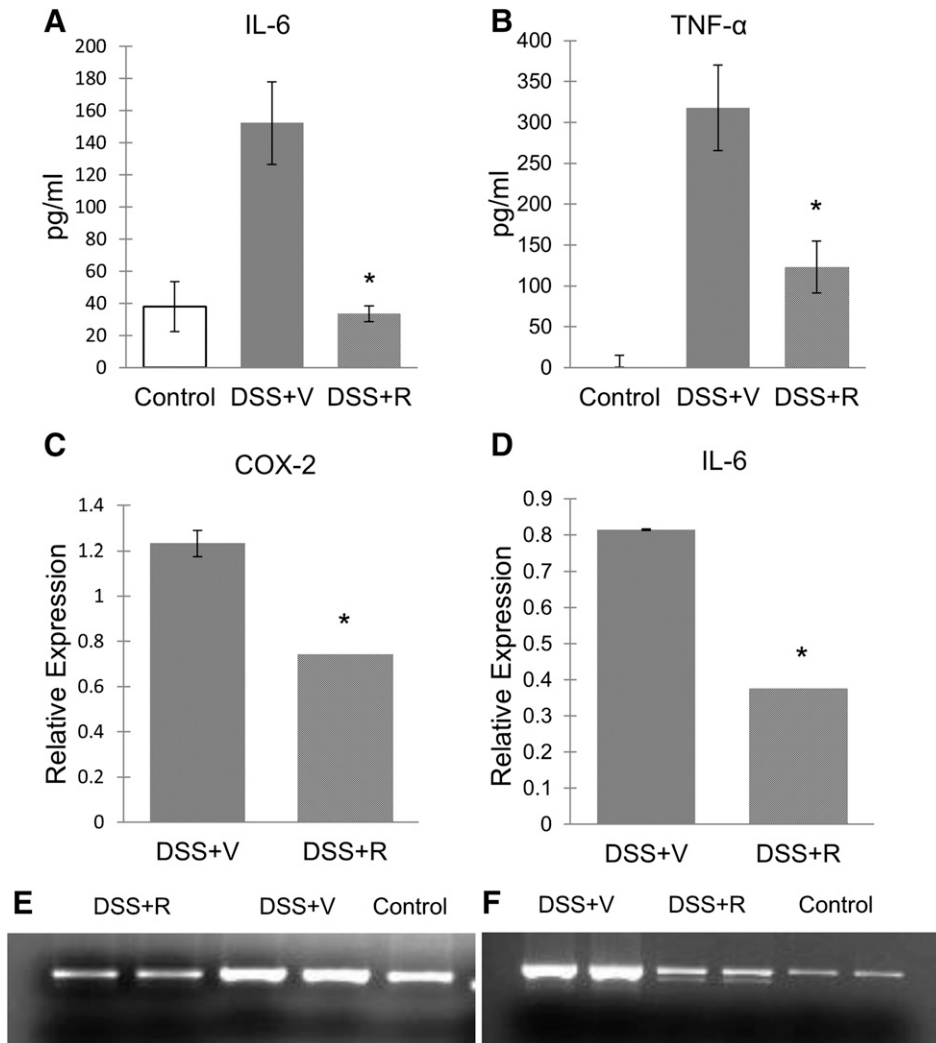


Fig. 3. Resveratrol reduces the concentration of proinflammatory cytokines in the small intestine. The effects of resveratrol compared with vehicle on the concentration of IL-6 (A) and TNF- α (B) in pooled sections 2 and 3 of the small intestine of DSS-exposed *Apc*^{Min/+} mice were analyzed by enzyme-linked immunosorbent assay. Values are averages \pm S.E.M. * $P < 0.05$ vs. DSS + V group. Resveratrol reduces COX-2 (C) and IL-6 (D) mRNA expression in mucosal scrapings from *Apc*^{Min/+} mouse small intestines. The corresponding densitometry is shown below each PCR for COX-2 (E) and IL-6 (F). Data are expressed as mean \pm S.E.M. and statistical significance calculated using the Student's *t* test. * $P < 0.05$ vs. DSS group.

The targets identified by IPA as having direct interactions with differentially expressed miRNAs were analyzed with ClueGo in Cytoscape to determine the relevant inflammatory pathways altered by resveratrol treatment (Supplemental Fig. 1) (Bindea et al., 2009). This analysis revealed that the resveratrol treatment of DSS-exposed *Apc*^{Min/+} mice predominantly targets expression of components involved in leukocyte differentiation and Toll-like receptor (TLR) pathways. The most relevant miRNAs modulated by resveratrol treatment that potentially could play a role in modulation of the immune response include miR-343, miR-130a-3p, let-7a-5p, miR-3909, miR-708-5p, miR-221-3p, miR-504, miR-874-3p, and miR-16-5. The microRNA targets with the greatest involvement in these pathways include ICAM1, IL-12A, CD4, S100A8, IL-10, AHR, CD3E, IL-6, IL-6St, TNF, PPAR γ , F2RL1, DDX58 (also called RIP1), CCR2, and TNFAIP2 (also called A20). T-cell activation, regulation of leukocyte activation, and regulation of the immune response are also significantly linked to targets of differentially expressed miRNAs. It is noteworthy that TNF, IL-6, and CD4 had numerous connections to involved pathways. AhR, IL-10, IL-12, and ICAM1 were also centrally featured targets.

Discussion

One bioactive property of resveratrol is its ability to reduce inflammation, a hallmark linked to every step of tumorigenesis (Wood et al., 2010). However, there are few studies examining resveratrol's benefits in rodent colon cancer models (Hudson et al., 2013) and even fewer exploring the role of microRNA in resveratrol-mediated anti-inflammatory effects. Therefore, the purpose of this investigation was to examine the effects of resveratrol on inflammation, immune regulation, polyp characteristics, and miRNA expression in the *Apc*^{Min/+} mouse model of intestinal tumorigenesis.

Animal studies reporting beneficial effects of resveratrol in cancer have recently been published (Athar et al., 2007). Resveratrol reduces colon adenomas, limits dysplasia, increases apoptosis, and decreases cell proliferation in *Apc*^{Min/+} mice following benzo(a)pyrene-induced colon carcinogenesis (Hudson et al., 2013). Resveratrol also reduces aberrant crypt foci in a chemically induced AOM/DSS model of colon cancer (Boddicker et al., 2011). Likewise, we demonstrated the chemopreventive effects of resveratrol in the AOM/DSS model; resveratrol reduced tumor incidence from 2.4 tumors to 0.2 per mouse (Cui et al., 2010).

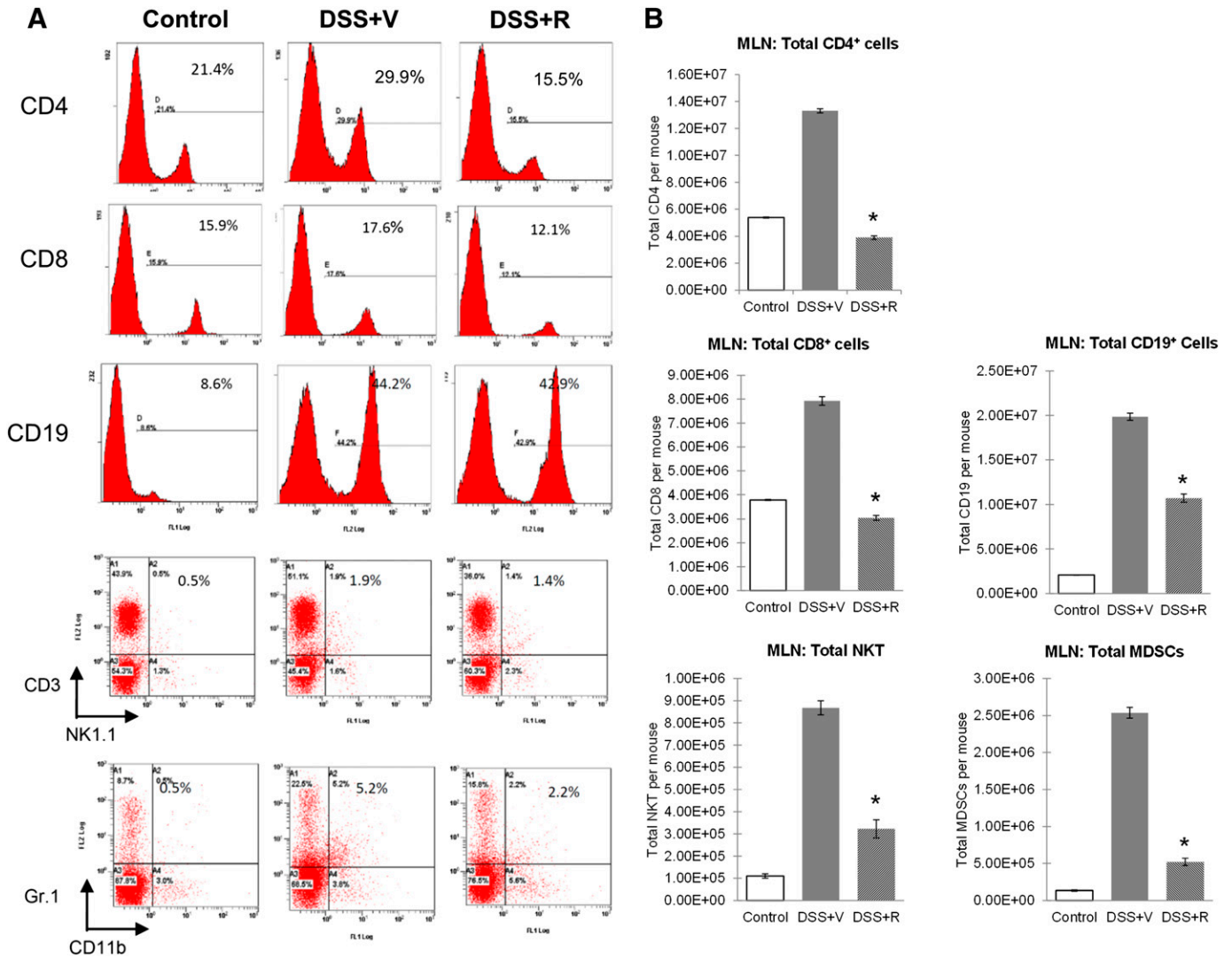


Fig. 4. Resveratrol diminishes the impact of DSS exposure on immune cell modulation in mesenteric lymph nodes. (A) Flow cytometry analysis of mesenteric lymph node cells from three groups of *Apc^{Min/+}* mice (control, DSS + V, and DSS + R). Cells were stained with anti-CD4 (CD4 T cells), anti-CD8 (CD8 T cells), anti-CD19 (B cells), anti-CD3 and anti-NK1.1 (NKT cells), or anti-Gr1 and anti-CD11b (MDSCs) antibodies. Results show that treatment with 100 mg/kg of resveratrol reversed the impact of DSS on percentages of each of the cell subsets measured. (B) Graphs depict the absolute numbers of these cells. Values are expressed as the average \pm S.E.M. * $P < 0.05$ vs. DSS + V group.

In the current study, we found that oral administration of resveratrol decreased the overall polyp burden in the colon of *Apc^{Min/+}* mice. When stratified by size, resveratrol specifically reduced the number of large polyps and the percentage of large polyps within the colon as well as histologic signs of cell damage. We interpret this to mean that resveratrol can affect both development and growth of polyps.

The cell cycle machinery is a recognized target of resveratrol action (Ahmadi et al., 2009). Thus, we examined the effects of resveratrol on cell proliferation. As expected, BrdU and PCNA immunohistochemical staining of the colonic mucosal epithelium indicated decreased proliferating cells following resveratrol treatment. This confirms the reported antiproliferative properties of resveratrol on several cancer cell lines and decreases in cell proliferation with resveratrol in *Apc^{Min/+}* mice following benzo(a)pyrene-induced colon carcinogenesis (Hudson et al., 2013).

Our results show that resveratrol treatment significantly represses the intestinal mucosal protein concentration of IL-6

and TNF- α and mRNA expression of COX-2 and IL-6. These are proinflammatory mediators with a well documented role in promoting colon cancer and known to be modulated by resveratrol (Oshima and Oshima, 2012). The literature supports a positive relationship between IL-6 and increasing tumor stage and size, metastasis, and decreased survival in colon cancer (Knüpfner and Preiss, 2010). Additionally, resveratrol reduced circulating IL-6 in an AOM/DSS model of colon cancer (Boddicker et al., 2011).

TNF- α is known to promote colon cancer (Flores et al., 2012) and is associated with human irritable bowel disease and colitis (Natsui et al., 1997). Resveratrol is reported to reduce TNF- α in colon cancer cell lines (Paul et al., 2009). Additionally, resveratrol inhibits COX-2, which is known to be overexpressed in gastric tumors, in the *Apc^{Min/+}* mouse model (Sale et al., 2005). In fact, resveratrol may directly inhibit COX-2 expression, as resveratrol suppressed growth of COX-2-positive colon cancer cells, whereas there was no response in COX-2-deficient cells (Zykova et al., 2008). Our

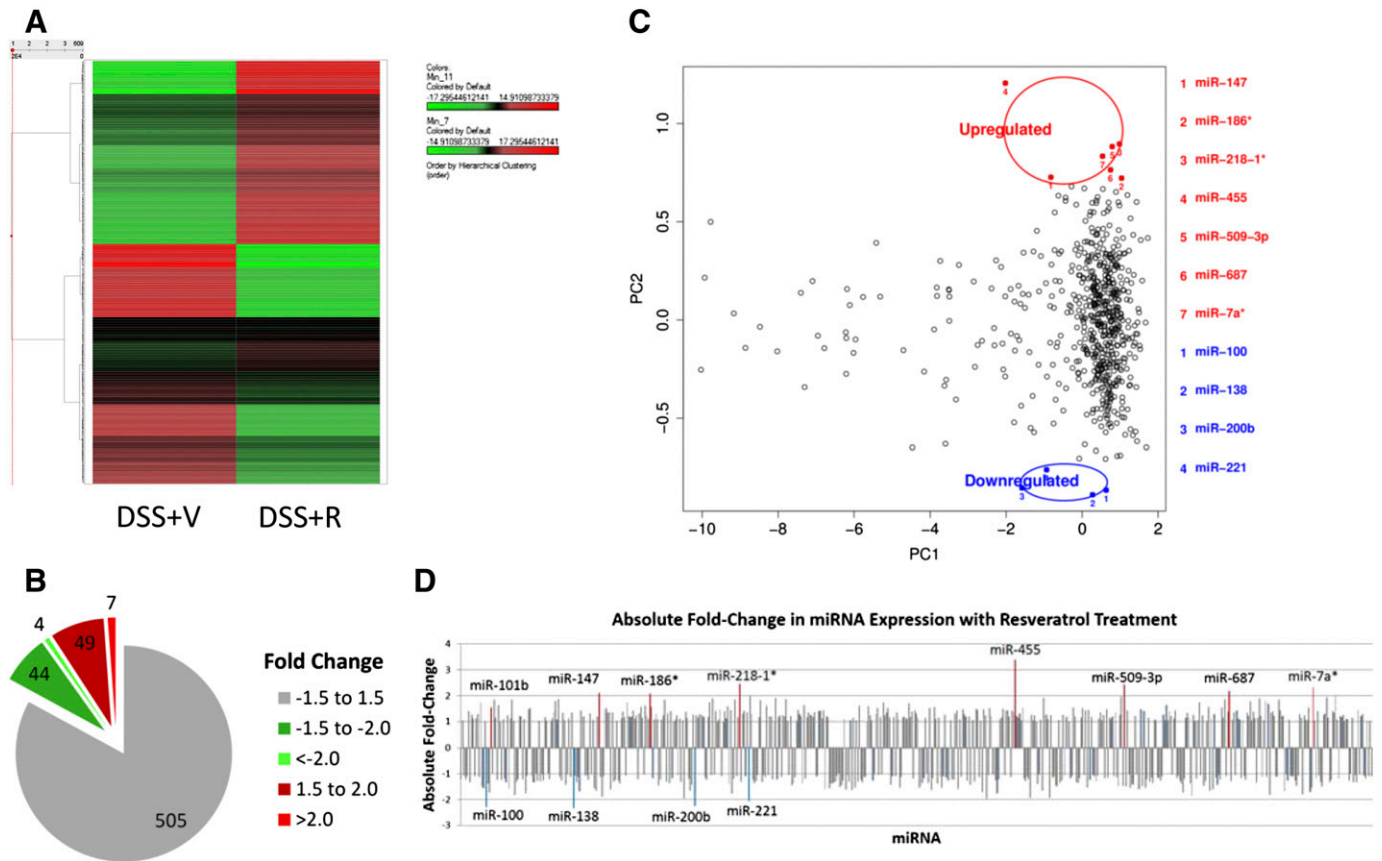


Fig. 5. Differences in miRNA expression profiles of DSS-exposed *Apc*^{Min/+} mice treated with vehicle or resveratrol. (A) Heat map of miRNA array performed on cell extracts from intestinal mucosal scrapings from two groups of DSS-exposed *Apc*^{Min/+} mice (DSS + V and DSS + R). Analysis demonstrated that a cluster of miRNAs are differentially expressed in mice treated with resveratrol. Red represents a positive change, green represents a negative change, and black represents no change. (B) Pie chart showing proportion of genes with <math><1.5</math>-fold change, 1.5- to 2.0-fold up- or downregulation, and >2-fold up- or downregulation. (C) The bar graph shows absolute fold change in expression of 609 miRNAs tested by Affymetrix microarray analysis of DSS-exposed *Apc*^{Min/+} mice treated with resveratrol compared with vehicle. (D) miRNAs that were >2-fold up- or downregulated are highlighted as well as miR-101b and -455, which were chosen for verification by RT-PCR. A principal component analysis plot was constructed with fold change >2 defined as upregulated and fold change <-2 defined as downregulated.

findings of a reduction in select inflammatory mediators (IL-6, TNF- α , and COX-2) following resveratrol treatment thus offer a plausible explanation for the reported benefits on polyp number and size.

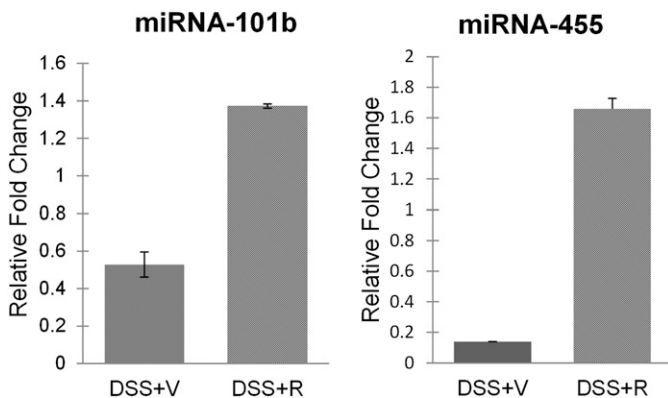
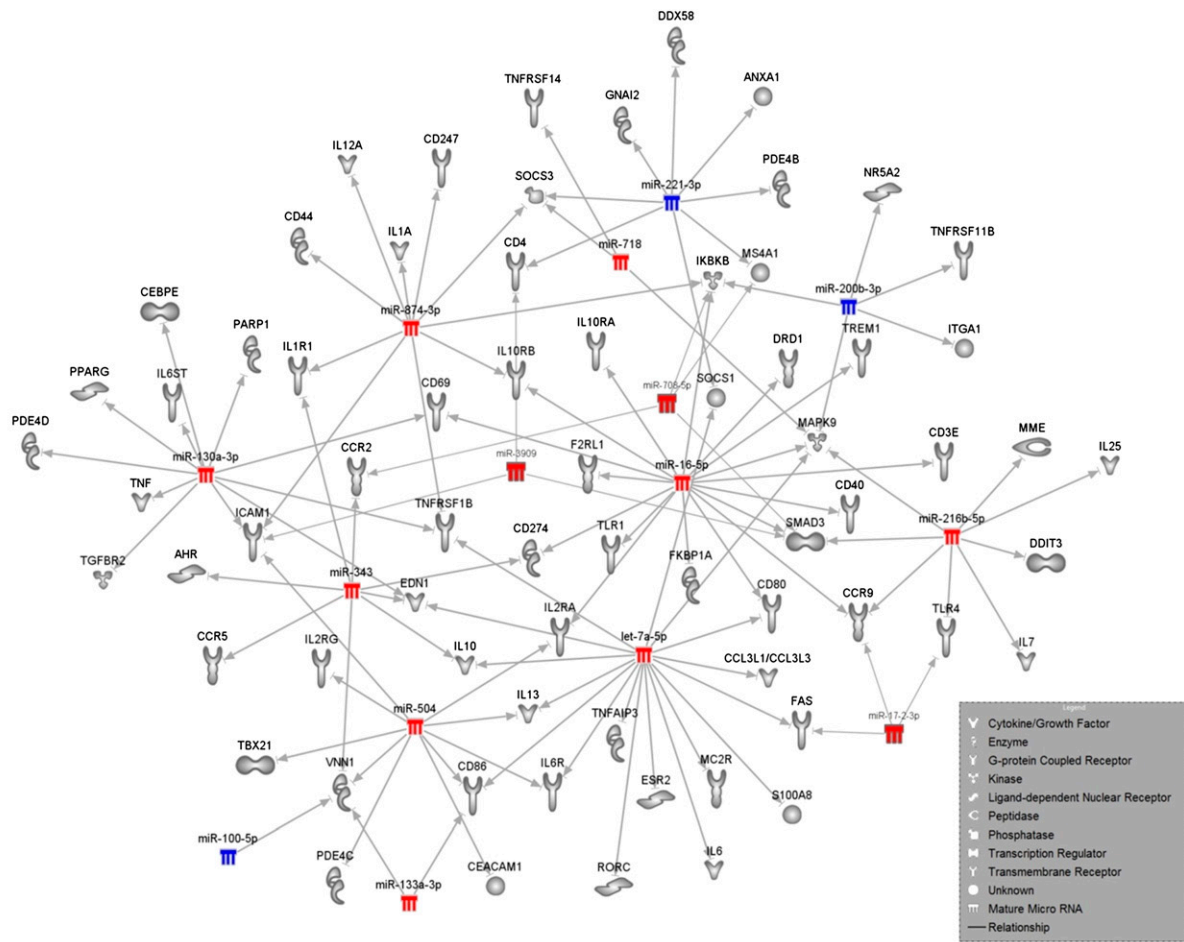


Fig. 6. Quantitative RT-PCR verifying upregulation of miRNA-455 and -101b expression in DSS-exposed *Apc*^{Min/+} mice treated with resveratrol. Quantitative RT-PCR shows that resveratrol treatment upregulates relative expression of miRNA-455 and -101b in mucosal tissue. Data are expressed as mean \pm S.E.M., and statistical significance was calculated using the Student's *t* test. **P* < 0.05 vs. DSS + V group.

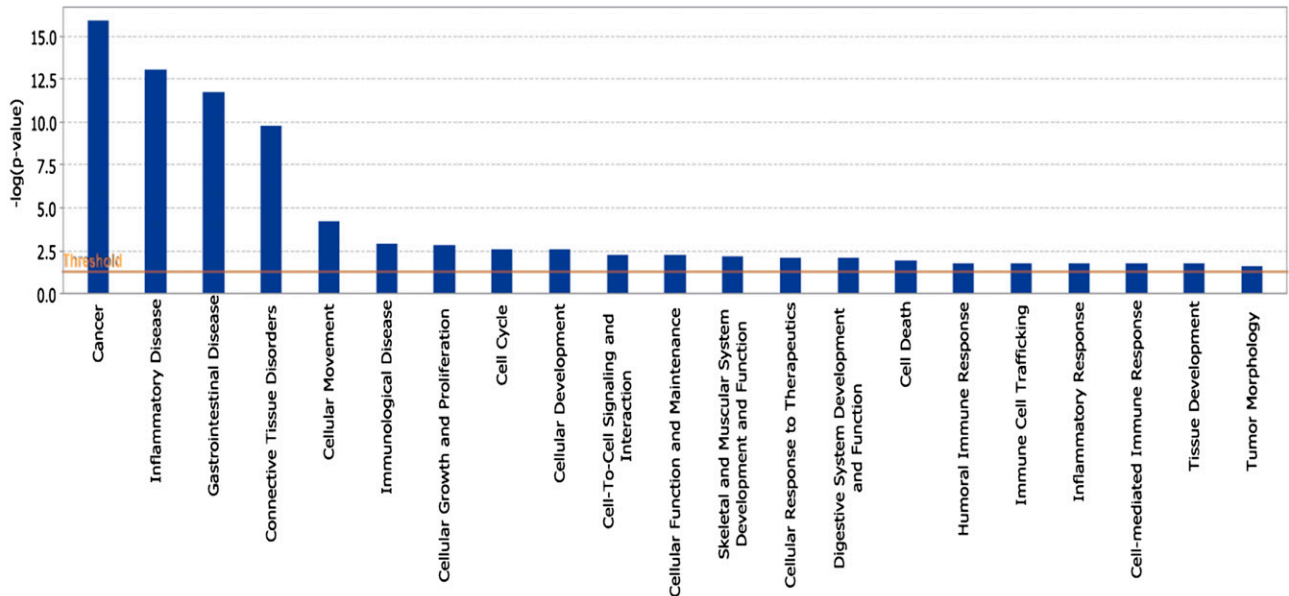
CD4⁺ T cells were examined, as they are reported to play a major role in the induction of irritable bowel disease and colitis-associated colon cancer. In fact, most of the intestinal damage caused by this disease is a result of CD4⁺ T cell-mediated injury (Elson et al., 1996). Further, RAG1-deficient mice, which lack B and T cells, do not develop tumors following AOM/DSS exposure, implicating a role for lymphocytes in the promotion of colon cancer (Becker et al., 2004). Our findings showed increases in the number of CD4⁺ T cells in MLNs, which was mitigated by resveratrol treatment. Importantly, the documented increase in the number of CD4⁺ T cells in this model was accompanied by marked elevation in inflammatory mediators, implicating a link between this cell population and inflammatory responses, which was reduced by resveratrol. Likewise, resveratrol treatment offset the increase in the number of CD8⁺ T cells and CD19⁺ B cells within MLNs.

We also measured MDSCs, as they are known to downregulate immune surveillance and antitumor immunity, thereby facilitating tumor growth (Ostrand-Rosenberg and Sinha, 2009). In the present study, we found an increase in MDSCs in MLNs. However, resveratrol treatment resulted in a significant downregulation in the absolute number of MDSCs. As MDSCs exert suppressive activity on cytotoxic

A



B



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Fig. 7. (A) Network wiring diagrams for miRNAs differentially regulated in DSS-exposed *Apc^{Min/+}* mice treated with resveratrol. Relationships for miRNAs with >1.5-fold-increased or -decreased expression with resveratrol treatment, for which mapping information was available and interaction experimentally demonstrated, were plotted. Relationships known to exist for immunomodulatory mRNAs interacting with the miRNAs of interest are shown. miRNAs in red are upregulated, and those in blue are downregulated. (B) Differential expression of pathway-specific microRNA following treatment of DSS-exposed *Apc^{Min/+}* mice with resveratrol. Canonical pathway of gene targets of upregulated or downregulated miRNAs. Pathways are sorted by score ($-\log P$ value). A higher score indicates that the pathway is more significantly associated with miRNAs of interest. The horizontal line represents statistically significant threshold limit.

T cells along with other immune cells, a reduction in this cell population with resveratrol would presumably result in a more favorable antitumor immune response.

Interestingly, NKT cells are implicated as both enhancers and suppressors of antitumor activity. We found that NKT cells were significantly increased in MLNs in this model and that resveratrol significantly offset this effect. However, given the dual role of NKT cells, these results should be interpreted with caution; type I NKT cells enhance tumor immunity by interferon- γ expression and NK cell activation (Yang et al., 2000) and are positively correlated with disease-free survival, whereas activation of type II NKT cells is associated with protumoral responses. Further investigation is needed to fully elucidate the effects of resveratrol on subsets of NKT cells in this model. Nonetheless, to our knowledge this is the first study to report a beneficial effect of resveratrol on immune regulation in a mouse model of intestinal tumorigenesis.

Resveratrol decreases the expression of several proinflammatory and/or oncogenic miRNAs and, conversely, upregulates miRNAs with anti-inflammatory and/or antitumor potential (Sonkoly and Pivarcsi, 2009). We found that 104 out of 609 miRNAs exhibited >1.5-fold change in expression with resveratrol treatment, with 56 miRNAs upregulated and 48 downregulated. We identified the putative targets of several of these miRNAs to be related to inflammatory processes. These include miRNA-455 and miRNA-101b, both upregulated following resveratrol treatment. In particular, miRNA-101b has a high-affinity binding site with the mRNA of Ptg2 (COX-2) (Strillacci et al., 2009). Upregulation of these miRNAs by resveratrol is a potential molecular mechanism for the decrease in TNF- α and COX-2 observed in this investigation.

IPA indicated that the miRNAs altered by resveratrol have important roles in cancer, inflammatory disease, and gastrointestinal disease. In fact, when these miRNAs and their target genes are graphically annotated using the IPA software and Cytoscape, we found direct associations between certain miRNAs with altered expression upon resveratrol treatment and numerous genes with immunomodulatory functions and known associations with colon cancer, including CD4, IL-6, TNF, ICAM1, PPAR γ , and CCR2 (Supplemental Fig. 2).

CD4 is a target of miR-3909, which is upregulated in resveratrol-treated mice. CD4⁺ effector T cells, which can release IL-6, are known to promote chronic inflammation in irritable bowel disease, leading to tumor initiation, promotion, and progression (Podolsky, 2002). As such, studies have linked IL-6 expression to sporadic and inflammation-associated colon cancer (Waldner et al., 2012). In the current study, miRNAs targeting IL-6 (let-7a-5p), IL-6R (miR-504 and let-7a-5p), and IL-6ST (miR-130a-3p) were upregulated in resveratrol-treated mice, thereby suggesting decreased induction of these inflammatory markers.

TNF- α is also associated with colorectal carcinogenesis, and blocking the expression of TNF- α in a murine model of colon cancer reduces tumor burden (Popivanova et al., 2008). Here, we show that resveratrol reduces TNF- α levels in DSS-exposed *Apc*^{Min/+} mice and that this may be achieved by upregulation of miR-3909 and miR-130a-3p. TNF- α signaling activates NF- κ B, which, in turn, induces the expression of COX-2, IL-6, IL-8, and TNF- α (Karin and Greten, 2005). Therefore, inhibition of TNF signaling by miRNA upregulation is a possible mechanism by

which resveratrol may reduce intestinal inflammation and tumorigenesis.

Additionally, pathway analysis revealed that several miRNAs upregulated with resveratrol treatment were strongly associated with TLR3 and -4 signaling pathways. The TLR4 pathway is required for COX-2 induction in DSS-treated mice. AOM/DSS treatment induced colon tumorigenesis and development of colitis-associated colon cancer in bone marrow chimera experiments (Fukata et al., 2009).

Although animal models allow for examination of stage-specific responses to various dietary strategies, it is important to note that our study design considers only the chemopreventive effects of resveratrol. This is consistent with the majority of animal studies on resveratrol's effects on colon cancer. Thus the chemotherapeutic effects of resveratrol are less clear. Given that polyp number generally does not increase after ~12 weeks of age in the *Apc*^{Min/+} mouse, we would expect that any therapeutic effect of resveratrol in this model would be reflected by a reduction in polyp size but not number.

In summary, we show that dietary resveratrol was associated with decreased cell proliferation, reduced inflammation, and suppression of the immune response. Consistent with these findings, miRNA analysis revealed alterations in pathways relevant to inflammation, cancer, and gastrointestinal disease with resveratrol treatment. Taken together, these data support further development of resveratrol as a potential chemopreventive strategy for patients at risk for development of colon cancer.

Authorship Contributions

Participated in research design: P. S. Nagarkatti, M. Nagarkatti.

Conducted experiments: Altamemi, Murphy, Catroppo, McClellan, Singh.

Contributed new reagents or analytic tools: P. S. Nagarkatti, M. Nagarkatti, Zhang.

Performed data analysis: Altamemi, Murphy, Catroppo, Zumbun, Zhang.

Wrote or contributed to the writing of the manuscript: Altamemi, Murphy, Zumbun, P. S. Nagarkatti, M. Nagarkatti.

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