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Immune chaperone gp96 drives the contributions of macrophages to inflammatory colon tumorigenesis

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Abstract

Macrophages are important drivers in the development of inflammation-associated colon cancers, but the mechanistic underpinnings for their contributions are not fully understood. Further, Toll-like receptors (TLR) have been implicated in colon cancer, but their relevant cellular sites of action are obscure. In this study, we show that the endoplasmic reticulum chaperone gp96 is essential in tumor-associated macrophages (TAM) to license their contributions to inflammatory colon tumorigenesis. Mice where gp96 was genetically deleted in a macrophage-specific manner exhibited reduced colitis and inflammation-associated colon tumorigenesis. Attenuation of colon cancer in these mice correlated strikingly with reduced mutation rates of β -catenin, increased efficiency of the DNA repair machinery and reduced expression of pro-inflammatory cytokines, including IL-17 and IL-23 in the tumor microenvironment. The genotoxic nature of TAM-associated inflammation was evident by increased expression of genes in the DNA repair pathway. Our work deepens understanding of how TAM promote oncogenesis by altering the molecular oncogenic program within epithelial cells, and it identifies gp96 as a lynchpin chaperone needed in TAM to license their function and impact on expression of critical inflammatory cytokines in colon tumorigenesis.

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Writing, review and revision of the manuscript: C. Morales, S. Rachidi and Z. Li.

Disclosure of Potential Conflicts of Interest

No potential conflict of interests disclosed.

Introduction

The multi-faceted roles of inflammation in cancer have been increasingly recognized (1, 2). One of the best pieces of evidence to support the link between un-resolving inflammation and cancer is the strong clinical association between inflammatory bowel disease and increased risk of colon cancer (3, 4). Using a colitis-associated colon tumorigenesis model in mice, it was found, in general, that pro-inflammatory responses via direct activation of epithelial cells is important for gut homeostasis and colon cancer progression (5-9). However, the roles and mechanisms of the host immune system, such as macrophages, in colon cancer development are far from clear (10). Depending on the model system examined, inflammation could be both friend and foe for oncogenesis. MyD88-mediated signaling drives colon cancer in the APC^{min} mouse model (8), but is protective against colitis-associated colon cancer induced by azoxymethane (AOM) and dextran sodium sulfate (DSS) (11). In addition, although microbial stimulation is important for inflammation-associated colon cancer (12), both cellular and molecular mediators in the process are yet to be defined. For example, it is unclear what specific roles macrophages play in the initiation of colon cancer, despite the well-recognized attributes of tumor-associated macrophages in promoting cancer invasion and metastasis in other models (13-16). In the case of innate microbial sensors, such as Toll-like receptors (TLRs), it has also been a challenge to tease out the specific contribution by macrophage-intrinsic TLRs in colonic oncogenesis, since TLRs are expressed by both gut epithelial cells and the host immune cells. The selective deletion of MyD88 in myeloid cells is not an ideal model (17) to address this question, inasmuch as MyD88 is the common adaptor for TLRs as well as the receptors for IL-1 and IL-18. Moreover, the MyD88-independent pathway may also contribute to TLR-mediated oncogenesis, an idea that has not been explored.

Glycoprotein 96 (gp96), also known as glucose regulated protein 94 (grp94) or HSP90b1, is a molecular chaperone in the lumen of the endoplasmic reticulum (ER). Although constitutively expressed in all cell types, gp96 is strongly induced in tumor-associated macrophages with unknown functional significance (18). As a major effector chaperone downstream of the unfolded protein response (UPR), gp96 surprisingly and selectively catalyzes the folding of a majority of TLRs except TLR3 (19). Deletion of gp96 genetically leads to categorical loss of responsiveness to all TLR ligands except dsRNA (20). We previously demonstrated that LysM-Cre mediated deletion of gp96 is highly macrophage-selective, without affecting other cell populations such as dendritic cells, B cells or T cells (21). Due to a relatively long half-life of gp96, cre-mediated deletion of its gene in neutrophils did not affect the protein expression level of gp96 during the life cycle of neutrophils. Only tissue macrophages in these mice were defective of gp96, and hence in TLR assembly. TLR signaling particularly through TLR4 was further compromised in gp96 null cells due to loss of β 2 integrins which play important roles in cross-talking with TLRs through membrane recruitment of TIRAP and MyD88 (22). In this study, we took advantage of these unique macrophage-specific gp96 knockout (KO) mice (referred to as *Mac96^{KO}* mice) to address the contribution of macrophages to colitis and colon tumorigenesis. Using the AOM/DSS-induced colon tumorigenesis model, we found that macrophages promote colitis and colitis-associated colon tumorigenesis in a gp96-dependent manner, which

correlated with increased levels of IL-17, IL-23, IL-12, IL-6 and IFN γ . Moreover, we discovered that macrophage-mediated inflammation promotes genotoxic insult resulting in the activation of the oncogenic machinery in the colon. Our study has thus demonstrated the importance of macrophage-intrinsic gp96 and its clientele in promoting both colitis and inflammation-induced colon tumorigenesis; it has revealed the critical roles of macrophages in directly initiating oncogenesis within the gut epithelium during chronic inflammation.

Materials and Methods

Animals

LysM^{Cre}Hsp90b^{fllox/fllox} mice were previously described (21). All animal experiments involving control or wild type (WT) (*LysM^{Cre}Hsp90b^{fllox/WT}*) and KO (*LysM^{Cre}Hsp90b^{fllox/fllox}*) mice were approved by our Institutional Animal Care and Use Committee, and guidelines were strictly followed. WT and KO mice were co-housed, and age matched mice were used for all experiments.

DSS Colitis

Mice were administered 3% DSS dissolved in water and fed *ad libitum* for five days. Fresh DSS was provided on day three. One cohort of mice was euthanized at day 5. Another cohort of mice was given regular drinking water from day 5 until day 8, at which point they were euthanized. Five mice per group were used. Mice were weighed, and their stool was scored daily. Colitis pathology score was obtained as follows: 0, normal epithelium, 1, minimal leukocyte infiltration, 2, moderate leukocyte infiltration, 3, severe leukocyte infiltration with no major architecture destruction, 4, severe leukocyte infiltration with destruction of normal architecture. Stool consistency was scored as follows: 0, well-formed stool; 1, semi-formed stool; 2, semi-formed stool that adheres to the anus; 3, liquid stool/diarrhea. Colon length was measured *ex vivo* from cecum to rectum.

AOM/DSS colon tumorigenesis

Ten to seventeen mice per group were given 12.5 mg/kg body weight AOM via ip injection on day one. DSS added to drinking water was given at 2.5% for 5 days on weeks two and five, and then at 2% for 4 days on week eight. Mice were weighed and monitored weekly for overall health, and bled every other week for serum analysis. Mice were euthanized at 19-21 weeks, at which point tissue was harvested. Colons were fixed in 10% paraformaldehyde overnight, and then stained with 0.2% methylene blue to facilitate the visualization of adenomas. Tumors were counted and measured under a dissecting microscope. Colons were then frozen as in a fashion of “Swiss rolls” in OCT for later histological analysis.

Histology

Tissue was fixed in 4% formalin at least overnight, and then switched to 30% sucrose-PBS overnight. Tissue was then frozen in OCT medium and kept at -80°C . 5 μm sections were cut on a Shandon Cryotome and mounted on charged slides (Fisher). Slides were processed by H&E staining and then mounted. H&E slides were scored blindly by a surgical pathologist, based on the percentage of high grade dysplasia in lesions: 1: 0%; 2: 1-25%; 3: 26-50%; 4: 51-75%; 5: 76-100%. For IHC, tissue was paraffin embedded.

Immunoblot

Immunoblot were performed as described previously (23, 24). In brief, cells or colonic tissues were lysed and homogenized respectively in radioimmunoprecipitation assay (RIPA) lysis buffer along with a cocktail of protease inhibitors. Total lysates were then resolved on 10% SDS-PAGE, and the proteins were transferred from the gel onto Immobilon-P membranes. The membrane was blocked with 5% nonfat milk, probed with different antibodies, followed by incubation with HRP-conjugated secondary antibody. Protein bands were visualized by using enhanced chemiluminescent substrate (Pierce, Rockford, IL). Intensity of the band on the scanned films was analyzed by Image J program. β -catenin antibody was purchased from Cell Signaling (Danvers, MA). Antibodies against MSH6 (ab92471) and MLH1 (ab92312) were obtained from Abcam (Cambridge, MA).

Immunohistochemistry (IHC)

Paraffin sections were cut and mounted on positively charged slides. Paraffin was removed by incubating the slides at 55°C for 30 minutes. Slides were dipped in xylene and then rehydrated through a series of ethanol and then water and PBS. For F4/80 IHC, antigen retrieval was performed by applying 20 μ g/ml proteinase K to the samples. For β -catenin IHC, antigen retrieval was carried out by boiling in sodium citrate buffer. Slides were washed and then incubated in 0.3% H_2O_2 + 0.3% normal goat serum diluted in PBS. Slides were again washed and then blocked with 2% BSA plus 10% goat serum diluted in PBS for 2 hours. Slides were then stained with anti-F4/80 antibody (Santa Cruz) or rat IgG2b isotype control antibody, or with β -catenin antibody or rabbit isotype control antibody, at 4°C overnight. Slides were washed, incubated with biotinylated anti-rat or rabbit antibody plus 2% goat serum diluted in PBS, washed again, and then streptavidin antibody diluted in PBS was applied (Peroxidase rat IgG Vectastain ABC kit). Slides were again washed and then developed with DAB substrate kit (Vector Labs). After staining, slides were counterstained with hematoxylin, washed, and then dehydrated in a series of ethanols followed by xylenes, and then mounted.

Lamina Propria Isolation

Small and large intestines were removed and flushed of content with PBS. Peyers patches were removed, the intestine was cut open longitudinally, and then cut into ~1cm pieces. Samples were washed three times in cold Hanks Balanced Salt Solution (HBSS) + 2% FBS and vortexed for 15 seconds to help remove the mucus layer. Samples were then spun at 100 rpm on a stir plate in HBSS + 5% FBS + 1 μ M DTT for 20 minutes at 37°C, and then vortexed. Samples were next spun as before in 1.3 mM EDTA + PBS at 37°C for 20-30 minutes, and then vortexed. This step was repeated to fully remove epithelium. Samples were washed twice in HBSS + 2% FBS and then once in RPMI, vortexing after each wash. Samples were further cut into smaller pieces, and then incubated twice with collagenase IV + 0.1mg/ml DNase I + 5% FBS + RPMI, and spun as above for 20 minutes. The solution was then passed through a 19 gauge syringe and a cell strainer, and washed with RPMI. Total lamina propria isolates were then analyzed for myeloid cells, or enriched for lymphocytes via Percoll centrifugation (44 & 66% 1X Percoll diluted in RPMI) at 2,800 rpm

for 20 minutes without braking. The interface was collected and washed in RPMI. Live cells were counted using a hemacytometer after Trypan blue exclusion.

Leukocyte stimulation and FACS

Leukocytes isolated from the lamina propria were stimulated with PMA (300 ng/mL) and Ionomycin (1 µg/mL) for 4 hours in RPMI complete media. Flow cytometry was performed as previously described (25).

qRT-PCR

RNA was extracted from colon tissue by TriZol reagent, and quantified with a Nanodrop. mRNA was then reverse transcribed into cDNA using Superscript II H. Reverse Transcriptase. cDNA was then diluted so that 5 ng of RNA was used per reaction. qRT-PCR was carried out with SYBR Green supermix (BioRad) using a BioRad iCycler. Data was exported into Excel for analysis using the delta Ct (the number of PCR cycles to reach the threshold of the product detection) method and normalized to β -actin as an internal control. Plots represent fold change in mRNA.

Culture of Bone Marrow-Derived Macrophages

Bone marrow (BM) cells were isolated from femurs and tibias. BM cells were cultured in DMEM medium supplemented with 10% FCS, 100 U/ml of penicillin, 100 mg/ml of streptomycin, 2 mM L-glutamine, 20% L929 conditioned medium for 6 days. Nonadherent cells were washed away. The adherent cells were harvested after trypsin digestion, which were mostly bone marrow derived macrophages (BMDMs) by phenotypic analysis.

cDNA array and pathway analysis

WT and gp96 KO BMDMs were stimulated with 200 ng/ml LPS (055:B5) from Sigma for 6 hours. Total RNA was extracted from WT and gp96 KO BMDMs using an RNeasy Mini Kit (Qiagen, Valencia, CA). Fluorescent anti-sense RNA (aRNA) target preparation was performed using an Eberwine-based amplification method with Amino Allyl MessageAmp II aRNA Amplification Kit. Cy5-labeled RNA targets were hybridized to Mouse Whole Genome OneArray® v2 (Phalanx Biotech Group, Belmont, CA), and slides were scanned by the Axon 4000 scanner (Molecular Devices, Sunnyvale, CA). The Cy5 fluorescent intensity of each spot was analyzed by Genepix 4.1 software (Molecular Devices, Sunnyvale, CA). Heat map was generated with the intensity value of each spot over the mean of the intensity of each gene from WT BMDMs in triplicates. Genes that were down-regulated in KO macrophages were classified using KEGG Pathway Database and those involved in Wnt signaling and cancer were highlighted. Heat map was generated demonstrating differential expression of genes in the WT and KO cells, using Gene Pattern program provided by the Broad Institute.

Gene sequencing

Genes were amplified by PCR from colon cDNA samples. PCR reactions were purified with Qiagen kits and then sent for sequencing by Genewiz. Sequencing results were then aligned

using Vector NTI software (Invitrogen). The sequences of β -catenin primers were: 300F gct gac ctg atg gag ttg ga, 526R gct act tgc tct tgc gtg aa (5).

Bone marrow transplantation

Using a Cesium irradiator, WT recipient C57BL/6 male mice were lethally irradiated at a dose of 1,100 cGy, split into 2 sessions (550 cGy each) 4 hours apart. Twenty-four hours after the first irradiation session, mice were injected with 2×10^6 bone-marrow cells from donor WT or *Mac96^{KO}* mice via the lateral tail vein. Donor bone marrow cells were obtained under sterile conditions by flushing the tibias and femurs with PBS, lysing red blood cells with ACK buffer, and suspending the cells at a concentration of 10^7 cells/ml. Twelve weeks after bone marrow transplant, mice were treated with the same AOM/DSS course as above, except that DSS was administered in weeks 2, 8, and 11 due to increased DSS-susceptibility at a different animal facility. After 16 weeks, mice were sacrificed.

Tissue culture

YAMC cells (26) were obtained from Dr. Philip Howe (Medical University of South Carolina) in December 2012. The normal gut epithelial cell morphology was verified microscopically and the expression of E-cadherin was confirmed by immunoblot. The temperature-sensitive growth at 25°C due to the expression of a temperature-sensitive mutant of the SV0-40 Large T antigen was confirmed. The cells were grown in DMEM medium (Sigma) supplemented with 10% FBS and 1% penicillin and streptomycin. Cells were grown at 33°C with 5% CO₂. For stimulation experiments, 0.3×10^6 cells were seeded in each well of a 12-well plate and left to adhere and grow overnight. Cells were then left untreated, or treated with a mixture of recombinant murine cytokines (5 ng/ml IL-1 β , 10 ng/ml IL-17A, and 1 ng/ml TNF α , all from Shenandoah) for 48 hours. Invasion assay was performed using Transwell system by seeding cells in the upper chamber of a 1% gelatin-coated Transwell membrane (Corning). At 15 hours, cells were fixed in 90% ethanol for 10 minutes and stained with 1% crystal violet for 10 minutes. Cells in the lower chamber were eluted with 10% acetic acid for 10 minutes and cell number was determined by OD at 595 nm. EMT markers were analyzed by immunoblot for E-cadherin and vimentin of whole cell lysates.

Quantification of serum cytokines by ELISA and cytokine bead arrays (CBAs)

Serum IL-1 α and IL-10 for the BMT experiment were quantified using cytokine bead arrays from Eve Technologies, Inc, Canada. Quantification for IL-6 and IL12 (p40) was performed by ELISA from R&D Systems. IL-17A ELISA antibodies were purchased from BD Pharmingen.

Statistical analysis

Two-sample student's T test was performed. Error bars represent either standard deviation or standard error, as indicated. For mouse weight in the BMT experiment, two-way ANOVA was used for statistical analysis.

Results

***Mac96^{KO}* mice are more resistant to chemical-induced colitis**

We reported previously that gp96 deletion is more pronounced in tissue macrophages in *Mac96^{KO}* mice due to the long half-life of gp96 (~ 7 days) (21). To verify the loss of gp96 in intestinal macrophages, lamina propria (LP) cells of WT and KO mice were isolated from both the small intestine and colon, and F4/80⁺ macrophages were analyzed via flow cytometry. Similar percentages of F4/80⁺ cells in the LP were demonstrated, indicating no defect in recruitment of KO macrophages into the lamina propria. Consistent with our previous findings, F4/80⁺ LP macrophages from both the small and large intestine of *Mac96^{KO}* mice showed significant reduction of gp96 expression by intracellular stain using a gp96 monoclonal antibody (Figure 1A).

To address the role of macrophage-specific gp96 in inflammation, we induced colitis by adding DSS in the drinking water. After treating WT and *Mac96^{KO}* mice with 3% DSS for five days and then analyzing at either day 5 or day 8, we saw weight loss in both groups of mice. Histological examination, however, demonstrated significantly more inflammation in the colon of WT mice compared to KO mice. This was evidenced by shortening of villi, infiltration of the colon mucosa with mononuclear cells including neutrophils, and destruction of crypt-villus structure (Figures 1B and 1C). As a further indication of inflammation, WT colons were significantly more shortened than KO colons on day 5 (Figure 1D). WT mice also had lower stool consistency score than KO mice (Fig. 1E). By qRT-PCR on colon samples, we found a general trend of increased cytokine mRNA levels in WT colons compared to KO ones, such as IL-1 β , IL-6, IL-17A, IL-17F, IL-22, IL-23 and IFN γ (Fig. 1F). However, macrophage density in the inflamed colon is similar between WT and KO colons as indicated by equal levels of F4/80 mRNA (Fig. 1F). Collectively, deletion of gp96 from macrophages renders mice less susceptible to DSS-induced colitis.

We next analyzed the cellular components of the lamina propria from WT and KO DSS-treated mice and examined the level of pro-inflammatory cytokines at the protein level. CD8⁺ cells in KO mice demonstrated a trend towards lower production of IFN- γ (Figures 1G and 1H). In the CD4 compartment, KO colons contained lower percentages of Th17 (Ror- γ t⁺ and IL17A⁺) and Th1 (Tbet⁺ and IFN- γ ⁺) helper T cells (Figures 1G, 1I, 1J, 1K, 1L, 1M, 1N and 1O). Intuitively, macrophages from KO mice secreted lower amounts of TNF- α (Figures 1G and 1P), IL-6 (Figures 1G and 1Q) and IL17A (Figures 1G and 1R).

***Mac96^{KO}* mice are protected against colitis-associated colon tumorigenesis**

We next studied the role of macrophage-intrinsic gp96 in the development of colitis-associated colon tumorigenesis by injecting WT and *Mac96^{KO}* mice with the carcinogen AOM, followed by chronic induction of colitis using three cycles of DSS. We found that WT mice suffered from increased tumor burden (number of tumors multiplied by the average tumor size) comparing with *Mac96^{KO}* mice (Figure 2). At week 20, WT mice had increased colonic tumors by gross pathological examination (Figure 2A). Histological analysis demonstrates that the colons were enriched with dysplasia and high-grade adenomas in the WT mice, while *Mac96^{KO}* mice had low-grade adenomas (Figures 2B and

2C). Importantly, KO mice had fewer (Figure 2D) and smaller tumors, especially fewer large tumors >2 mm in diameter (Figure 2E). The decrease in both tumor number and size in *Mac96^{KO}* mice translated into a significant reduction in tumor burden (Figure 2F).

Therefore, we conclude that deletion of gp96 in macrophages renders mice resistant against colitis-associated colon cancer.

gp96 reduction in M ϕ protects gut epithelium from β -catenin mutation and the activation of the canonical Wnt pathway

The Wnt pathway, important in cell growth and embryogenesis, has been definitely linked to the development of colon cancer (27). By analyzing the colon samples from AOM and DSS-treated mice, we found that Wnt target molecules, including Axin, β -catenin, c-Myc, Cox-2, Cyclin D1, Fzd1, and p53, were significantly increased compared to untreated mice (Figure 3A). Of note, colon from *Mac96^{KO}* mice expresses significantly less mRNA for the Wnt receptor Fzd1 and the downstream target gene p53. Interestingly, we also found significantly less β -catenin mRNA levels basally in the colon of KO mice, indicating that loss of gp96 in macrophages might have effect on baseline mucosal biology. To determine if inflammation accelerates AOM-induced mutations in β -catenin, thus driving oncogenesis, we sequenced exon 3 of β -catenin, which contains GSK3 β phosphorylation sites. Mutations were absent in both untreated WT (n=3) and *Mac96^{KO}* (n=3) mice. However, while 67% of WT mice (6 of 9 mice) treated with AOM/DSS harbored mutations in exon 3, we found no mutations in AOM/DSS-treated KO mice (0 of 14 mice) (Figure 3B). When we closely examined the mutation pattern in each mouse, we found that WT mice contained anywhere from 0-6 mutations each (Figure 3C). These mutations in exon 3 are indeed clustered around the GSK3 β phosphorylation sites (Capitalized and underlined bases respectively) (Figure 3D). By immunohistochemistry, β -catenin nuclear translocation was observed (Figure 3E). We therefore conclude that gp96 deletion from macrophages alone without alteration of any other cellular compartment protects colonic epithelium from β -catenin mutation and the activation of the canonical Wnt pathway.

Inflammation activates β —catenin and DNA repair pathways

In addition to the increased β -catenin mutation rate in WT colons, we also found that inflammation *per se*, without an exogenous mutagen (AOM) being present, is capable of inducing β -catenin expression (Figure 3F). Furthermore, the higher prevalence of β -catenin mutation in WT colons suggests that macrophages and their mediators exert genotoxic pressure during the process of oncogenesis. If this surmise were true, one would expect to see the induction of components of the DNA repair pathway during inflammation. To examine this possibility, we looked at the mismatch repair (MMR) and base excision repair (BER) pathways, both of which have been implicated in the pathogenesis of colon cancer in human and mouse (28-30). We used qRT-PCR to examine the mRNA levels of DNA repair molecules during acute inflammation induced by DSS alone and during chronic inflammation induced by both AOM and DSS. We found that DSS alone indeed turned on the MMR pathway, evidenced by increased mRNA levels of MLH1 and MSH6 (Figure 3G). We also found that AOM/DSS caused significant up-regulation of AAG mRNA, a key component of the BER pathway. On the protein level, acute inflammation induced MLH1 and MSH6 expression in both WT and KO mice (Figure 3H and 3I). However, in the

absence of an acute inflammatory insult; 2 months after the latest DSS exposure and 5 months after AOM exposure, KO mice maintained higher levels of both MLH1 and MSH6 than WT mice (Figures 3H and 3I), which could potentially contribute to the lower prevalence of β -catenin mutation rates as well as the lower tumor burden in KO mice.

Taken together, we conclude that inflammation is genotoxic, and suggest that deletion of gp96 in macrophages protects mice from inflammation-associated colon tumorigenesis partially due to a reduced genotoxic pressure, as well as an increased DNA repair mechanism.

Deletion of gp96 in M ϕ results in altered cytokine milieu in the tumor microenvironment

gp96 deletion did not significantly alter the level of tissue macrophages as evidenced by the comparable density of F4/80+ cells in WT and KO colonic tumors by immunohistochemistry (Figure 4A). This was also supported by similar levels of F4/80 on the mRNA level (Figure 4B). We next focused on the potential difference between WT and KO macrophages in producing oncogenic inflammatory mediators that might contribute to the distinct susceptibility to colon tumorigenesis in WT and KO mice. After AOM and DSS treatment, we found a significant induction of many pro-inflammatory cytokines in the colon tissues in both WT and KO mice, including IL-1 β , IL-6, IL-17A, IL-17F, IL-18, IL-22, IL-23, IFN γ and TNF α (Figure 4B). When the induction of cytokine levels was compared between WT and KO mice, the levels of selective cytokines, such as IL-17A, IL-17F, IL-23, and TNF α , were significantly reduced in KO mice.

To further characterize the impact of loss of macrophage gp96 in gut immunity during colon tumorigenesis, we isolated lamina propria cells and performed immunophenotypic analysis to probe cytokine production at the protein level. No differences in CD4 and CD8 distribution were observed between WT and KO mice (Figures 5A and 5B). However, there were fewer IL-17A-producing CD4+ cells in the LP of untreated KO mice, in response to stimulation by PMA and ionomycin (Figures 7C and 7D). KO mice also possessed lower levels of IFN- γ + CD4 cells at baseline and after AOM/DSS treatment (Figures 5C and 5E). Consistent with this observation, serum from KO mice at week 20 (upon sacrifice) after AOM+DSS treatment demonstrated lower levels of multiple cytokines, including GM-CSF, TNF- α , IL-2, IL-7, IL-12, IFN- γ , IL-10, as well as chemokines CCL3 and CCL4 (Figure 5F). Importantly, the systemic level of IL17A, TNF α and IL12p40 was significantly reduced in the KO mice comparing with the WT mice during the induction phase of the colon cancer (before Day 70) (Figure 5G). Taken together, deletion of gp96 in macrophages causes selective reduction of a number of cytokines including IL-23, IL-17 and IFN γ . Given the established oncogenic roles of IL-17 in the AOM/DSS-induced colon cancer model (31), our data strongly favors the idea that macrophages can promote colon tumorigenesis via a gp96-TLR-IL23-IL17 axis.

***Mac96*^{KO} bone marrow cells protect WT mice from increased susceptibility to inflammation-associated colon cancer**

So far we have used LysM-Cre-mediated gp96 deletion to address the role of macrophage-intrinsic gp96 in colitis and colon tumorigenesis. This strategy is expected to delete gp96

from other tissue-resident APCs such as radio-resistant Langerhans cells (32). There was also a possibility of cre-expression in other non-hematopoietic parenchymal tissues to complicate the interpretation of our data. To address this possibility, we next reconstituted lethally irradiated WT mice with bone-marrow (BM) cells from WT or *Mac96^{KO}* mice. This strategy resulted in greater than 90% donor chimerism in the gut-associated lymphoid tissues. We then treated these chimeric mice with AOM and DSS. Twenty weeks later, mice were sacrificed for analysis. As expected, we found that F4/80+ macrophages in the peritoneal exudate cells (PECs) from KO→WT BM chimeric mice had nearly 100% knock-down of gp96 by intracellular stain (Figure 6A). Similar to the straight *Mac96^{KO}* mice, the KO chimeric mice had lower tumor burden compared to WT→WT mice (15 mm² in KO compared to 33 mm² in WT mice, $p=0.04$, Figure 6B). During the course of tumor induction, WT mice lost significantly more weight than KO mice in the AOM/DSS-treated group (Figure 6C). Additionally, we observed significantly higher levels of serum IL-1 α and lower levels of IL-10 in KO mice compared to WT mice (Figure 6D). Immunophenotypic analysis of the gut-associated lymphoid tissues also demonstrated decreased CD4+ Foxp3⁺ Treg cells in KO mice (21% of CD4+ cells) compared to WT mice (25% of CD4+ cells, $p=0.03$) (Figure 6E). As expected, macrophage density in AOM/DSS treated mice was similar in WT and KO tumors (Figure 6F). This experiment provided further evidence to support the important role of macrophage-intrinsic gp96 in promoting colitis-associated colon tumorigenesis.

Inflammatory cytokines induce cancer pathways

A strong causative correlation exists between inflammation and increased susceptibility to cancer. Of interest, activation of WT but not gp96 KO macrophages by TLR ligands (LPS) upregulates not only the proinflammatory genes but also genes that control cell growth and survival, such as Wnt receptor, PI3K-AKT and NF κ B (Figure 7A). Given the differences in IL-1 β , IL-17A, and TNF α between WT and *Mac96^{KO}* mice, we asked if inflammatory cytokines could directly induce cancer pathways and promote oncogenesis. To this end, we treated normal colonic murine epithelial cells (YAMC) (26) with a mixture of the three cytokines mentioned above. We found that the cytokine mixture can induce significant epithelial-mesenchymal transition (EMT) and invasion, but not the expression of DNA mismatch repair genes (Figures 7B and 7C). Exposure of normal epithelial cells to this cytokine cocktail as short as 48 hours led to reduction of E-cadherin and induction of vimentin, which was accompanied by cell cycle arrest but increased invasion. Testing those cytokines individually showed that TNF- α and IL-1 β could independently induce YAMC cell invasion, but IL-17A could not (Figure 7C).

Discussion

The macrophage possesses excellent phagocytic and cytotoxic functions (33); it expresses multiple pattern recognition receptors such as TLRs, scavenger receptors, cytosolic NOD and NOD-like receptors (34); it is armed with both antigen-presenting (35) and co-stimulatory molecules; and it produces proinflammatory cytokines during infection. Many functions of macrophage are regulated by TLRs, including cytokine production, bactericidal activity, phagocytosis, and even self vs. non-self discrimination in that TLR ligand-“tainted”

antigen is preferentially presented to MHC class II (36). However, the roles of macrophage in cancer have not been completely resolved (1, 37). gp96 is a master chaperone for TLRs and integrins (19-21). Loss of gp96 from macrophage results in profound disturbance of their function (21). Thus, by selectively deleting gp96 from macrophages, we were finally able to tease out the roles of this important cell type in inflammation and cancer. This work is also significant since gp96 is strongly induced in tumor-associated macrophages with unknown functional consequences (18). In the current study, we specifically focused on the roles of macrophages in colitis and colitis-associated colon tumorigenesis. We demonstrated the critical roles of macrophages via gp96 and its clientele in conferring intestinal inflammation, inducing genetic instability in the colonic epithelium, and promoting colitis-associated colon tumorigenesis. Since oncogenesis occurs in the wild type epithelium, our study has conclusively demonstrated for the first time that macrophage-intrinsic gp96 plays critical roles *in trans* in promoting inflammation-associated cancer in the gut mucosa.

One fundamental question is the mechanism by which macrophage-derived inflammatory mediators drive oncogenic program. Our study suggests that the upregulated Wnt activity is the key missing link based on the following considerations. First, consistent with findings that the AOM/DSS model is associated with mutations within exon 3 of β -catenin (5), we found a high rate of mutation in colon tissue in the AOM/DSS-treated WT mice. Strikingly, however, no mutation was found in that of KO mice. Since these mutations are in and around the GSK3 β phosphorylation sites, β -catenin is no longer phosphorylated in WT mice and will thus be liberated from the β -catenin-destruction complex, contributing to the increased Wnt activity in WT mice. Second, looking at other members of the Wnt pathway, we further see a reduction of the Wnt receptor Fzd1 and the downstream target p53 in KO mice. This decreased Wnt pathway activation in KO mice is consistent with less tumor burden, as Wnt activation is also seen in colon cancer patients (38). Third, both IL-1 β and TNF α have been linked to Wnt activation in a variety of cancer models (39-42). These two cytokines were significantly reduced in *Mac96*^{KO} mice. Taken together, our data favors a model to support the roles of macrophage-intrinsic gp96 in promoting cancer via activating Wnt pathway in colonic epithelial cells.

We have examined the possibility of compromised DNA repair pathway as an alternative mechanism for macrophage-associated carcinogenesis. The MMR pathway of DNA repair has been associated with both human and murine colon cancer (28, 29, 43, 44). Even though the BER pathway is not as highly associated with spontaneous colon cancer as the MMR pathway, it has been shown that AAG^{-/-} mice are more susceptible to AOM/DSS-induced colon cancer (28, 30). Furthermore, the MMR pathway is responsible for fixing the O⁶-methylguanine adducts formed by AOM (43). Of interest, we saw that DSS alone without exposure to carcinogen induced the expression of MMR machinery, indicating indeed that inflammation imposes genotoxic pressure. Importantly, upon treatment with AOM and DSS, we found that both MLH1 and MSH6 were maintained at higher level in KO comparing with WT colonic epithelium, raising an intriguing possibility that chronic inflammation destabilizes the DNA repair machinery.

The reduced tumor burden in *Mac96*^{KO} mice correlated significantly with decreased IL-17A, IL-17F, TNF α , and IL-23. We also demonstrated that the treatment of normal

colonic epithelial cells with a combination of IL17, TNF α and IL1 β could cause EMT. To note, Langowski *et al.* demonstrated that IL-23, but not IL-12, was overexpressed in various human cancers, including colon cancer. They further showed that IL-23 promoted tumorigenesis in the 9,10-dimethyl-1,2-benzanthracene and 12-O-tetradecanoyl-phorbol acetate model of skin papillomas, while IL-12 proved protective (45). The tumor promoting abilities of IL-23 have also been proven in the 3'-methylcholanthrene fibrosarcoma model (46). Besides cancer, IL-23 acting upon T cells proved important for promoting inflammatory colitis in the T cell adoptive transfer model (47). IL-23 is also important clinically, where both the cytokine and its receptor are elevated in patients with ulcerative colitis and Crohn's disease (48). A correlation is further seen between inflammatory bowel diseases and mutations in IL23R (49). Even IL-17 has been shown to aid tumor growth by promoting angiogenesis (50), and its expression is elevated in patients with ulcerative colitis (48). Taken together, our data strongly suggest that macrophage-intrinsic gp96 promotes colon tumorigenesis via inducing IL-17 and IL-23 in the tumor microenvironment.

Few studies link macrophage specific TLRs to cancer. Given that gp96 is a master chaperone for TLRs, our study indicate the roles of TLR sensing on myeloid cells in oncogenesis. However, it is important to point out that gp96 client network is not restricted to TLRs, but also include other important receptors such as integrins (19-21, 25, 51), insulin-like growth factors (52) and others. Additionally, gp96 is a major effector chaperone of UPR and plays important roles in regulating ER homeostasis (53). Therefore, the immediate downstream mechanism of gp96 in regulating macrophage biology and cancer is likely complex. Nevertheless, our study has positioned gp96 as a strategically important chaperone in integrating stress and innate immunity, both of which could play important roles in macrophage biology and the tumor microenvironment to promote oncogenesis. Thus, macrophage-directed inhibition of gp96 may prove to be an attractive strategy for treatment of cancer in the future.

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Figure 1. *Mac96*^{KO} mice are more resistant to chemical-induced colitis and demonstrated lower activation status of CD8+, CD4+ cells and macrophages in the lamina propria of the colon

A, Lamina propria cells were isolated from both the small intestine (SI) and colon (C), stained for surface F4/80, and then intracellular gp96. F4/80+ cells were gated (Top, number denotes percent), and then analyzed for gp96 (Bottom, number denotes mean fluorescent intensity). Isotype (iso) control was included. B, H&E staining of colon cross-section from untreated (UT) mice, or from either day 5 or 8 mice (D5, D8) after DSS treatment. C, H&E staining in B was scored. D, Colon lengths were measured *ex vivo*. E, Stool consistency was scored daily after DSS administration. F, Various cytokines were analyzed by qRT-PCR from colon samples. β -actin was used as the control gene. Data are representative of two separate experiments with n=5 per group. Error bars represent the standard error of the mean. *p<0.05. G, Bar graphs representing the different cellular populations within each of the CD4+, CD8+ and F4/80+ cellular compartments. H-R, Flow cytometry plots of representative samples from WT and KO mice for each of the cellular populations. 6 WT and 4 KO mice were used in all panels. Error bars represent standard deviation.

Figure 2. *Mac96*^{KO} mice are protected against colitis-associated colon cancer

A, Gross photograph of whole colons after AOM and DSS treatment that were flushed and opened longitudinally. B, H&E staining of colons from AOM and DSS treated mice. C, H&E staining in (B) was scored. D, The average number of tumors within each size range was quantitated. E, The average total number of tumors per mouse. F, Tumor burden (number of tumors multiplied by average tumor size) was scored for each individual mouse. Data are representative of two separate experiments with n=7-10 mice per group. Error bars represent the standard deviation. *p<0.05, **p<0.005.

Figure 3. gp96 reduction in Mφ protects gut epithelium from β-catenin mutation and stabilizes DNA repair pathway

A, Wnt pathway molecules were analyzed by qRT-PCR from colon samples of both untreated (UT) and AOM+DSS treated mice (7 WT UT, 7 KO UT, 9 WT AOM and 14 KO AOM mice). B, Exon 3 of β-catenin was sequenced from colon samples, and the number of mice harboring mutations were quantitated. Results are shown as percentage. C, The number of mutations in exon 3 of β-catenin was enumerated from individual WT mice treated with AOM+DSS. In B and C, mutation analysis was done with 3 pairs of untreated WT and KO mice. For AOM/DSS-treated mice, 9 WT and 14 KO mice were analysed. D, A depiction of exon 3 of β-catenin showing GSK3β phosphorylation sites (underlined) and mutated nucleotides found (red). E, β-catenin IHC of colon samples from both untreated (UT) and AOM+DSS treated mice (One representative image from 5 mice per group). *p<0.05, **p<0.005. F, Acute DSS-induced colitis triggers the Wnt signaling pathway as evidenced by increased β-catenin expression. G, DNA repair molecules were analyzed by qRT-PCR from colon samples of either untreated (UT, n=7 per group) or mice treated with DSS only (n=5 per group) or AOM+DSS (9 WT and 14 KO). H, Immunoblot of MLH1 in the colon epithelium of WT (W) and KO (K) mice, at the baseline (untreated), or after treatment with DSS (day 7), or AOM+DSS (week 20). I, Immunoblot of MSH6 expression in the colon epithelium of WT and KO mice treated with indicated conditions. Data in F, H and I are representative of 5 mice per group. Error bars represent standard error of the mean. *p<0.05, **p<0.005.

Figure 4. Deletion of gp96 in M ϕ results in decreased local cytokine levels in tumor-bearing mice

A, IHC of colon samples for F4/80+ cells in both untreated (UT) or AOM+DSS treated mice. B, qRT-PCR for various cytokines was performed on colon samples from mice treated with AOM+DSS (7 WT and 7 KO untreated mice, and 9 WT and 14 KO AOM/DSS-treated mice). Error bars represent standard error of the mean. * $p < 0.05$, ** $p < 0.005$.

Figure 5. *Mac96*^{KO} mice display decreased Th17 and Th1 cells

A, and B, Lamina propria lymphocytes were stained by Flow Cytometry for CD4 and CD8 expressing cells in either untreated (UT) or AOM+DSS treated mice. C, Lamina propria lymphocytes were stimulated *ex vivo*, and then stained for surface CD4 and intracellular IFN- γ and IL-17A. Numbers denote percent of cells in the gated CD4+ population. D, and E, Quantification of the populations displayed in C. Data is representative of 2 experiments, with 3 mice per group. Error bars represent standard deviation. F, Systemic cytokine level in the sera from AOM/DSS-treated WT and KO mice at week 20. G, Serum cytokine levels of IL-17A, IL-6 and IL-12 (p40) in WT and KO mice 70 days after AOM injection. * $p < 0.05$, ** $p < 0.005$.

Figure 6. *Mac96*^{KO} bone marrow cells protect WT mice from inflammation-associated colon cancer

WT mice were lethally irradiated and reconstituted with bone marrow from either WT or KO mice, followed by induction of tumorigenesis by AOM+DSS. A, Peritoneal exudate cells were isolated and stained by flow cytometry for surface F4/80 and intracellular gp96. B, Tumor burden (number of tumors multiplied by average tumor size) was calculated based on number and size of tumors. C, Weight change of mice after AOM treatment. D, Serum cytokine levels from WT and KO bone marrow chimeras. *p<0.05. E, Single cell suspensions from MLN were harvested and stained by flow cytometry for CD4 and FoxP3. Double positive cells were quantified as percentage of CD4+ cells. F, Macrophage density by F4/80 immunohistochemistry in colon cancer from WT and KO chemic mice. Error bars represent standard deviation. Four WT and 6 KO untreated, and 12 WT and 12 KO AOM/DSS-treated mice were used.

Figure 7. Inflammation induces cancer pathways

A, WT and gp96 KO BMDMs were stimulated with LPS for 6 hours followed by mRNA extraction, reverse transcription and cDNA array analysis using the whole mouse cDNA array. Shown are genes involved in Wnt signaling and/or carcinogenesis which are down-regulated in KO macrophages using KEGG Pathway Database analysis. Heat map shows raw expression levels of 3 independent samples from each of the WT and KO cells. Scales represent the intensity value of each spot over the mean of the intensity of each gene from WT BMDMs in triplicates. B, and C, YAMC cells were treated with the following cytokines: 5 ng/ml IL-1 β , 10 ng/ml IL-17A, and 1 ng/ml TNF α for 48 hours, alone or in combination. Cells were then analyzed for EMT markers by immunoblot (B). Invasion assay was performed by a Transwell assay (C). Western blot represents data from 3 experiments. Invasion assay was performed in triplicate. Error bars represent standard deviation.