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Role of resveratrol-induced CD11b⁺ Gr-1⁺ myeloid derived suppressor cells (MDSCs) in the reduction of CXCR3⁺ T cells and amelioration of chronic colitis in IL-10^{-/-} mice

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Abstract

Resveratrol, a naturally occurring polyphenol has received significant attention as a potent anti-inflammatory agent. Inflammatory bowel disease (IBD) is a chronic intestinal inflammation caused by hyperactivated effector immune cells that produce pro-inflammatory cytokines. Myeloid derived suppressor cells (MDSCs) are a heterogeneous population characterized by the co-expression of CD11b⁺ and Gr-1⁺ and have long been known for their immunosuppressive function. We report that resveratrol effectively attenuated overall clinical scores as well as various pathological markers of colitis in IL-10^{-/-} mice by down regulating Th1 responses. Resveratrol lessened the colitis-associated decrease in body weight and increased levels of serum amyloid A (SAA), CXCL10 and colon TNF- α , IL-6, RANTES, IL-12 and IL-1 β concentrations. After resveratrol treatment, the percentage of CXCR3 expressing T cells was decreased in the spleen, mesenteric lymph nodes (MLN), and intestinal lamina propria (LP). However, the percentage and absolute numbers of CD11b⁺ and Gr-1⁺ cells in the lamina propria (LP) and spleen were increased after resveratrol treatment as compared with the vehicle treatment. Co-culture of resveratrol induced CD11b⁺ Gr-1⁺ cells with T cells, attenuated T cell proliferation, and most importantly reduced IFN- γ and GM-CSF production by LP derived T cells from vehicle treated IL-10^{-/-} mice with chronic colitis. The current study suggests that administration of resveratrol into IL-10^{-/-} mice induces immunosuppressive CD11b⁺ Gr-1⁺ MDSCs in the colon, which correlates with reversal of established chronic colitis, and down regulation of mucosal and systemic CXCR3⁺ expressing effector T cells as well as inflammatory cytokines in the colon. The induction of immunosuppressive CD11b⁺ Gr-1⁺ cells by resveratrol during colitis is unique, and suggests an as-yet-unidentified mode of anti-inflammatory action of this plant polyphenol.

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Keywords

Inflammation; Resveratrol; Colitis; CXCR3; CD11b⁺ and Gr-1⁺ and MDSCs

1. Introduction

Inflammatory bowel disease (IBD) are chronic, relapsing, and remitting gastrointestinal diseases characterized by inflammation in the intestine (Strober et al., 2002). IBD is characterized by two major forms Crohn's disease (CD) and ulcerative colitis (UC). It has been suggested that human IBD is associated with infiltration of the intestinal lamina propria (LP) by mononuclear cells such as macrophages or lymphocytes that over-express NF- κ B-regulated target genes, such as the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 (Podolsky, 2002). Murine models of colitis that resemble human IBD provide important tools to develop novel therapeutic strategies for this disease (Strober et al., 2007). Similar to human IBD, it has been reported that persistent intestinal inflammation in mice results from massive immune cell infiltration, associated with either enhanced or aberrant immunologic responsiveness to the normal gut flora (Singh et al., 2007b). It has been shown that T cells are key players in tissue injury mediated by both immune and non-immune cells, and occurs in the areas that are heavily infiltrated with activated T cells (Maynard and Weaver, 2009).

Distinct macrophage populations have been identified which are prominent in active disease and play an important role in the pathogenesis of Th1 mediated IBD (Kamada et al., 2010). MDSCs are heterogeneous populations of cells composed of precursors of macrophages, granulocytes, dendritic cells, and myeloid cells at earlier stages of differentiation (Kusmartsev et al., 2003). MDSCs show their ability to differentiate under the influence of selected cytokines and differentiation factors into more mature cell types both *in vivo* and *in vitro* (Kusmartsev et al., 2003). Several cytokines have been reported to play a key role in the recruitment of MDSCs to peripheral organs; these include granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), macrophages colony-stimulating factor (M-CSF), and IL-6 (Hegde et al., 2010; Serafini et al., 2006). More recently, it has been reported that the frequency of MDSCs dramatically increased during intestinal inflammation in mice and that these cells suppressed IFN- γ release by T cells (Haile et al., 2008). Furthermore, MDSC frequency increases in the peripheral blood of patients with active colitis, possibly halting the development of more severe and possibly fatal colitis. Together, these studies identify MDSCs as a previously unexplored important immune regulatory system in IBD.

The CXCR3 ligand, CXCL10, is an immediate-early gene that is induced by IFN- γ and expressed by epithelium, fibroblasts, keratinocytes, natural killer (NK) cells and monocytes (Farber, 1997). We have shown that CXCR3 ligands are upregulated at the sites of colitis in IL-10^{-/-} mice (Singh et al., 2003b). Additionally, in human IBD patients, the number of CD4⁺ CXCR3⁺ T cells in the LP has been shown to be higher as compared with normal, healthy donors (Yuan et al., 2001). Moreover, our laboratory and others have shown that blocking of CXCL10 expression prevents the development of symptomatic chronic colitis in IL-10^{-/-} mice (Hyun, 2005; Singh et al., 2003a).

The polyphenolic phytoalexin, resveratrol (3,5,4'-trihydroxy-trans-stilbene), is a naturally occurring stilbene found in peanuts, grapes, and red wine that exert several biological activities (Gholam et al., 2007). Resveratrol been shown to extend the life span of yeast and mice (Howitz et al., 2003) and regulate tumor growth (de la Lastra and Villegas, 2005; Singh et al., 2011). Recent studies from our laboratory and elsewhere suggested that resveratrol exhibits strong anti-inflammatory properties and suppresses lipopolysaccharide-

induced airway inflammation (Birrell et al., 2005), experimental model of multiple sclerosis (Singh et al., 2007a), osteoarthritis (Elmali et al., 2005), colitis and colon cancer (Cui et al., 2010; Martin et al., 2004; Singh et al., 2010), and allograft rejection (Wu et al., 2006). Resveratrol mediates these anti-inflammatory effects through multiple pathways.

Conventional treatment of colitis by infliximab and adalimumab can reduce periods of active disease and help to maintain remission, but these treatments often bring marginal results and the disease becomes refractory. Unfortunately, the side effects associated with these treatments could result in adverse reactions or poor responses by the patients, thereby limiting their clinical use (Mouser and Hyams, 1999). For this reason, many colitis sufferers turn to unconventional treatments in the hope of abating symptoms of active disease. It is estimated that 40% of IBD patients use some form of megavitamin therapy or herbal or dietary supplement (Head and Jurenka, 2004). While recent studies have shown that resveratrol can suppress colitis (Cui et al., 2010; Martin et al., 2004; Singh et al., 2010), the role of regulatory cells in this process has not been investigated. Here, we provide data demonstrating that oral administration of resveratrol not only ameliorates the established chronic colitis in IL-10^{-/-} mice but also results in the significant induction of immunosuppressive CD11b⁺ Gr-1⁺ cells that may further contribute to the anti-inflammatory effects of resveratrol treatment. Based on this induction, we propose that resveratrol-induced CD11b⁺ Gr-1⁺ cells may suppress the local effector T cell responses in LP, thus inhibiting inflammation and leading to reversal of chronic colitis.

2. Materials and Methods

2.1 Animals

Female IL-10^{-/-} mice on BL/6 background and BL/6 wild-type mice aged ~10 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were housed and maintained in micro-isolator cages under conventional housing conditions at the University of South Carolina School of Medicine animal facility. Experimental groups consisted of six mice each and the study was repeated three times.

2.2 Fecal sample collection

Fecal samples were collected immediately before sacrifice of the mice after the week 28 at experimental endpoint. Fecal samples were dissolved in PBS containing 0.1% sodium azide (e.g., 1 ml per 100 mg of fecal pellet), vortexed, centrifuged, and the supernatant was collected for analysis.

2.3 Spontaneous chronic colitis development in IL-10^{-/-} mice

Under conventional housing conditions, IL-10^{-/-} mice develop spontaneous colitis with 100% of incidence by the week 12 and develop chronic colitis by the week ~18. The histological feature presented by these mice shows similarities to human IBD patients. We followed similar criteria to determine the onset of spontaneous chronic colitis based on our previous study, based on the level of serum amyloid A (SAA) and IL-6 which reached to 300 µg/ml and 5.0 ng/ml respectively, with at least a 15% decrease in the original body weight (Singh et al., 2003a). To determine, whether resveratrol can suppress the established colitis following onset of chronic colitis after 18th week, IL-10^{-/-} mice received either 100 µl of 10, 50 or 100 mg/kg body weight dose of resveratrol by oral gavage as described previously (Singh et al., 2007a) or saline (termed as vehicle treated group), every second day until the termination of the experiment. As other controls, normal BL/6 mice also received similar treatments till the end-point of the experiment. After 28th week of treatment, mice were sacrificed and blood, fecal sample, spleen, MLN and LPL were collected for measuring all analysis. The 10 and 50 mg/kg body weight dose did not show any significant

improvement in colitis severity or in body weight as compared to vehicle treated mice while the 100mg/kg dose did. Therefore, we used the 100mg/kg body weight dose in our subsequent experiments. The body weight of each mouse was monitored twice a week and presented as mean for each week. Resveratrol (3,4,5-Trihydroxy-trans-stilbene), a purified compound with the molecular formula $C_{14}H_{12}O_3$ was obtained from Sigma Chemical Co (St. Louis, MO). The certificate of origin indicated that it was originally purified and extracted from the bushy knotweed plant and was found to be greater than 99% pure by both gas chromatography and thin-layer chromatography. At the end of the experiment, colon samples were washed with phosphate-buffered saline (PBS), cut longitudinally, fixed in formalin, and embedded in the paraffin.

2.4 Cell isolation

Spleens and MLN from individual mice were mechanically dissociated. RBCs were lysed with lysis buffer (Sigma St. Louis, MO). Single cell suspensions of spleen and MLN were passed through a sterile wire screen (Sigma St. Louis, MO). Cell suspensions were washed twice in RPMI 1640 (Sigma St. Louis, MO) and stored in media containing 10% FBS on ice until used within two hours. The small intestine was cut into 1-cm strips and stirred in PBS containing 1mM EDTA at 37°C for 30 min. The cells from intestinal LP were isolated as described previously (Singh et al., 2003a). In brief, the LP was isolated by digesting intestinal tissue with collagenase type IV (Sigma St. Louis, MO) in RPMI 1640 (collagenase solution) for 45 min at 37°C with moderate stirring. After each 45 min interval, the released cells were centrifuged, stored in complete media and mucosal pieces were replaced with fresh collagenase solution at least two times. LP cells were further purified using a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient collecting at the 40–75% interface. Lymphocytes were maintained in complete medium, which consisted of RPMI 1640 supplemented with 10 ml/L of nonessential amino acids (Mediatech, Washington, DC), 1 mM sodium pyruvate (Sigma), 10 mM HEPES (Mediatech), 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin (Elkins-Sinn, Inc., Cherry Hill, NJ), 50 µM mercaptoethanol (Sigma) and 10 % FCS (Atlanta Biologicals).

2.5 In vitro proliferation assay

At termination of the experiment (after 28th week), both resveratrol and vehicle treated IL-10^{-/-} mouse splenocytes, LPL (under healing micro-environment) were collected and stained for CD11b⁺ Gr-1⁺ cells and analyzed by flow cytometry. The double positive CD11b⁺ Gr-1⁺ cells (MDSCs) were sort-purified by using a FACS-AriaTM (Becton Dickinson) (purity >90%) cell sorter. These sorted CD11b⁺ Gr-1⁺ cells express high levels of ARG-1 but very little NOS-2. To demonstrate the proliferation, purified T cells from MLN of normal BL/6 mice were cultured either alone, or co-cultured with purified CD11b⁺ Gr-1⁺ at T cell: CD11b⁺ Gr-1⁺ cell ratios of (10:1, 5:1, and 1:1). These cultures were stimulated with CD3 and CD28 Abs (BD PharMingen) at 37°C in 5% CO₂. Proliferation of co-culture cells was measured by the 5-Bromo-2'-deoxy uridine (BRDU) absorption and detection (Roche Diagnostics; Dusseldorf, Germany). In brief, after 72 hour of culture, 10 µl of BrdU labeling solution (10 µM/ml final concentration per well) was added and incubated for 18 h at 37°C with 5 % CO₂. The cells were then fixed and incubated with 100 µl of nuclease in each well for 30 minute at 37°C. Next, cells were pulsed with BrdU labeling solution and incorporation was detected by ELISA assay (Roche Molecular Biochemical). To demonstrate whether, vehicle or resveratrol induced CD11b⁺ Gr-1⁺ cells have the potential to suppress the T lymphocyte response, the LP T cells obtained from vehicle or resveratrol treated normal or IL-10^{-/-} mice, were co-cultured with purified CD11b⁺ Gr-1⁺ cells at T cell: CD11b⁺ Gr-1⁺ cell ratios of 5:1. The culture supernatants were collected after 3 days and assayed for cytokine (IFN-γ and GM-CSF) secretion by Luminex assay.

2.6 Flow cytometry analysis

Cells from the spleen, MLN, and LP were freshly isolated as described above from each experimental group as well as from normal BL/6 mice treated with resveratrol. The MDSCs were sort-purified using a FACS Aria using double positive of CD11b⁺ Gr-1⁺ cells (Becton Dickinson). For cell surface antigen staining, cells were pre-blocked with Fc receptors for 15 min at 4°C. The cells were then washed with FACS staining buffer (PBS with 1% BSA) followed by staining with Abs. The cells were stained with CY-, FITC- or PE-conjugated - CD4 (H129.19), CD11b (M1/70), Gr-1 (BD-PharMingen, San Diego CA), and/or-CXCR3 (CXCR3- clone173), CD69 (H1.2F3) from (Bio-Legend, San Diego), mouse anti-ARG-1 (BD Transduction Laboratories), Alexa Flour (647) conjugated NOS-2 C-11 from (Santa Cruz Biotechnology, Inc) APC conjugated CXCL10 (R& D system) for 30 minutes with occasional shaking at 4°C. The cells were washed two times with FACS staining buffer. Lymphocytes were again washed thoroughly with FACS staining buffer and analyzed by flow cytometry (FC 500, Beckman Coulter Fort Collins Co).

2.7 Cytokine quantitation by Luminex™ analysis

T helper cell-derived cytokines IL-1β, RANTES, IL-6, IL-12, IFN-γ, GM-CSF, and TNF-α in the colon tissue supernatant were assayed using Beadlyte™ mouse multi-cytokine detection system kit (Bio-Rad, Hercules, CA). Filter bottom ELISA plates (Bio Rad Hercules, CA) were rinsed with 100 μl of bioplex assay buffer and removed using a Millipore™ Multiscreen Separation Vacuum Manifold System (Bedford, MA), set at 5 mm Hg. Analyte beads in assay buffer were added into wells followed by 50 μl of serum or standard solution and incubated for 30 minutes at RT with continuous shaking (at setting #3) using a Lab-Line™ Instrument Titer Plate Shaker (Melrose, IL). The filter bottom plates were washed as before and centrifuged at 300× g for 30 seconds. Subsequently, 50 μl of anti-mouse IFN-γ, IL-1β, RANTES, IL-12, IL-6, or TNF-α Ab-biotin reporter solution was added in each well followed by incubation with continuous shaking for 30 minutes, centrifugation, and washing. Next, 50 μl streptavidin-phycoerythrin solution was added and incubated with continuous shaking for 10 minute at RT. 125 μl of Bio-Plex assay buffer was added and Beadlyte™ readings were measured using a Luminex™ System (Austin, TX) and calculated using Bio Rad Bioplex™ software. The cytokine Beadlyte™ assays were capable of detecting > 5 pg/ml for each analyte.

2.8 Abs, quantification by luminex assays

Total IgG and IgA Abs in fecal extracts were measured with a luminex assay kit (Millipore Corporation, MA USA). Analyte beads in assay buffer were added into pre-wet vacuumed wells followed by 25 μl of serum or standard solution, 25 μl of assay buffer, and 25 μl of assay beads, and incubated overnight at 4°C with continuous shaking (at setting #3) using a Lab-Line™ Instrument Titer Plate Shaker (Melrose, IL). The filter bottom plates were washed as before and centrifuged at 300× g for 30 seconds. Subsequently, 25 μl of anti-mouse detection Ab was added to each well and incubated for 1 hour at room temperature. Next, 25 μl streptavidin-phycoerythrin solution was added and incubated with continuous shaking for 30 minute at RT. 200 μl of wash buffer was added and Milliplex™ readings were measured using a Luminex™ System (Austin, TX) and calculated using Millipore software. The Ab Milliplex™ MAP assays were capable of detecting > 10 pg/ml for each analyte.

2.9 Acute phase (SAA) ELISA

SAA level was determined by ELISA (Biosource International, Camarillo, CA) in the serum after termination of the experiment. In brief, 50 μl of SAA-specific mAb solution was used to coat micro-titer strips to capture SAA. Serum samples and standards were added to wells

and incubated for 2 hours at RT. After washing in the assay buffer, the HRP-conjugated anti-SAA mAbs solution was added and incubated for 1 hour at 37° C. After washing, 100µl TMB (Biosource International, Camarillo, CA), substrate solution was added and the reaction was stopped after incubation for 15 minutes at RT. After adding the stop solution, plates were read at an optical density of 450_{nm}.

2.10 Serum CXCL10 ELISA

Serum concentrations of CXCL10 were determined by ELISA assay (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. In brief, samples and standards were added to the wells and plates were incubated for 2 hrs at room temperature (RT). Next, conjugate-detection solutions were added to each well, and the plates were further incubated for 2 hrs at RT. Then, substrate solutions were added to each well and plates were incubated for 30 mins. Finally, stop solution was added to each well and the plates were read at an optical density of 450 nm after 30 mins using a λ correction of 540 and 570 nm. ELISA assays were capable of detecting > 10 pg/ml of CXCL10.

2.11 Histology

Mice were sacrificed at the experimental end-point (at 28th week) and colons were fixed in 10% buffer neutral formalin and embedded in paraffin. Fixed tissues were sectioned at 6 µm and stained with hematoxylin and eosin. Two independent pathologists, blinded to the study design, gave an inflammation score to samples taking into account the number of lesions as well as severity of the disease. A score (0 to 4) was given based on established criteria (Singh et al., 2003a). Each segment of colon were scored 0 to 4 based on the following well establish criteria. Grade 0, no change from normal tissue; grade 1, 1 or few multifocal mononuclear cell infiltrates in the LP; grade 2, intestinal lesion involved several multifocal cellular infiltrates in LP; grade 3, lesions involved moderate inflammation and epithelial hyperplasia; grade 4, inflammation involved most of the intestinal sections. The summation of scores per mouse provided the total colonic disease score. Summation of these disease scores provided a total colonic disease score with graded lesions in proximal, middle, and distal colon segments.

2.12 Statistics

The data were expressed as the mean $\pm$ SEM and compared using a two-tailed paired Student's *t*-test or an unpaired Mann Whitney U test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Inc., Berkeley, CA) and Microsoft Excel (Microsoft, Seattle, WA). Single-factor variance ANOVA and Duncan's Multiples range test analyses were used to evaluate changes in groups. Results were considered statistically significant if *p* values were < 0.05 between the control and experimental groups.

3. Results

3.1 Resveratrol reduces weight loss and elevated SAA levels typically associated with colitis

Because resveratrol has previously been shown to be a potent inhibitor of the inflammatory process, we wanted to assess its role in spontaneous established chronic colitis. We first examined the effect of resveratrol on body weight loss and SAA concentration in IL-10^{-/-} and normal BL/6 wild-type mice. Vehicle-treated IL-10^{-/-} mice developed chronic colitis as shown by significant weight loss, diarrhea, ruffled fur, and occasional rectal bleeding. Associated with the development of colitis was a 15% to 20% reduction in initial body weight. We did not notice any rectal prolapse in mice during our entire study. Body weights of the vehicle-treated group of mice continued to decline throughout the study (Fig. 1A). In

contrast, resveratrol treatment caused significant improvements in body weight of IL-10^{-/-} mice, as compared to vehicle-treated IL-10^{-/-} mice (Fig. 1A). We did not notice any significant changes in body weight of resveratrol treated normal BL/6 mice (Fig. 1A).

We have previously shown that SAA levels serve as indicators of severity of colitis progression (Singh et al., 2003a). SAA concentrations at the termination of experiment in the vehicle-treated IL-10^{-/-} mice were elevated significantly and these levels were markedly decreased following resveratrol treatment (Fig. 1B). At this point, we also compared SAA levels with naïve BL/6 mice to provide a normal SAA range. These basal levels were not altered after resveratrol treatment of normal BL/6 mice (Fig. 1B). Together, these findings suggested that resveratrol was able to lessen the colitis-induced weight loss and caused a decrease SAA concentration in IL-10^{-/-} mice.

3.2 Resveratrol reduces fecal IgG and IgA responses during chronic colitis

To test the hypothesis that resveratrol treatment mediates a Th-1/Th-2 biased humoral responses during chronic colitis, we measured total fecal IgG and IgA to determine correlative changes in the intestinal Ab level after resveratrol treatment in mice with chronic colitis. A significant decline in both fecal IgA and IgG Abs was observed in IL-10^{-/-} mice that received resveratrol treatment as compared to vehicle-treated IL-10^{-/-} mice (Fig. 2 A & B). We did not notice any major changes in IgG and IgA concentration in naïve or resveratrol-treated BL/6 mice (Fig. 2A & B). These results illustrated that resveratrol treatment attenuated the excretion of both IgA and IgG Abs from the intestinal mucosa into the lumen during colitis.

3.3 Resveratrol decreases inflammatory cytokines associated with colitis

TNF- α , IL-6 and IL-1 β are often over-produced during a variety of inflammatory diseases, including both UC and CD (Powrie et al., 1994). We therefore determined if resveratrol treatment of IL-10^{-/-} mice would lead to decreased local and systemic levels of these cytokines. Resveratrol treatment decreased IFN- γ , TNF- α , IL-6, RANTES, IL-12, and IL-1 β levels in the colon tissues supernatant of mice with chronic colitis as compared to vehicle treated IL-10^{-/-} mice (Fig. 3). Concentrations of these cytokines were also reduced in the serum of resveratrol-treated IL-10^{-/-} mice (data not shown). We did not notice any significant changes in these cytokines in naïve or resveratrol-treated BL/6 mice (Fig. 3). Thus, resveratrol treatment was able to decrease both mucosal and systemic inflammatory cytokine responses in mice with chronic colitis.

3.4 Resveratrol increases CD11b⁺ Gr-1⁺ cell frequency and number during colitis progression

Macrophages are important early mediators of protective immunity and they are present in elevated numbers in the mucosa of both CD and UC patients. A distinct population of macrophages was found during active disease, but was absent from normal gut mucosa. MDSCs are immature precursors of macrophages that have potent immunosuppressive functions. Interestingly, resveratrol treatment in IL-10^{-/-} mice caused a significant increase in CD11b⁺ Gr-1⁺ percentage as well as numbers in the spleen and LP when compared to vehicle-treated IL-10^{-/-} mice (Fig. 4 A and B). The CD11b⁺ Gr-1⁺ frequency in MLN of IL-10^{-/-} mice was low and resveratrol treatment caused a modest decrease (data not shown). Also, we did not see any significant changes in the frequency and numbers of CD11b⁺ Gr-1⁺ derived from spleen, MLN and LP from naïve or resveratrol-treated BL/6 mice (data not shown). Taken together, these data suggested that resveratrol treatment increased the proportion and absolute numbers of CD11b⁺ Gr-1⁺ cells in the spleen and LP, leading to decreased effector T cell function, that correlated with the reversal of chronic colitis progression.

3.5 Functional characterization of resveratrol-induced CD11b⁺ Gr-1⁺ cells during colitis as MDSCs

For CD11b⁺ Gr-1⁺ cells to be characterized as MDSCs, it is critical to demonstrate that they exhibit immunosuppressive properties. Also, MDSCs are well known to inhibit effector T cell function through a variety of mechanisms, the best characterized of which include ARG-1 and NOS-2 production. We wanted to determine if either of these pathways was used by CD11b⁺ Gr-1⁺ cells induced by resveratrol. We therefore analyzed the expression of ARG-1 and NOS-2 in CD11b⁺ Gr-1⁺ cells isolated from the LP of IL-10^{-/-} that received vehicle or resveratrol. As shown in (Figure 5A & B), 81.2% of CD11b⁺ Gr-1⁺ cells isolated from LP of vehicle-treated and 95.2% cells from resveratrol-treated IL-10^{-/-} mice expressed ARG-1. Also, a very small percentage of such cells (2.2%) in vehicle-treated mice expressed NOS-2 and this increased slightly to 4.2% in resveratrol-treated groups. To analyze the suppressive capacity of these CD11b⁺ Gr-1⁺, we co-cultured MLN T cells from normal BL/6 mice activated using anti-CD3 and anti-CD28 Abs with decreasing concentrations of sorted purified CD11b⁺ Gr-1⁺ cells both from vehicle and resveratrol treated IL-10^{-/-} mice. The results shown in (Figure 5C) illustrated that CD11b⁺ Gr-1⁺ cells from both vehicle and resveratrol treated groups significantly reduced T cell proliferation at T cell: MDSC ratios of 5:1 and 1:1. Further, we tested whether these cells suppressed the cytokine production by LPL, such as IFN- γ and GM-CSF and the data revealed that resveratrol induced CD11b⁺Gr-1⁺ caused significantly higher levels of suppression of IFN- γ and GM-CSF when compared to vehicle-induced CD11b⁺ Gr-1⁺ cells (Figure 5D). These cytokines were not significantly induced in LPL cultures of naïve or resveratrol-treated BL/6 mice. Together, these data indicated that the CD11b⁺ Gr-1⁺ cells induced by resveratrol were indeed MDSCs that possessed potent immunosuppressive functions in LP.

3.6 Resveratrol treatment affects CD4⁺ CXCR3⁺ and CD4⁺ CXCR10⁺ T cells and systemic CXCL10 after colitis

The association between CXCR3 expression and Th1-dependent immunity has been observed in several models of inflammatory diseases and CXCR3 expressing T cells have been shown to produce predominantly Th1 cytokines (Bonecchi et al., 1998). Previously we have shown that CXCR3 ligand blockade abrogated spontaneous colitis in IL-10^{-/-} mice (Singh et al., 2003a). In the present study, we found that resveratrol treatment reduced the percentage and numbers of CD4⁺T cells that expressed CXCR3⁺ in the spleen, MLN and LPL, after spontaneous chronic colitis development (Fig. 6A,B). Similarly, we noticed that resveratrol also reduced CXCL10⁺ T cell numbers from LP as well as the systemic levels CXCL10 (ligand for CXCR3) when compared to vehicle-treated mice (Fig. 6 C and D). It is important to note that there were more activated T cells in the LP during colitis as compared to other cell types, such as NK and NKT cells (data not shown). Taken together, our data suggested that CXCR3⁺ T cells are a source of inflammatory cytokines in the LP during severe colitis, and that resveratrol impairs the mobilization of these inflammatory lymphocytes into the LP from both inductive and effector sites.

3.7 Resveratrol reduces activated T cell numbers in spleen, MLN and LP

To determine whether resveratrol has any effect on activated T cells in our colitis model, we enumerated the percentage of CD4⁺CD69⁺ T cells from spleen, MLN and LP by flow cytometry. We observed a significant decline in the percentage and number of CD69⁺T cells in the spleen, MLN and LP of IL-10^{-/-} mice that received resveratrol as compared to vehicle treated mice (Fig. 7A &B).

3.8 Resveratrol modulates the severity of colitis

Based on these data, we next examined the effects of resveratrol treatment on the reversal of colitis severity. To this end, we analyzed colonic sections from naïve or resveratrol-treated BL/6 mice and vehicle or resveratrol treated IL-10^{-/-} mice. As expected, we observed an increase in leukocyte infiltrate (Fig.8A middle panels) and a distortion of glandular architecture in the colon during colitis in vehicle-treated IL-10^{-/-} mice. In contrast, IL-10^{-/-} mice that received resveratrol showed a significant reduction in colon inflammation, reduced leukocytic infiltration, and partially restored glandular and goblet cell architecture as compared to control mice (Fig.8 A lower panels). The leukocytic infiltrates consisted of lymphocytes and occasionally small numbers of neutrophils. Multinucleated enlarged epithelial and elongated glandular cells were also present in vehicle-treated IL-10^{-/-} mice (Figure 8 A middle panels). Pathologic changes included leukocyte infiltrates in the LP of the colon of vehicle-treated IL-10^{-/-} mice, which was reduced following resveratrol treatment (Fig. 8A lower panels). The mean histological scores of IL-10^{-/-} mice with colitis that received vehicle were significantly higher than those mice treated with resveratrol (Fig. 8B). The naïve and resveratrol-treated normal BL/6 mice showed normal morphology of the colon (Fig. 8A upper panels). Taken together, our results showed a marked reduction in intestinal inflammation associated with colitis after resveratrol treatment.

4. Discussion

In IBD patients, clinical use of immunomodulatory agents that target key cytokine pathways, such as anti-TNF- α (Infliximab), is frequently compromised by the development of other serious complications or opportunistic infections. Therefore, additional treatment options capable of altering the balance of functional T cell subsets and attenuation of IBD are needed. In the present study, we show that oral administration of resveratrol, an ingredient commonly found in red grapes, into IL-10^{-/-} mice after the onset of chronic colitis, reduced the course of the disease. Resveratrol treatment lessened the loss of body weight, reduced SAA, and decreased local and systemic inflammatory cytokine concentrations. Our results suggested that the percentage and numbers of mucosal and systemic CXCR3⁺T cells as well as mucosal and systemic CXCL10 during chronic colitis were reduced by resveratrol treatment. Moreover, resveratrol induced an increase in both the percentage and numbers of CD11b⁺ Gr-1⁺ cells in the spleen and LP that correlated with decrease in colitis severity. Taken together, these results suggested that resveratrol eliminates activated CXCR3⁺ effector T cells, and most importantly induces CD11b⁺ Gr-1⁺ MDSCs that may also suppress the local effector T cell responses by inhibiting inflammatory cytokines produced by T lymphocytes leading to suppression of established chronic colitis in IL-10^{-/-} mice.

In IBD, distinct macrophages populations that lack the expression of cell-surface markers and specifically expressed by monocytes, macrophages or DCs, and comprise a mixture of cells are reported. These macrophages (CD11b⁺), along with Gr-1⁺ cells known as MDSCs, have well known for their immunosuppressive function. These macrophage populations have been found prominent in active disease, but are absent from normal mucosa. MDSCs are a heterogeneous population of cells that expand during cancer and are well characterized for their ability to suppress anti-tumor immunity (Jiang et al., 2010). Recent studies have suggested that MDSCs may play a critical role in down-regulating the T cell response, similar to the regulatory T cells (De Santo et al., 2008; Gabrilovich and Nagaraj, 2009). MDSCs are characterized by the co-expression of antigen Gr1 and CD11b (Peranzoni et al., 2010) in mice, and normal mouse spleens exhibit 2–3% of CD11b⁺ Gr-1⁺ cells. It has been reported that very little or no MDSCs are present in the lymph nodes (Haile et al., 2008). In the present study, CD11b⁺ Gr-1⁺ cells were found to be in very low numbers in the MLN. However, in the spleen and LP, the percentage and especially the numbers of these CD11b⁺

Gr-1⁺ cells increased significantly after resveratrol treatment. Further studies revealed that such CD11b⁺ Gr-1⁺ cells were immunosuppressive inasmuch as they were able to decrease the proliferation and cytokine production by T cells from mice with colitis. There are very limited studies on the role of MDSCs in colitis. A recent study indicated that the frequency of MDSCs dramatically increased during intestinal inflammation in mice and that these cells suppressed IFN- γ release by T cells (Haile et al., 2008).

A variety of factors trigger MDSCs. These include G-CSF, GM-CSF, TGF- β , inflammatory cytokines, etc. It has been shown that CD4⁺ T cells and Paneth cells of the intestinal epithelium secrete GM-CSF (Fukuzawa et al., 2003). Therefore, increased concentration of GM-CSF may be an important factor for MDSCs expansion in colitis. Recent studies from our laboratory demonstrated that activation of cannabinoid receptors triggers massive numbers of CD11b⁺ Gr-1⁺ MDSCs through induction of G-CSF (Serafini et al., 2006)). In this context, it is interesting to note that high-dose therapy with G-CSF has been shown to cause marked decrease of proinflammatory mediators in experimental colitis (Hommes et al., 1996) and it can be speculated that this results from induction of MDSCs. Further, it has been reported that MDSCs down-regulate L-selectin levels on naive T cells, decreasing their ability to home to sites where they would be activated (Hanson et al., 2009). In contrast, treatments that reduce MDSC levels, such as Ab depletion of Gr-1⁺ cells or treatment with chemotherapeutic drugs, activate T cells (Pan et al., 2008). These observations are consistent with our data that CD11b⁺ Gr-1⁺ cells increased in resveratrol-treated mice, which correlated with decreased levels of chronic inflammation as measured by a wide range of parameters.

Most studies have shown that MDSCs mediate the immunosuppressive properties through direct cell–cell contact with T cells (Ostrand-Rosenberg, 2010). The most well characterized mechanism of action of MDSCs involves metabolism of L-arginine, which serves as a substrate for two enzymes, iNOS (which generates NO) and ARG-1 (which converts L-arginine to urea and L-ornithine) (Rodriguez and Ochoa, 2008). This leads to enhanced L-arginine catabolism, which depletes this amino acid from the microenvironment thereby inhibiting T-cell proliferation. In the current study, we noted that majority of the MDSCs present at sites of colitis, expressed ARG-1 while expressing minimal levels of iNOS. In fact, the MDSCs from resveratrol-treated mice expressed slightly lower levels of ARG-1 when compared to the vehicle controls. Nonetheless, such MDSCs from resveratrol-treated mice were more potent at suppressing T cell functions than those from vehicle-treated mice. This suggested that resveratrol-treated MDSCs may use additional mechanisms to cause immunosuppression. Because MDSCs constitute a heterogeneous population, they have been known to use a wide range of mechanisms to suppress the immune response including ROS and TGF- β (Ostrand-Rosenberg and Sinha, 2009). In addition, MDSCs down-regulate L-selectin (CD62L) as well as block T cell activation by depriving the environment of cysteine, an amino acid that is essential for T cell activation (Ostrand-Rosenberg and Sinha, 2009). Moreover, MDSCs also express differential levels of inhibitory molecules. For example, monocytic MDSC do not produce ROS but use NO to mediate T cell suppression whereas granulocytic MDSC suppress through ROS production and do not express NO (Ostrand-Rosenberg and Sinha, 2009). It has been reported that MDSCs also indirectly affect T cell activation by inducing T regulatory cells (Tregs), which in turn down-regulate T cell-mediated immunity (Huang et al., 2006; Serafini et al., 2008).

The role of MDSCs to suppress inflammation *in-vivo* can conclusively be established through adoptive transfer experiments. Depletion studies *in-vivo* using antibodies are not feasible because there are no specific markers for MDSCs. In fact, in a recent study from our laboratory, we noted that MDSCs could prevent hepatitis upon adoptive transfer (Hegde et al., 2011). However, it should be noted that in the current study involving IL-10 deficient

mouse model of chronic colitis, it is not feasible to perform such adoptive transfers. Upon *in-vivo* transfer, MDSCs stay functional for two days only, and it will take repeated adoptive transfer of MDSCs for several weeks, involving a large number of donor IL-10^{-/-} mice and cells to see any effect. While the adoptive transfer of these cells into an active IBD model may yield valuable data on the therapeutic efficacy of these cells, the timing of administration, the cells numbers required and the stage of disease administered (at the genesis, initiation or later stages of the IBD responses) would have to be worked out to yield useable and reproducible results. Together, this study indicates that immunosuppressive MDSCs could potentially become a therapeutic target for colitis in human patients.

The mucosal and systemic humoral immunity in IL-10^{-/-} mice with chronic colitis is significantly different from that found in control animals. Mice with chronic colitis displayed excretion of IgA and IgG in fecal extracts as compared to controls. There is no known active transport mechanism for IgG secretion by epithelial cells into the lumen. Chronic inflammation in mice leads to increase Ag-specific mucosal Abs, and heightened CD4⁺T cell proliferative and cytokine responses in both mucosal compartments. Treatment with resveratrol abrogated these heightened mucosal responses, and brought the IgG and IgA levels, in particular, back towards the baseline.

The precise cytokine signals required for S-IgA production are not completely understood and studies suggest that both Th1- and Th2- type cell-derived cytokines are important for S-IgA. We have previously shown that chemokines like lymphotactin and RANTES also induce S-IgA (Lillard et al., 2001). The present study clearly shows enhanced mucosal Ab responses as indicated by high IgA fecal secretions in mice with colitis. The sharp IgA Ab response generated by these mice also correlates with the predominant Th1 cytokine response and the presence of higher levels of IFN- γ generation. Importantly, resveratrol treatment reduced IgA levels in the feces, indicating that it could lessen the excessive mucosal Ab responses associated with colitis.

Pro-inflammatory cytokines are known to be critical mediators of IBD pathogenesis. For example, elevated TNF- α production is closely associated with both human IBD and murine colitis. Previous studies in mice have indicated that TNF- α is an important promoter of TNBS-induced chronic colitis (Kim et al., 2004). SAA is elevated during many inflammatory disease conditions like CD, rheumatoid arthritis, and chronic sepsis (De Beer et al., 1982). IL-1 α and IL-1 β are potent pro-inflammatory cytokines that are produced by and activate many immune and inflammatory cells. In patients with IBD, activated macrophages are known to be an important source of increased IL-6 and IL-1 β expression (Casini-Raggi et al., 1995). In the present study, we have demonstrated that local expression of TNF- α , IL-6, RANTES, IL-12, IL-1 β , and IFN- γ was decreased by resveratrol treatment in IL-10^{-/-} mice. These results are in agreement with previously published *in-vivo* and *in-vitro* findings that resveratrol reduces the level of inflammatory cytokines and inflammatory cell infiltrates in the colon (Martin et al., 2006).

In this study, resveratrol treatment reduced the percentages of activated T cells in MLN and LP during colitis, clearly indicating that resveratrol has potent anti-inflammatory effects that parallel the reduction in colitis-induced T-cell expansion. This is supported by our findings that the percentage of colitis-induced CD69⁺ cells was reduced (Fig. 6). The preferential reduction in effector T-cell responses was associated with a decrease in colon TNF- α and IFN- γ levels, and concomitant reductions in mucosal IL-1 β and IL-6 (Fig. 3). We have previously reported that resveratrol induces apoptosis in activated T cells (Singh et al., 2007a). Thus, resveratrol may decrease the numbers of activated T cells during colitis either by induction of apoptosis or by increased cell migration from the colon to the inductive site.

CXCR3 is expressed by epithelial, endothelial, and lymphoid derived cells. CXCR3 ligands, such as CXCL9 and CXCL10, are upregulated in many inflammatory diseases and we have reported that the CXCR3 ligands are upregulated during murine colitis specifically (Singh et al., 2003b). We have also shown that antibody-mediated neutralization of the CXCR3 ligand CXCL10 inhibits chronic colitis in IL-10^{-/-} mice (Singh et al., 2003a). As previously stated, CXCR3 ligands mainly attract activated T cells of the Th1 phenotype that express high levels of CXCR3 (Bonecchi et al., 1998). It is also known that colitis is largely mediated by cytokine-producing Th1 cells that infiltrate the mucosa (Davidson et al., 1996). CXCR3 ligands further enhance the polarization of Th1 and Th2 cells (Loetscher et al., 2001), and the CXCR3 ligands themselves are induced by IFN- γ , indicating the presence of a positive feedback loop that generates more Th1 responses and more inflammation. Together, these findings clearly indicate the importance of CXCR3 ligands in colitis development. In the present study, resveratrol reduced the number of CXCR3⁺ T cells in the spleen, MLN and LP. We have reported that IL-10^{-/-} mice display large amounts of CXCL10 in the colon during the initial phase of colitis, resulting in the recruitment of more CXCR3⁺ leukocytes to the colon to propagate disease progression. Interestingly, in the present study we noticed that resveratrol also reduced serum CXCL10 levels as well as absolute cell numbers of CXCL10⁺ cells as compared to vehicle treated mice. This has also been observed in other murine models of colitis (Scheerens et al., 2001).

In summary, present study suggests that resveratrol acts as an anti-inflammatory agent by targeting multiple pathways. While many mechanisms of resveratrol-mediated immunosuppression have been identified, the data at our disposal demonstrated that resveratrol induced immunosuppressive CD11b⁺ Gr-1⁺ cells that express ARG-1, which correlated with reversal of chronic colitis severity. The unique correlation between induction of immunosuppressive CD11b⁺ Gr-1⁺ cells and reversal of chronic colitis can be interpreted to suggest that these cells may also contribute towards reversal of chronic colitis in IL-10^{-/-} mice. Taken together, this study suggests that resveratrol induced CD11b⁺ Gr-1⁺ cells play immunosuppressive role in this T-cell dependent mouse model of intestinal inflammation. Further studies in other suitable model are needed to investigate whether targeted expansion of CD11b⁺ Gr-1⁺ cells might become a therapeutic option for IBD patients. These studies not only indicate the potential use of resveratrol in the treatment of colitis but also suggest that CD11b⁺ Gr-1⁺ cells (MDSCs) may constitute a novel target for therapeutic intervention in patients with IBD.

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Abbreviations

TNF-α	Tumor Necrosis Factor-Alpha
IBD	Inflammatory Bowel Disease
UC	Ulcerative Colitis
CD	Crohn's Disease
MDSCs	myeloid derived suppressor cells
NF-κB	nuclear transcription factor kappa B

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Fig. 1.

Change in body weight and serum amyloid A (SAA) level after resveratrol treatment in IL-10^{-/-} mice. IL-10^{-/-} mice, on a BL/6 background and normal BL/6 mice (●) received 100 μ l of resveratrol (100 mg/kg body weight) suspended in distilled water (□) or vehicle (■) every second day, starting at the beginning of week 18 when mice had lost about 15% of their body weight and attained a peak in SAA levels. The treatment was continued until the mice were sacrificed at week 28. The body weight of the IL-10^{-/-} mice was recorded every week, and the change from initial body weight was expressed as a percentage of the weight at 2 weeks before the onset of colitis (week-2) minus the weight at week divided by the weight before the onset of colitis (panel A). Resveratrol treatment led to significant reversal of the loss of body weight in IL-10^{-/-} mice ($p < 0.05$). SAA levels were measured at the end of the experiment at week 28 (panel B). The statistical significance (★) $P < 0.05$ between values from vehicle vs resveratrol treatment was assessed using Student's t-test. Data represent the mean $\pm$ SEM of three independent experiments involving six mice per group.

Fig. 2.

Change in fecal Ab response after resveratrol treatment in IL-10^{-/-} mice. IL-10^{-/-} mice with chronic colitis received resveratrol or vehicle as described in Fig.1. Following sacrifice after 28 weeks, fecal Ab responses of mice were determined by Milliplex™MAP ELISA kit capable of detecting >10 pg/ml of IgA and IgG Abs. Panels A and B show IgA and IgG Ab levels in fecal secretions. The data presented are the mean concentration of IgG subclass, or IgA ± SEM of three separate experiments. Asterisks (★) indicate statistically significant differences $p < 0.05$ using ANOVA followed by Duncan's Multiples range test ($p < 0.01$) between vehicle vs resveratrol treated IL-10^{-/-} mice.

Fig. 3.

Resveratrol reduces the local levels of IL-1 β , RANTES, IL-12, IL-6, TNF- α , and IFN- γ after chronic colitis. Colon supernatant cytokines were measured at 28 weeks post colitis induction by ELISA analysis. The data presented are the mean concentrations in the colon supernatant from six mice $\pm$ SEM. The statistical significance ($\star$) $P < 0.05$ between values from vehicle vs resveratrol treated IL-10^{-/-} mice was assessed using Student's t-test.

Fig. 4.

Resveratrol induces CD11b⁺ Gr-1⁺ cells in mice with chronic colitis. Splenic and LP lymphocytes were isolated from the two groups of IL-10^{-/-} mice (panel A). Changes in the numbers of MDSCs (panel B) were determined by flow cytometry and expressed as the mean percentage of cells/mice $\pm$ SEM. Data shown are from a representative experiment; three independent experiments involving six mice/group yielded similar results. The statistical significance (★) $P < 0.05$ between values from vehicle vs resveratrol treated IL-10^{-/-} mice was assessed using Student's t-test.

Fig. 5. Phenotypic and functional characterization of resveratrol-induced CD11b⁺ Gr-1⁺ cells. CD11b⁺ Gr-1⁺ cells were sorted from LP isolated from the IL-10^{-/-} mice that received vehicle (A) or resveratrol (B). CD11b⁺ Gr-1⁺ cells were gated and analyzed for expression of ARG-1 and NOS-2 (panel A & B). CD11b⁺ Gr-1⁺ cells from two groups were sorted and co-cultured with T cells from MLN activated with anti-CD3 and anti-CD28 Abs. Proliferation of T cells was measured by BrdU incorporation (C). LP lymphocytes were also cultured with sorted CD11b⁺ Gr-1⁺ cells, at 1:5 ratio, obtained from vehicle or resveratrol-treated IL-10^{-/-} mice with chronic colitis at termination of experiment. IFN-γ and GM-CSF cytokine levels were measured three days thereafter from the culture supernatant by ELISA (D). Data shown are from a representative experiment; three independent experiments involving six mice/group yielded similar results. The statistical comparisons were made between various groups using ANOVA and Student's t-test as indicated in the figure and $p < 0.05$ were indicated with an asterisk (★).

Fig. 6.

Resveratrol treatment reduced percentage and numbers of CXCR3⁺ T cells *in vivo*. Cells from the spleen, MLN and LP were isolated from vehicle- or resveratrol treated IL-10^{-/-} mice and stained for CD4⁺ CXCR3⁺ T cells using flow cytometry. A representative experiment showing percentages of cells is shown in (panel A), and absolute numbers of CD4⁺CXCR3⁺ cells in various lymphoid organs are shown in (panel B). At termination of the experiment (28 week), absolute numbers of CD4⁺CXCL10⁺ were determined by flow-cytometry and serum CXCL10 levels by ELISA as shown in panel C and D. The data presented are mean absolute numbers and concentration of CXCL10 ± SEM of three separate experiments (panel C and D). The statistical significance (★) p<0.05 was compared between vehicle vs resveratrol treated IL-10^{-/-} mice, which was assessed using Student's t-test.

Fig. 7.

Resveratrol regulates activated T cells in the colon. Spleen, MLNs and LP lymphocytes were isolated from vehicle- or resveratrol treated IL-10^{-/-} mice and stained for CD4⁺ CD69⁺ T cells. Changes in the mean percentage of CD69 expression by CD4⁺ T cells from MLNs and LPs were compared between the two groups (Panel A) and absolute numbers of CD4⁺ CD69⁺ T cells in spleen, MLN and LPL are shown in (panel B.) The statistical significance (★) P<0.05 between vehicles vs resveratrol treated IL-10^{-/-} mice were assessed using Student's t-test.

Fig. 8.

Effect of resveratrol on histopathology of colon in IL-10^{-/-} mice with colitis. Colon sections from the naïve, resveratrol-treated BL/6, and vehicle or resveratrol-treated IL-10^{-/-} mice with colitis were histologically evaluated at 28 weeks. Upper panel shows normal colon from naïve mice and colon of normal mice that received resveratrol. The middle panels show vehicle-treated IL-10^{-/-} mice with colitis showing large numbers of cellular infiltrates, enlarged epithelial and distorted crypts. Lower panels show IL-10^{-/-} mice that received resveratrol, showing significant decrease in cellular infiltrates as well as some normal epithelial cells. The inflammation score of these four groups is presented in Fig 8 B. Representative sections from three separate experiments (10× and 40× magnification) are shown, with each group containing six mice. The statistical significance (★) $p < 0.01$ between vehicle- and resveratrol-treated IL-10^{-/-} mice was assessed by Student's t-test.