Downregulation of Hematopoietic MUC1 during Experimental Colitis Increases Tumor-Promoting Myeloid-Derived Suppressor Cells

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Abstract

Purpose: MUC1 is a tumor-associated antigen that is aberrantly expressed in cancer and inflammatory bowel disease (IBD). Even though immune cells express low MUC1 levels, their modulations of MUC1 are important in tumor progression. Consistent with previous clinical data that show increased myeloid-derived suppressor cells (MDSCs) in IBD, we now show that downregulation of MUC1 on hematopoietic cells increases MDSCs in IBD, similar to our data in tumor-bearing mice. We hypothesize that MDSC expansion in IBD is critical for tumor progression.

Experimental Design: To mechanistically confirm the linkage between Muc1 downregulation and MDSC expansion, we generated chimeric mice that did not express Muc1 in the hematopoietic compartment (KO→WT). These mice were used in two models of colitis and colitis-associated cancer (CAC) and their responses were compared with wild-type (WT) chimeras (WT→WT).

Results: KO→WT mice show increased levels of MDSCs during colitis and increased protumorigenic signaling in the colon during CAC, resulting in larger colon tumors. RNA and protein analysis show increased upregulation of metalloproteinases, collagenases, defensins, complements, growth factors, cytokines, and chemokines in KO→WT mice as compared with WT→WT mice. Antibody-mediated depletion of MDSCs in mice during colitis reduced colon tumor formation during CAC.

Conclusion: Development of CAC is a serious complication of colitis and our data highlight MDSCs as a targetable link between inflammation and cancer. In addition, the lack of MUC1 expression on MDSCs can be a novel marker for MDSCs, given that MDSCs are still not well characterized in human cancers. Clin Cancer Res; 19(18); 1–14. ©2013 AACR.

Introduction

Inflammatory bowel disease (IBD) is a chronic, idiopathic inflammatory syndrome that involves deregulated homeostasis between the gut microbiota and the mucosal immune system in genetically susceptible individuals. IBD encompasses both ulcerative colitis (affects the colon and/or rectum) and Crohn’s disease (affects any part of the gastrointestinal tract). A serious complication of ulcerative colitis is the development of colitis-associated cancer (CAC). However, any significant inflammation in the colon, whether as a result of ulcerative colitis or Crohn’s disease, can lead to colon cancer, especially if the inflammation involves a large part of the colon. As such, there is a need for continued clinical, genetic, and animal studies for determining the appropriate clinical, genetic, serologic, and fecal biomarkers (1–3) that could be used to diagnose and stratify IBD, as well as provide information toward the most ideal therapeutic strategy for the patient. Despite identification of susceptibility genes for IBD from genome-wide association studies, it is important to follow up with functional studies given that many of these genes have different functions in epithelial and immune cells, both of which play a major role in IBD pathogenesis. A genome-wide association study has recently identified MUC1 (MUC1 in humans, Muc1 in mice) as a potential susceptibility gene for Crohn’s disease (4). The role of MUC1 in IBD is interesting – the ability to elicit an immune response to the tumor form of MUC1, together with its known oncogenic role in the colonic mucosa, would make it an optimal candidate for immunotherapy to reduce cancer risk in patients suffering from chronic IBD (5). Muc1 knockout (KO) mice have been shown to be more resistant to dextran sulfate sodium (DSS)-induced colitis with a thickening of the mucus layer.

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Translational Relevance

Chronic inflammatory damage to the colon during colitis has long been assumed to be the classic, primary cause for eventual progression to colitis-associated cancer (CAC). As such, therapies have been designed toward the prevention of the inflammatory process. We have discovered that the expansion of myeloid-derived suppressor cells (MDSC) during chronic colitis is responsible for altering the colonic microenvironment toward colon tumor development and represents an important therapeutic target. Our data further highlight the importance of monitoring patients with colitis for immunosuppressive profiles such as MDSC expansion and/or T-cell dysfunction that could be responsible for the eventual development of cancer in genetically susceptible individuals. In addition, we also show that the lack of MUC1 on MDSCs can be a novel marker for MDSCs, given that MDSCs are still not well characterized in human cancers.

and less infiltration of T cells (6). However, absence of Muc1 has also been recently shown to result in the exacerbation of chronic inflammation in both T-helper 1 (T\textsubscript{H1}-) and T\textsubscript{H1}2-mediated colitis models (7). These studies highlight the important role of Muc1 in the complicated etiology of IBD but do not take into account the significant contribution of Muc1 as expressed (and its function) in the hematopoietic compartment with regards to inflammatory signaling.

In the epithelium, MUC1 plays a multifaceted role ranging from signal transduction in oncogenesis to protecting the epithelium against pathogenic infections (8–10). Originally identified as a result of aberrant overexpression in cancer (11), MUC1 has been identified as number 2 on the National Cancer Institute (Bethesda, MD) cancer vaccine target antigen prioritization list (12). Compared with its high levels of expression in the epithelium, MUC1 is expressed at a much lower level in immune cells, such as T cells, where MUC1 has nevertheless been shown to act as an important adaptor molecule for T-cell activation (13, 14). Although it is also expressed on other hematopoietic cells such as dendritic cells, natural killer (NK) cells, B cells, hematopoietic stem, and progenitor cells in the bone marrow (15–21), the function and expression pattern of MUC1 in these cells are still relatively unknown.

We observe that in patients with IBD, there is an increase in CD11b\textsuperscript{+}HLA DR\textsuperscript{+}Gr1\textsuperscript{+} cells in the peripheral blood that do not express any MUC1. It has previously been shown that increased levels of tumor-promoting myeloid-derived suppressor cells (MDSCs) are found in the peripheral blood during IBD and have been phenotyped to be CD11b\textsuperscript{+}HLA DR\textsuperscript{+}Gr1\textsuperscript{+} (22). We further confirmed our observations in a murine ulcerative colitis model where we show an expansion of Muc1 low expressing CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells during colitis. We have previously shown that the lack of Muc1 in the bone marrow from Muc1 knockout mice resulted in an increased expansion of CD11b\textsuperscript{+}Gr1\textsuperscript{+} MDSCs that are immunosuppressive and promote tumor growth (23). Our current study shows that deletion of hematopoietic Muc1 in chimeric mice (KO→WT) increased expansion of immunosuppressive CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells during chronic colitis, with reduced inflammation in the colon. In our mouse model of CAC, these CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells were also responsible for the development of larger colon tumors in KO→WT mice, as antibody-mediated depletion of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells reduced tumor burden. Our data suggest that tumor progression during CAC is not entirely due to the inflammatory damage to the colon. Rather, tumor progression is also dependent on the immunosuppressive milieu that is a result of deregulated immune signaling in IBD, which can result in MDSC expansion (22).

Here, we propose that an increase in MUC1 low expressing MDSCs in the peripheral blood in IBD can be responsible for eventual tumor development during CAC. Prolonged circulation of immunosuppressive CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells during colitis can activate protumorigenic pathways to colorectal cancer. Given that MUC1 is currently a vaccine candidate for cancer and IBD (5, 24), knowledge of the function and expression of MUC1 on immune cells would be very useful in preventing inappropriate downregulation of MUC1 on immune cells by vaccine strategies and can be a useful aid for diagnosis and prognosis of MDSC levels in patients with IBD. In addition, our data also further highlight the need to functionally study IBD susceptibility genes in the context of epithelial and immune signaling.

Materials and Methods

Peripheral blood was collected from 12 consenting patients with IBD (5 patients with Crohn’s disease and 7 patients with ulcerative colitis) with approval from the Institutional Review Board (IRB) at the Mayo Clinic (Scottsdale, AZ). Patients enrolled in the study were diagnosed under standard criteria as having active flare of disease. Peripheral blood mononuclear cells (PBMCs) were separated on a Ficoll gradient and residual red blood cells were lysed with Pharm Lyse (BD Biosciences). Cells were stained with antibodies to CD14 and HLA DR (BD Pharmingen) and MUC1 (anti-CT2; Mayo Clinic) for flow cytometry analysis.

Bone marrow transplant

Eight- to 12-week-old wild-type (WT) female mice were given 11 Gy irradiation split into two doses, separated by 4 hours. After irradiation, 20 × 10\textsuperscript{5} male donor knockout bone marrow cells were intravenously injected into female irradiated WT recipients to generate KO→WT chimeras and WT bone marrow cells were injected to generate WT→WT chimeras. Chimerism was monitored after 30 days using PCR analysis of DNA from PBMCs for presence of the Y chromosome gene product in the female irradiated recipient mice (23, 25) as well as the Muc1 and LacZ gene (26) for WT→WT and KO→WT chimeras, respectively (Supplementary Fig. S1).
Experimental model of colitis and CAC

Chimeric mice (WT→WT and KO→WT) were given 3% DSS (MW 36–50 kDa; MP Biochemicals) in their drinking water for 4 days, followed by 14 days of regular water. This cycle was repeated for two more times to mimic the pathology of chronic ulcerative colitis after which mice were sacrificed for histologic analysis of inflammatory lesions in the colon. Azoxymethane (AOM) is used to induce colorectal cancer in mice (27) and was used as the carcinogen in our CAC model. Chimeric mice were injected intraperitoneally with 12.5 mg/kg of AOM (NCI Chemical Resource Repository, Midwest Research Institute, Kansas City, MO) 3 months after the bone marrow transplant was conducted. Five days later, colitis was induced in the mice by administration of 2% DSS in the drinking water for 4 days, followed by another 14 days of regular water. This cycle was repeated again, followed by a final administration of 2.5% DSS in drinking water for 5 days (Fig. 3A). DSS is a well-characterized colonic irritant which upon repeated administration causes chronic inflammation that mimics the pathology of ulcerative colitis, which would enhance the incidence of AOM-induced tumors only in the colon (28). Four months after injection of AOM, mice were sacrificed for evaluation of tumors. Colons were opened longitudinally and washed with PBS before fixing in formalin for paraffin embedding. Histologic analysis of the percentage of colon tissue that had tumors after 4 months and inflammatory lesions in the colon at day 47 (1 day after the last DSS-drinking cycle; Fig. 3A) was evaluated on hematoxylin and eosin (H&E)-stained sections by a veterinary pathologist and a second independent viewer. For analysis of collagen, Masson’s Trichome stain was conducted on colon slide sections.
Immunohistochemistry for RAGE and Gr1

Paraffin colon sections were heated in antigen retrieval solution (Dako) and blocked in 10% fetal calf serum (FCS) with an avidin–biotin blocking kit (Vector Laboratories), followed by overnight incubation at 4°C with either anti-receptor for advanced glycation end products (anti-RAGE; Abcam) or anti-Gr1 (eBioscience). Biotinylated secondary antibodies to anti-RAGE or anti-Gr1 were added to slides for 1 hour, followed by incubation with streptavidin–horseradish peroxidase (BD Pharmingen). Slides were developed using a 3,3'-diaminobenzidine (DAB) kit (Vector Laboratories) and counterstained in hematoxylin (Sigma).

Isolation of lamina propria cells

One day after the last DSS-drinking cycle in the CAC model (day 47; Fig. 3A), mice were sacrificed and colons were dissected and washed in ice-cold PBS. Isolation of lamina propria cells from colons was conducted as previously described (29). Lamina propria cells were analyzed by flow cytometry using anti-CD11b phycoerythrin (PE), anti-LY6C fluorescein isothiocyanate (FITC), and anti-LY6G V450.

Flow cytometry

A total of 10^6 cells were isolated and stained in 1x PBS with 0.5% FCS using the following antibodies at 1 μg/mL: anti-B220 FITC, anti-CD11b antigen-presenting cell (APC), anti-Gr1 PE (BD Pharmingen), anti-F4/80 allophycocyanin (APC) (eBioscience), and anti-CT2 (Mayo Clinic Arizona). Anti-CT2 detects the Muc1/MUC1 cytoplasmic tail and cells are permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization solution (BD Pharmingen) overnight before staining with anti-CT2. Acquisition was carried out on a Dako CyAn flow cytometer and analysis was conducted on Summit 4.3. At least 20,000 events were isolated.

Cytokine array analysis

WT→WT and KO→WT mice were sacrificed on day 47 of the AOM + DSS experimental model, 1 day after the final duration of 4 days of DSS-treated drinking water,
and 0.5 mL of blood was collected and loaded on a Ficoll gradient. Plasma was collected and the cytokine content in the plasma was analyzed using the RayBio Mouse Cytokine Antibody Custom Array (Ray Biotech) customized for 20 cytokines known to be involved in inflammation and immunosuppression, as per the manufacturer’s instructions.

**Microarray and protein analysis**

Two groups of WT—WT and KO—WT mice [either preinjected with AOM and having undergone three cycles of DSS (AOM + DSS), or nontreated control mice, n = 3 per group] were sacrificed on day 47 of the AOM + DSS experimental model, 1 day after the last DSS-drinking cycle. Colons were opened longitudinally, washed in PBS, and the mucosa from each colon was scraped and individually snap-frozen in liquid nitrogen. For Western blot analysis, mucosal cells were lysed with 200 µL radio-immunoprecipitation assay (RIPA) lysis buffer, freeze-thawed once and centrifuged at 13 000 rpm for 20 minutes at 4°C. The supernatant was used for Western blot analysis with anti-C1q, anti-RAGE (Abcam), and anti-MMP-10 (Abnova). For analysis of matrix metalloproteinase (MMP) activity, mucosal cells were lysed overnight in 200 µL of lysis buffer containing 50 mmol/L Tris 7.5, 10 mmol/L CaCl2, 150 mmol/L NaCl, 0.05% w/v Brij-35 before loading onto a precast gelatin zymogram gel (Bio-Rad) and developed according to the manufacturer’s instructions. Samples were loaded alongside recombinant mouse MMP-2 (R&D Systems) activated with 4-Aminophenylmercuric acetate (APMA) (Sigma).

RNA from colonic mucosa was isolated using the RNeasy Mini Kit (Qiagen). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent). Cy3-labeled cRNA was generated from 1 µg RNA per sample, using the Quick Amp Labeling Kit (Agilent). Quantification and dye incorporation of the labeled cRNA was conducted using the NanoDrop (Thermo Scientific). Labeled cRNA (1.65 µg) was hybridized onto a 4 x 44 whole mouse genome slide, washed, scanned, and feature extracted on an Agilent scanner as per the manufacturer’s instructions. Microarray data were loaded onto GeneSpring 11.5 for analysis. During
Figure 3. KO→WT mice show less inflammation postcolitis after induction of CAC, as compared with WT→WT mice. Reduced inflammation in KO→WT mice was accompanied by increased levels of CD11b⁺Gr1⁺ cells and sRAGE in peripheral blood and reduced RAGE expression in the colonic mucosa. A, flow chart of CAC experimental model using AOM and DSS. Mice were sacrificed 1 day after the end of the third DSS-drinking cycle (day 47) for (B) analysis of the absolute numbers of CD11b⁺Gr1⁺ cells in the PBMCs of WT→WT and KO→WT mice and (C) for histologic analysis of H&E colon sections for the percentage of colon that had inflammatory lesions. Incidence of inflammation was similar between WT→WT and KO→WT experimental mice (7 of 8 WT→WT experimental mice and 7 of 7 KO→WT experimental mice showed at least 10% or more of the colon that had inflammatory lesions), so only experimental mice that developed inflammation in the colon were compared. D, plasma derived from the tail bleeds conducted 1 day after the end of the third DSS cycle (day 47) were used in a cytokine antibody array for 20 cytokines known to be involved in immunosuppression. Boxed areas are spotted with antibodies to RAGE and are representative of results from arrays conducted in duplicate for WT→WT and triplicate for KO→WT mice treated with AOM + DSS. Densitometry graph shows the average signal intensity for the RAGE positive spots over replicate experiments. E, lysates from colonic mucosa of WT→WT and KO→WT mice treated with AOM and DSS were made for Western blot analysis against RAGE as described in Materials and Methods. β-Actin was probed for as a loading control. F, immunohistochemical staining of RAGE in inflammatory lesions of WT→WT (n = 8) and KO→WT (n = 6) was conducted and data shown are representative of all stained sections. Images were taken at ×200 magnification. i.p., intraperitoneal.
analysis, the gene list data were filtered to remove compromised signals. Unpaired t test was conducted between samples from control and AOM–DSS–treated mice within each group of chimeras to determine the genes that were significantly altered ($P < 0.05$) upon treatment between WT→WT and KO→WT mice. Genes that were altered less than 2-fold upon treatment with AOM + DSS were subsequently excluded from the gene list. To develop a gene signature that could further explain the larger tumors that eventually developed in these mice, GeneGo analysis was used to further categorize genes that were mutually or exclusively altered in WT→WT or KO→WT mice. Microarray data can be accessed at Gene Expression Omnibus (GEO), accession number GSE47487.

Figure 4. One day after the last DSS-drinking cycle in the CAC and colitis model, KO→WT mice show increased levels of CD11b$^+$Gr1$^+$ cells in the lamina propria as compared with WT→WT mice. Four months after the start of the AOM + DSS experimental model, KO→WT mice show increased colon tumors as compared with WT→WT mice that could be reduced with anti-Gr1 treatment during each DSS cycle. A, WT→WT and KO→WT mice were sacrificed 1 day after the last DSS-drinking cycle of the CAC model and colons were either fixed in formalin and embedded in paraffin for immunohistochemical staining of Gr1$^+$ cells (left most; images taken at ×200 magnification; black arrows indicate some of the cells that stained positive for Gr1) or used for isolation of lamina propria cells. Lamina propria cells were analyzed with antibodies to CD11b and the two subtypes of Gr1 (LY6C and LY6G) via flow cytometry. WT→WT and KO→WT mice were also sacrificed 1 day after the last DSS-drinking cycle of the 3% DSS colitis model and lamina propria cells were similarly analyzed for CD11b and LY6C (right most). No significant difference in CD11b$^+$LY6G$^+$ cells were observed between the lamina propria cells from WT→WT and KO→WT on the 3% DSS colitis model, hence the data were not shown. Lamina propria data reflect pooled cells from at least $n = 5$ for each group of WT→WT and KO→WT mice analyzed. WT→WT and KO→WT mice were sacrificed after 4 months after the start of the AOM + DSS experimental model for (B) histologic analysis of percentage of colon tissue that had developed adenomas. Tumor incidence was similar between WT→WT and KO→WT experimental mice (6 of 7 WT→WT and 5 of 6 KO→WT mice developed colonic tumors), so only experimental mice that had developed tumors are shown. C, measurement of colon length in all chimeric mice subjected to the AOM + DSS experimental model. D, KO→WT mice were injected with 100 µg anti-Gr1 on the second day after starting each DSS cycle in the AOM + DSS experimental model and sacrificed 4 months later for analysis of the percentage of colon tissue that had developed tumors. Graph shows mice that did or did not develop tumors and reflects a reduction in tumor incidence and size upon treatment with anti-Gr1.
RT-PCR validation of microarray results

The same RNA samples (n = 3 per treatment or control group) derived from colonic mucosa that were used for microarray analysis were also used for validation of the microarray results. One microgram of CDNA was made from these RNA samples and loaded onto TaqMan (Applied Biosystems) microfluidic cards with TaqMan reaction mix for quantitative real-time PCR (qRT-PCR) analysis as per the manufacturer’s instructions. The cards are loaded and run on a 7900HT RT-PCR system. The TaqMan microfluidic cards are spotted with 191 sets of primers in duplicate, with an endogenous control (18S). The selected primers are against candidate genes that were derived from our microarray data. Most of the candidate genes assayed for on the TaqMan microfluidic cards have at least two different primer sets spotted on the cards. Data were analyzed using the SDS software package, using an automatic baseline and a manual threshold of 0.2 to record the cycle threshold.

Immunosuppression assay

CD11b+Gr1+ PBMCs were sorted by flow cytometry from chimeric mice that had under gone three rounds of 3% DSS-drinking cycles. A total of 10^6 CD11b+Gr1+ cells sorted from each chimeric mice were incubated with 10^5 Carboxyfluorescein succinimidyl ester (CFSE)-labeled T cells that were sorted from the spleen with magnetic beads to CD4 and CD8 (Miltenyi Biotec). These CFSE-labeled T cells were stimulated with 1 μg/mL soluble anti-CD3 and 0.5 μg/mL soluble anti-CD28. T-cell proliferation was analyzed 7 days later via flow cytometry for change in CFSE fluorescence. Percentage of total T cells in each division of the T-cell population as reflected by changes in CFSE fluorescence was analyzed using the Flowjo software.

Statistical analysis

All statistical analysis was conducted using unpaired Student t test unless otherwise stated.

Results

Low MUC1 expression on MDSCs in human and mouse peripheral blood during IBD

Low levels of MUC1 were detected on increased levels of CD14+HLA DR−/−/I-Bα PBMCs (Fig. 1A and B), a previously described MDSC population (22), from patients with IBD who were enrolled at Mayo Clinic Arizona in accordance with IRB guidelines. We also detected increased levels of CD11b+Gr1+ cells, an MDSC phenotype, in C57BL/6 WT mice treated with 3% DSS (Fig. 1C). Further analysis revealed similarly low levels of Muc1 on these CD11b+Gr1+ cells (Fig. 1D), as reflected by a lack of shift in fluorescence intensity in the flow cytometry histogram. The constitutively low expression of Muc1 on increased levels of CD11b+Gr1+ cells further diluted the overall observed levels of Muc1 on PBMCs (Fig. 1D).

Functional impact of Muc1 downregulation during colitis and CAC

To determine the functional effect of low levels of Muc1 on hematopoietic cells during colitis, we conducted bone marrow transplants on C57BL/6 WT recipient mice using WT or Muc1 knockout bone marrow to generate chimeric mice that either did or did not express Muc1 in the hematopoietic compartment only (WT→WT and KO→WT, respectively). We have previously published that Muc1 knockout mice are viable (26) with no significant changes in the immune cell compartment at steady state (23). Interestingly, KO→WT mice were significantly healthier (as shown by reduced weight loss) than WT→WT mice when subjected to three drinking cycles of 3% DSS (Fig. 2A), with significantly smaller inflammatory lesions after the third drinking cycle (Fig. 2B).

Deleting Muc1 only in the hematopoietic compartment also altered the immune cell phenotype seen during chronic colitis. There was a significant increase in CD11b+Gr1+ cells but no significant decrease in B220+ cells in the KO→WT mice as compared with WT→WT (Fig. 2C). We did not observe significant changes in other cell types such as T cells and NK cells in both WT→WT and KO→WT mice. As we had previously published that a lack of Muc1 in the bone marrow promoted the expansion of CD11b+Gr1+ MDSCs during tumor growth (23), we analyzed the suppressive ability of CD11b+Gr1+ cells obtained from the PBMCs of KO→WT mice during colitis. Indeed, deletion of MUC1 from the hematopoietic compartment prompted an increase (Fig. 2C) in CD11b+Gr1+ cells that were able to suppress T-cell proliferation in vitro (Fig. 2D). CD11b+Gr1+ cells from colitic KO→WT mice were able to significantly suppress proliferation to a greater extent as compared with CD11b+Gr1+ cells from colitic WT→WT mice (Fig. 2D).

Figure 5. Postcolitic KO→WT mice showed increased ECM remodelling in the colonic mucosa as compared with similarly treated WT→WT mice. Analysis of gene regulation was conducted on RNA derived from the colonic mucosa of treated and untreated chimeric mice 1 day after the end of the third DSS cycle (day 47). A, left, microarray data are depicted in heatmaps that show the transcriptional regulation of MMPs with (A, right) validation of MMP genes derived from the microarray analysis as conducted with the TaqMan microfluidic cards as described in Materials and Methods. Blanks in heatmaps indicate that the genes were not altered by at least 2-fold and were thus not considered during analysis. B, lysates from colonic mucosa of WT→WT, KO→WT, and KO→WT mice treated with anti-Gr1 were made as described in Materials and Methods for Western blot analysis of pro and active MMP-10 levels. All lysates were run on the same gel and the vertical line on gels indicates scans of different areas of the same gel. C, WT→WT and KO→WT mice were sacrificed 1 day after the end of the third DSS cycle on the 3% colitis model and MMP-2 activity was analyzed via gelatin zymography. Samples were run alongside activated recombinant MMP-2 (rMMP-2). D, top, microarray data are similarly depicted in heatmaps that show the transcriptional regulation of collagen genes with (D, bottom) validation of collagen genes derived from the microarray analysis as conducted with the TaqMan microfluidic cards. E, representative colon sections of WT→WT and KO→WT mice treated with AOM + DSS that were stained with Masson’s Trichome stain to reflect increased collagen deposition (stained blue) in the colons of treated KO→WT mice. Colon sections show typical inflammatory lesions from both WT→WT and KO→WT mice treated with AOM + DSS (images were taken at ×200 magnification).
Relative quantification (mRNA) of various genes in WT and KO mice treated with AOM/DSS.

- **A**: Bar graph showing relative quantification of Defa20, C1qc, and C3 in WT and KO mice.
- **B**: Western blot analysis of C1q and β-Actin levels in WT and KO mice treated with α-Gr1.
- **C**: Heatmap showing relative quantification of various genes in WT and KO mice treated with AOM/DSS.
- **D**: Bar graph showing relative quantification of various genes in WT and KO mice.
MDSCs correlate with tumorigenic advancement of CAC

The increase in CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs would account for the reduced inflammatory lesions in the colon of KO→WT mice and their overall improved weight as compared with WT→WT mice. MDSCs have been observed in patients with IBD in other studies (22); however, their origin and function in IBD remain unknown. We hypothesized that prolonged circulation of MDSCs in patients with colitis might render genetically susceptible patients more likely to develop colon cancer. To test our hypothesis, we put our mouse chimeras (WT→WT and KO→WT) on a CAC model that uses AOM as a procarcinogen with multiple drinking cycles of DSS to mimic the chronic colitis observed in humans.

We reduced the percentage of DSS used in this model (2% DSS for 4 days, 14 days regular water; ± 5% DSS for 5 days; Fig. 3A), as using 3% DSS as previously shown resulted in high mortality rates when combined with use of AOM. In the CAC model, both WT→WT and KO→WT mice showed significant increases in CD11b<sup>+</sup>Gr1<sup>+</sup> cells after three DSS-drinking cycles (Fig. 3B). However, there seems to be a trend of higher levels of CD11b<sup>+</sup>Gr1<sup>+</sup> cells in KO→WT mice, with higher mean values and lower P values for the CD11b<sup>+</sup>Gr1<sup>+</sup> dataset in KO→WT mice as compared with WT→WT. More importantly, the significant increase in CD11b<sup>+</sup>Gr1<sup>+</sup> cells in KO→WT mice was accompanied by significantly reduced inflammatory lesions in the colon (as compared with similarly treated WT→WT mice) after three drinking cycles of DSS (Fig. 3C).

We conducted a cytokine array (against 20 cytokines known to be involved in immunosuppression) on the plasma of WT→WT and KO→WT mice that were obtained after three drinking cycles of DSS and observed a significant increase in the levels of soluble RAGE (sRAGE; Fig. 3D). Interestingly, there was a corresponding decrease in RAGE levels in the colonic mucosa of KO→WT mice, which could be reduced upon treatment with anti-Gr1 (Fig. 5B). Gelatin zymography also showed increased MMP-2 activity in the colonic mucosa of KO→WT mice as compared with WT→WT (Fig. 5C). Further evidence of increased extracellular matrix (ECM) remodeling was seen by the increased transcription and protein levels of collagen in the colon as shown by microarray, qRT-PCR (Fig. 5D), and Masson’s Trichome stain of colon sections (Fig. 5E).

Interestingly, microarray (Fig. 6A, left) and confirmatory qRT-PCR (Fig. 6A, right) analysis showed increased transcription of defensin and complement genes in KO→WT mice on the CAC model after three DSS-drinking cycles. From the microarray data, the growth factor, chemokine, and interleukin genes that were confirmed by further qRT-PCR analysis are Cxcl3, Ccl4, Ccl2, Ccr2, Cxcl10, Il1rl1/ST2, Il41, Gcsfr, Tnf, and Igf1 (Fig. 6D), which have been shown to be involved in tumor development (31–35).

Discussion

Role of hematopoietic Muc1 during acute and chronic inflammation and its role in the progression to CAC

We show here for the first time that patients with IBD have increased levels of CD14<sup>+</sup>HLA DR<sup>+</sup>/I0 MDSCs that...
express low levels of MUC1. In mice, downregulation of MUC1 on PBMCs during colitis indicates an expansion of CD11b+Gr1+ MDSCs that express low levels of Muc1. We have previously shown by using Muc1 knockout mice that deletion of Muc1 on hematopoietic cells promotes expansion of CD11b+Gr1+ MDSCs during tumor development (23). We show here that similar downregulation of Muc1 on hematopoietic cells also promotes the expansion of immunosuppressive CD11b+Gr1+ cells during colitis that enhanced colon tumor development in KO→WT mice. We observed similar levels of Gr1+ cells in the inflammatory lesions of WT→WT and KO→WT mice, however, flow cytometry of the colonic lamina propria cells revealed that more of these cells were CD11b+Gr1+ in KO→WT mice as compared with WT→WT. CD11b+Gr1+ cells decreased steadily to normal levels during the recovery period post-DSS-induced colitis (data not shown), but the increase in CD11b+Gr1+ cells during colitis was sufficient to alter the colonic microenvironment such that more tumors eventually developed in KO→WT mice on the CAC model as compared with WT→WT mice. Even though MDSCs have been shown to be present in patients with IBD (22), this is the first time that the transient inflammatory expansion of CD11b+Gr1+ MDSCs during colitis has been shown to promote a protumorigenic milieu in the progression of CAC.

In our model, we observed an increase in sRAGE in the plasma of KO→WT mice that was concomitant with the increase in CD11b+Gr1+ cells (as compared with WT→WT) after three DSS-drinking cycles. The increase in sRAGE could be a result of cleavage of RAGE (36), given that we observed a reduction of RAGE levels in the colonic mucosa of KO→WT mice treated with AOM + DSS. RAGE activation can activate NF-kB signaling, leading to transcription of proinflammatory factors (37). Therefore, it is likely that the anti-inflammatory effects observed in KO→WT mice during colitis are a result of reduced RAGE signaling in the colon. In addition, MDSCs have been shown to express RAGE (38). It is possible that the increased level of sRAGE in the plasma of KO→WT mice treated with AOM + DSS is also a result of additional cleavage of RAGE from the increased levels of CD11b+Gr1+ MDSCs. Preliminary data in our studies seem to indicate that treatment with anti-Gr1 during the DSS-drinking cycles can reduce the levels of sRAGE in the plasma (data not shown). Despite the anti-inflammatory effects of reduced RAGE signaling in the colon, our microarray analysis of the colonic mucosa uncovered a gene signature in the colons of KO→WT mice treated with AOM + DSS that was more protumorigenic than in similarly treated WT→WT mice. This indicates that tumor progression can still proceed in the presence of lower levels of colonic inflammation during colitis in KO→WT mice, as compared with similarly treated WT→WT mice. These findings further emphasize the significance of the IBD-associated deregulation of immune signaling toward protumorigenic immunosuppression, for example, via the generation of MDSCs.

Transcriptional network involved in tumorigenesis in the absence of hematopoietic Muc1

We did not observe any significant difference between KO→WT and WT→WT untreated mice, indicating that the differences in gene signatures observed are purely due to the induction of CAC in these mice. Given the larger tumors that eventually developed in KO→WT mice, it was not surprising that the colonic mucosa of postcolitic KO→WT mice reflected a larger amount of ECM remodeling, of which increased transcription of collagen and MMP genes predominated. These data were further confirmed by the increased collagen deposition with Masson’s Trichome stain and increased MMP-2 activity. Increased levels of MMP-2 have been found in biopsy specimens of Crohn’s disease and ulcerative colitis (39). Increased levels of pro and active MMP-10 were observed in the mucosa of colitic KO→WT mice. MMP-10 has been described to be produced by infiltrating myeloid cells during colitis (40) and our data confirm that observation by showing that the levels of pro and active MMP-10 could be reduced with anti-Gr1 treatment.

Intriguingly, our microarray data reflect a dramatic increase in innate immune signaling, as indicated by upregulation of α-defensin and complement genes, in KO→WT treated mice, as compared with WT→WT mice. Defensins are antimicrobial peptides and expression of α-defensins has been shown to be upregulated in colonic tumor mucosa (41). We observed upregulation of a variety of α-defensins in our microarray data but were only able to spot primers for Defa 1, 4, and 20 on our TaqMan microfluidic cards. Of these three Defas, only upregulation of Defa 20 in postcolitic KO→WT mucosa was validated in our TaqMan microfluidic card analysis. Defa 20 was observed to be similarly upregulated in a mouse model of 1,2-dimethylhydrazine (DMH)-induced colorectal cancer and thought to be associated with tumorigenesis (42). Increased activation of the complement pathway in the postcolitic KO→WT mucosa also parallels studies by others that show elevation of the complement effector protein, C3, in the colon of patients with IBD (43). In addition, activation of the complement pathway is also a potential immunosuppressive mechanism that could promote tumor formation in CAC (44). C1q has been shown to promote canonical Wnt signaling and aging-related phenotypes (45). Wnt signaling is important in the progression of colon cancer (46), which could be significant in our data given that we observe C1q upregulation in the colonic mucosa of KO→WT mice during CAC that could be reversed with anti-Gr1 treatment.

We observed marked upregulation of Ccl2 and Ccr2 in the mucosa of postcolitic KO→WT mice. Blocking of Ccl2 signaling has been shown to be able to reduce tumor incidence in CAC (32). Increased growth signaling as seen by the increased transcription of Gcsfr, Tnf, Tgf, and Igf signaling in postcolitic colonic mucosa of KO→WT chimeras can help promote tumor growth in these mice. Tnf-α can regulate the trafficking of macrophages to the site of inflammation and blocking of the Tnf-α/Tnf receptor axis reduced colorectal carcinogenesis (34). In addition, Gcsfr
The role of Muc1/MUC1 loss on hematopoietic cells in the tumorigenic progression of malignantly transformed cells in the colon

This is the first time that downregulation of Muc1 on PBMCs has been shown to be indicative of an increase in Muc1 low expressing CD11b+Gr1+ MDSCs during colitis in mice. Similarly, we have also shown that CD14+ HLA DR−/lo cells, purported to be indicative of the MDSC population in IBD (22), also do not express MUC1. We observe a lack of reduction of B220+ cells in the peripheral blood during colitis in KO→WT mice as compared with WT→WT. The deletion of hematopoietic Muc1 prevented the down-regulation of B220+ cells in KO→WT mice during colitis, either at the B-cell level or through a lack of differentiation of B220+ progenitor cells. This may be related to the increased expansion of MDSCs observed in the KO→WT mice as an effect of disease-associated shunting of immune developmental pathways and could be important for the changes we observe in inflammation and tumor development. However, for this article, we chose to focus on the role of the expansion of Muc1 low expressing CD11b+Gr1+ MDSCs for two reasons: (i) MDSCs are found in patients with IBD (22) but their functions remain unknown, and (ii) we have shown a direct link between deletion of Muc1 in mice and MDSC expansion during tumor development (23). MDSCs in colitis do not seem to contribute to alleviation of inflammation in patients, as all our patient samples were derived from patients having an active flare of disease; however, they can play a significant role in promoting eventual tumor development, which is what we have shown phenotypically and mechanistically in this article.

MDSCs expand in part due to the deregulated cytokine production during colitis and can result in an altered protumorigenic colonic microenvironment in genetically susceptible individuals. KO→WT mice exhibit increased peripheral levels of MDSCs with reduced inflammation during colitis. Depletion of MDSCs in KO→WT mice reduced tumorigenesis indicating that CAC progression is dependent on MDSCs. Furthermore, we have shown that levels of MMP-10 and the complement C1q can be modulated by MDSCs as depletion with anti-Gr1 blocked the increase in levels of these proteins in KO→WT mice.

Our data show that tumor progression in CAC is not entirely dependent on the inflammatory colonic damage caused during colitis. In fact, deregulated immune signaling as a result of the colitic inflammation is an important component for tumor progression. Our data indicate a need to consistently analyze the levels of peripheral MDSCs and other immune cell populations during colitis. Inappropriate skewing of the immune profile during colitis toward a protumorigenic phenotypic (e.g., accumulation of MDSCs) might be responsible for promoting CAC, even after recovery from colitic inflammatory damage in the colon.

Our data highlight a crucial link for CD11b+Gr1+ MDSCs in the transition from inflammation to cancer in CAC, independent of inflammatory damage in the colon. Preventing this process could be an important pathway to target. Furthermore, MDSCs are not well characterized in humans across different diseases and downregulation of MUC1 might be a further prognostic marker for MDSCs in cancer and inflammation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Downregulation of Hematopoietic MUC1 during Experimental Colitis Increases Tumor-Promoting Myeloid-Derived Suppressor Cells

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