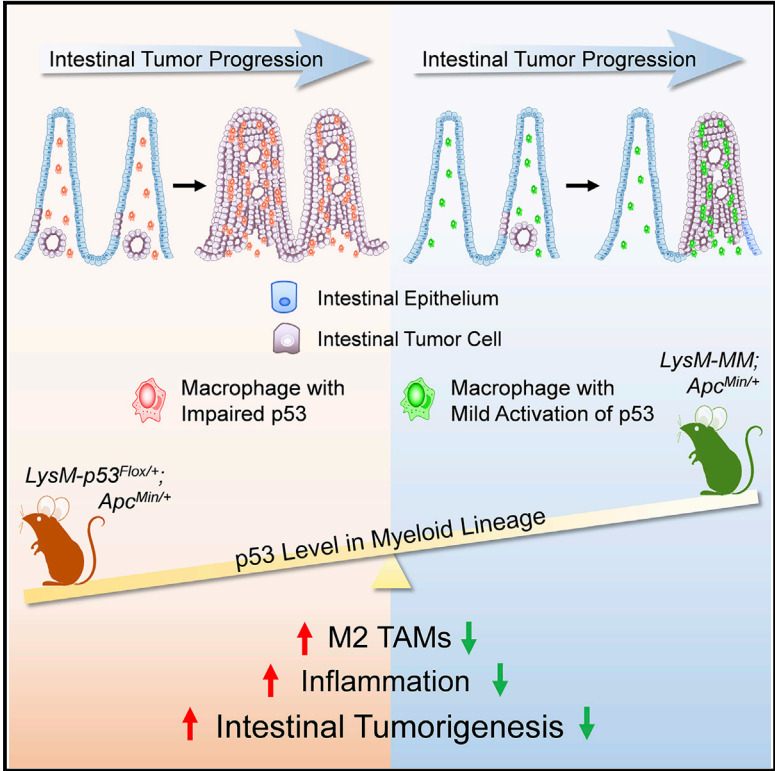# Cell Reports

p53 in the Myeloid Lineage Modulates an Inflammatory Microenvironment Limiting Initiation and Invasion of Intestinal Tumors

Graphical Abstract

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In Brief

He et al. show that p53 in the myeloid lineage plays critical roles in modulating the tumor microenvironment and suppressing intestinal tumor development in mice.

Highlights

- Impaired p53 in myeloid linage stimulates initiation of adenomas in $Apc^{Min/+}$ mice

- Mild p53 activation in the myeloid linage attenuates tumor development and invasion

- p53 attenuates the inflammatory response and alternative polarization of macrophages

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CellPress

p53 in the Myeloid Lineage Modulates an Inflammatory Microenvironment Limiting Initiation and Invasion of Intestinal Tumors

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SUMMARY

Chronic inflammation promotes the development and progression of various epithelial tumors. Wild-type p53 suppresses inflammation, but it is unclear whether the role of p53 in suppression of inflammation is linked to its tumor suppression function. Here, we established mouse models of myeloid lineage-specific p53 deletion or activation to examine its role in inflammation-related intestinal tumorigenesis. Impaired p53 in the myeloid linage resulted in elevated levels of inflammatory mediators and stimulated adenoma initiation in $Apc^{Min/+}$ mice. In contrast, mice with mild p53 activation in the myeloid lineage attenuated the inflammatory response and were more resistant to intestinal tumor development and invasion, which were initiated through $Apc^{Min/+}$ mutation or carcinogen and promoted by colitis. Furthermore, p53 activation also suppressed alternative (M2) macrophage polarization together with c-MYC downregulation. Therefore, as a regulator of macrophage function, p53 is critical to protection against tumorigenesis in a non-cell-autonomous manner.

INTRODUCTION

Both clinical and experimental studies have established that chronic inflammation promotes development and/or progression of various epithelial tumors (Mantovani et al., 2008). Colitis-associated cancer (CAC) is a classic inflammation-driven cancer, and mouse models of CAC have proved valuable for understanding general mechanisms connecting inflammation and cancer. Immune cells infiltrating tumor stroma, such as tumor-associated macrophages (TAMs), engage in an extensive and dynamic crosstalk with tumor cells (Balkwill and Mantovani, 2001). Cytokines and chemokines, including interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), which are produced by activated immune cells (especially TAMs) in the tumor microenvironment, control the viability and growth of surrounding epithelial cells and their malignant derivatives through the necrosis factor κB (NF-κB) and STAT3 pathways (Bollrath and Greten, 2009).

As a major tumor suppressor that responds to stress signals such as DNA damage and oncogene activation, p53 activation promotes cell-cycle arrest, apoptosis, or senescence (Vousden and Prives, 2009). Dysfunctional p53 signaling or p53 mutations are present in over 80% of all human cancers, including colorectal cancer (Lozano, 2007). Both MDM2 and MDM4 are negative regulators of p53, exhibiting non-overlapping and synergistic roles in inhibiting p53 (Terzian et al., 2007). Under cellular stress, p53 activation is achieved through disruption of the p53-MDM2/MDM4 interaction. As a small-molecule antagonist of the p53-MDM2 protein interaction, Nutlin-3 has been shown to have activity against human xenografts in preclinical models (Vassilev et al., 2004). Moreover, restoration of p53 function in p53-deficient tumors or increasing p53 activity in p53 wild-type tumors has been used to help develop p53-based anticancer treatments (Lane et al., 2010).

Although the cell-autonomous function of p53 on tumor suppression has been extensively studied, a few studies also suggest that stromal p53 has an inhibitory effect on cancer growth (Kiaris et al., 2005; Schauer et al., 2013), angiogenesis (Nishizaki et al., 1999), and invasion and metastasis (Kang et al., 2009; Moskovits et al., 2006). In a mouse model of prostate cancer, epithelial tumorigenesis imposes a strong selective pressure for loss of p53 in tumor-associated fibroblasts (Hill et al., 2005). Furthermore, ablation of p53-dependent senescence in hepatic stellate cells increases cirrhosis and reinforces the transformation of adjacent epithelial cells into hepatocellular carcinoma (Lujambio et al., 2013). Therefore, it seems that p53 could render tumor microenvironment less conducive for tumor development.

A number of reports point to the potential involvement of p53 in inflammation control in processes including collagen-induced arthritis (Yamanishi et al., 2002) and bleomycin-induced lung fibrosis (Ghosh et al., 2002). Higher levels of induction of various pro-inflammatory factors were found in macrophages from *p53*-null mice (Zheng et al., 2005). Mechanisms of p53 in control of immunity may involve its direct repression of promoter activity of NF-κB subunit p65, as well as negative regulation of the transcriptions of NF-κB-dependent genes, including *Il-6*, *Cox-2*, *Nos2*, and others (Cooks et al., 2014).

It remains to be determined whether the role of p53 in inflammation is involved in modulating the tumor microenvironment and tumor suppression. In this study, we established genetic

mouse models with p53 deletion or activation specifically in the myeloid lineage during intestinal tumorigenesis. Our results reveal a functional role for p53 in tumor suppression in a non-cell-autonomous manner through modulation of the tumor inflammatory microenvironment.

RESULTS

p53 Deficiency in Myeloid Lineage Accelerated Tumorigenesis in $Apc^{Min/+}$ Mice

In order to study the potential role of p53 in myeloid lineage on mouse intestinal tumorigenesis, we first deleted *p53* in myeloid lineage by crossing *LysM-Cre* (Clausen et al., 1999) and *p53^{Flox/Flox}* (Jonkers et al., 2001) mice. Recombination of *p53* allele was present in peritoneal exudate cells (PECs) from *LysM-Cre; p53^{Flox/+}* (*LysM-p53^{Flox/+}*) mice (Figure S1A). With deletion efficiency at around 80% (Figure S1B), *LysM-p53^{Flox/Flox}* mice developed normally and did not exhibit overt phenotypes up to 4 months of age (Figure S1C). However, higher expression of inflammatory cytokines and *Nos2* both in naive PECs (Figure 1A) and PECs under stimulation of lipopolysaccharide (LPS) (Figure 1B) were found in *LysM-p53^{Flox/Flox}* mice, as compared with the *p53^{Flox/Flox}* control littermates. Next, we crossed *LysM-p53^{Flox/Flox}* mice with the adenoma prone *Apc^{Min/+}* mice (Moser et al., 1990). Strikingly, at 100 days of age, loss of one allele of *p53* in myeloid lineage was able to increase the total number of intestinal adenomas by nearly 2-fold (Figures 1C and 1D). Higher tumor burdens in *Apc^{Min/+}; LysM-p53^{Flox/+}* mice were found throughout the small intestine covering proximal to distal regions (Figure 1E). A small cohort of *Apc^{Min/+}; LysM-p53^{Flox/Flox}* mice examined also exhibited an increased adenoma incidence in the small intestine, similar to *Apc^{Min/+}; LysM-p53^{Flox/+}* mice (Figure 1D).

Although tumor size distribution, tumor cell proliferating rate, and macrophage infiltration were comparable between 100-day-old *Apc^{Min/+}; p53^{Flox/+}* and *Apc^{Min/+}; LysM-p53^{Flox/+}* mice (Figures S1D–S1F), quantification of microadenomas in 4- to 5-week-old mice indicated much higher adenoma initiation in *Apc^{Min/+}; LysM-p53^{Flox/+}* mice (Figure 1F), possibly accounting for their increased tumorigenesis observed at 3–4 months of age. In addition, markedly increased expression of mRNAs for inflammatory cytokines and chemokine was found in tumors from *Apc^{Min/+}; LysM-p53^{Flox/+}* mice (Figure 1G).

Collectively, impaired p53 in myeloid lineage promoted elevated level of inflammatory cytokines and led to enhanced adenoma initiation and higher tumor burdens in *Apc^{Min/+}* mice.

Mild p53 Activation in Myeloid Lineage Attenuated Tumor Progression in $Apc^{Min/+}$ Mice

Under chronic inflammatory conditions such as ulcerative colitis (UC), p53 could be activated through phosphorylation at serine 15 (Hofseth et al., 2003). We also found myeloid cells with activated p53 in the stroma of UC tissues from mice treated with 3% of dextran sulfate sodium (DSS) (Figure S2A). Tissue-specific *Mdm2* and *Mdm4* double heterozygous mice can be used to model moderate p53 activation elicited by stress signals (Zhang et al., 2012). We therefore generated mice heterozygous for *Mdm2* in the *Mdm4^{+/−}* background (Grier et al., 2006; Xiong

et al., 2006) specifically in myeloid lineage. The recombined *Mdm2* allele could be detected in bone marrow of *LysM-Cre; Mdm2^{FM/+}; Mdm4^{+/−}* (*LysM-MM*) mice (Figure S2B). In addition, genetic recombination as indicated by X-gal staining in a *Rosa* 26 reporter background from these mice were found limited in the intestinal epithelial lumen where myeloid cells resided (Figure S2C) and in the PEC cells (Figure S2D). The *LysM-MM* mice were viable, with similar hemogram parameters and histological features compared with the control mice (Figures S2E and S2F). The number of total and mature macrophages was comparable with the *LysM-p53^{Flox/+}* or control mice (Figures S2G, S2H, and S2J). Fluorescence-activated cell sorting (FACS) analyses confirmed similar cell-cycle and survival status of these macrophages (Figure S2I). As expected, a moderate elevation of both p53 protein levels (Figure S2K) and the expression of its common downstream targets (Figure S2L) was found in PECs from the *LysM-MM* mice. Under LPS stimulation, the *LysM-MM* PECs exhibited reduced levels of IL-6, IL-1β, and IL-12p40 as well as mRNA level of *Nos2* (Figures S2M and S2N), suggesting anti-inflammatory roles for p53 activation in myeloid cells.

The *LysM-MM* mice did not exhibit any tumorigenic phenotypes in GI tract up to 13 months of age (Figure S3A). When these mice were crossed to the *Apc^{Min/+}* background, dramatic reductions (about 50%) in the number of intestinal adenomas were found in both the small intestine and colon of 2- to 4-month-old *Apc^{Min/+}; LysM-MM* mice, compared with the *Apc^{Min/+}; Mdm2^{FM/+}; Mdm4^{+/−}* (*Apc^{Min/+}* control) mice (Figures 2A–2C). On average, *Apc^{Min/+}* control mice died within 200 days of age from complications of intestinal tumors (Moser et al., 1990). In contrast, the overall survival of *Apc^{Min/+}; LysM-MM* mice was prolonged to 293 days (Figure 2D). β-Gal immunostaining indicated specific Cre-mediated recombination in the infiltrated myeloid cells of tumors from *Apc^{Min/+}; Rosa26; LysM-MM* mice (Figure S3B). Although adenoma initiation seemed to be similar in both genotypes of mice at 4–5 weeks of age (Figures 2E and 2F), an increase in small size tumors (<1 mm in diameter) and a decrease in larger size tumors (2–3 mm in diameter) were found in *Apc^{Min/+}; LysM-MM* mice at the age of 3–4 months (Figures 2G and S3C), suggesting that p53 activation affected tumor progression in this setting. The incidence and size of a tumor can be influenced by a balance of cell proliferation and death within the tumor mass. Higher apoptotic index (an 82% increase) and lower proliferative index (a 56% decrease) were found in the size-matched tumors from *Apc^{Min/+}; LysM-MM* mice, compared with *Apc^{Min/+}* control mice (Figures 2H and 2I). In addition, there were markedly reduced expressions of *Cox-2* as well as pro-inflammatory cytokines and chemokine in *Apc^{Min/+}; LysM-MM* tumors (Figure 2J). Immunofluorescence staining confirmed the attenuated COX-2 expression in the infiltrating macrophages from *Apc^{Min/+}; LysM-MM* tumors (Figure 2K). Similar frequency of myeloid cells infiltration in tumors (Figure 2K) also suggested that the differences in cytokine expression might not result from the difference of macrophage recruitment.

Therefore, p53-dependent signaling in myeloid cells was critically involved in the downregulation of critical pro-inflammatory cytokines and modifier genes, together with an inhibition of tumor growth in *Apc^{Min/+}* mice.
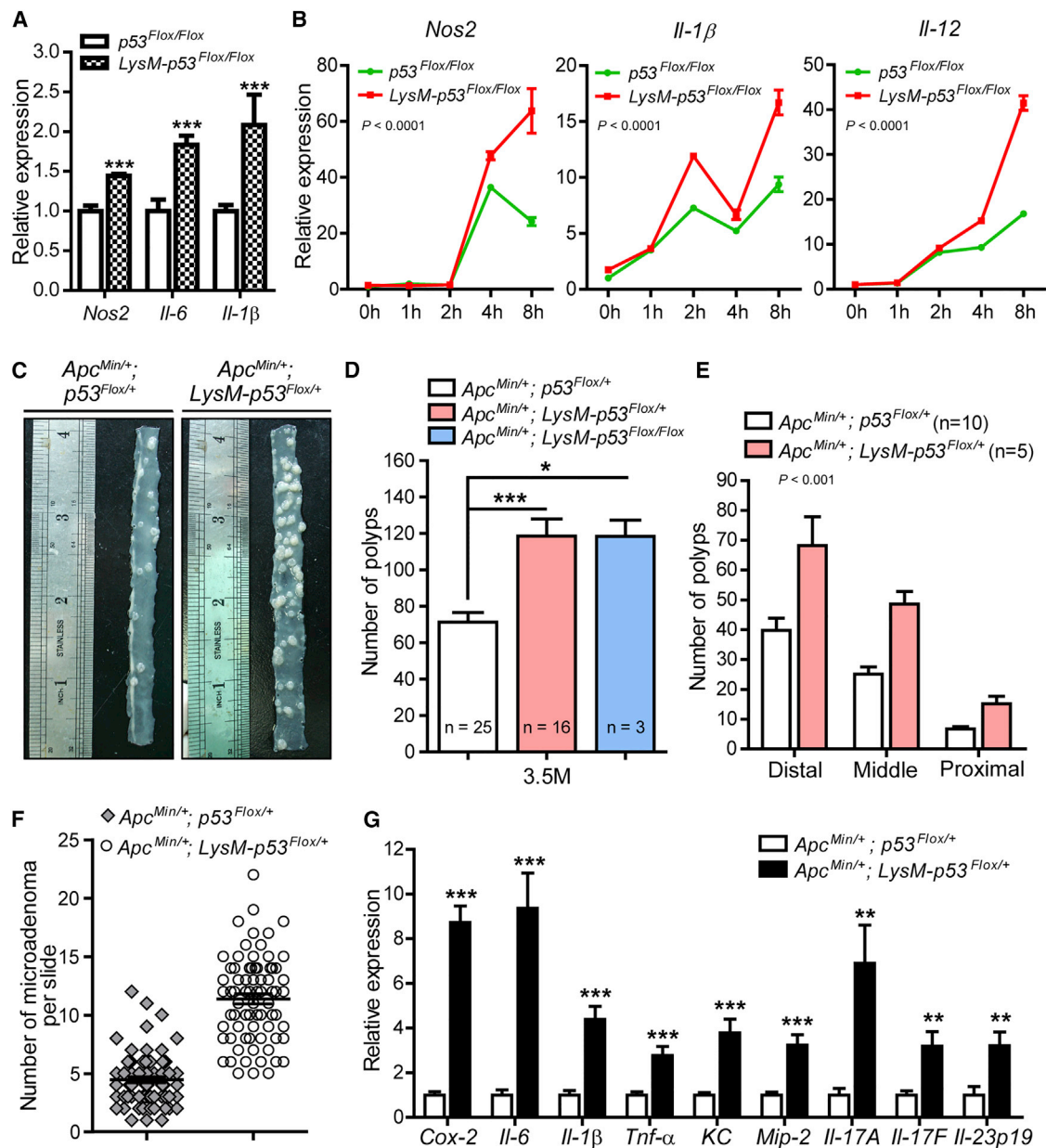
Figure 1. p53 Deficiency in Myeloid Lineage Accelerated Tumorigenesis in $Apc^{Min/+}$ Mice

(A) Relative gene expression normalized to $Hprt$ in naive PECs from $p53^{Flox/Flox}$ and $LysM$-$p53^{Flox/Flox}$ mice.

(B) Expressions of $Nos2$, Il-1β, and $Il12$ in LPS-stimulated PECs from $p53^{Flox/Flox}$ and $LysM$-$p53^{Flox/Flox}$ mice at different time points were normalized to $Hprt$. Values are means ± SEMs, p < 0.001 (two-way ANOVA).

(C) Representative small intestinal segments with visible polyps from $Apc^{Min/+}$; $p53^{Flox/+}$ and $Apc^{Min/+}$; $LysM$-$p53^{Flox/+}$mice at 100 days of age.

(D) Quantifications of polyps on longitudinal sections throughout small intestines. Values are means ± SEMs, *p < 0.05; ***p < 0.001 (one-way ANOVA with Dunnett's multiple comparisons).

(E) Numbers of the polyps in $Apc^{Min/+}$; $p53^{Flox/+}$ and $Apc^{Min/+}$; $LysM$-$p53^{Flox/+}$ mice stratified by small intestinal region. Values are means ± SEMs, p < 0.001 (two-way ANOVA).

(F) Microadenomas were quantified over H&E-stained sections from 4- to 5-week-old mice throughout the intestine.

(G) Relative mRNA expressions in size-matched polyps of 100-day-old mice. Values are means ± SEMs, **p < 0.01; ***p < 0.001 (t test).

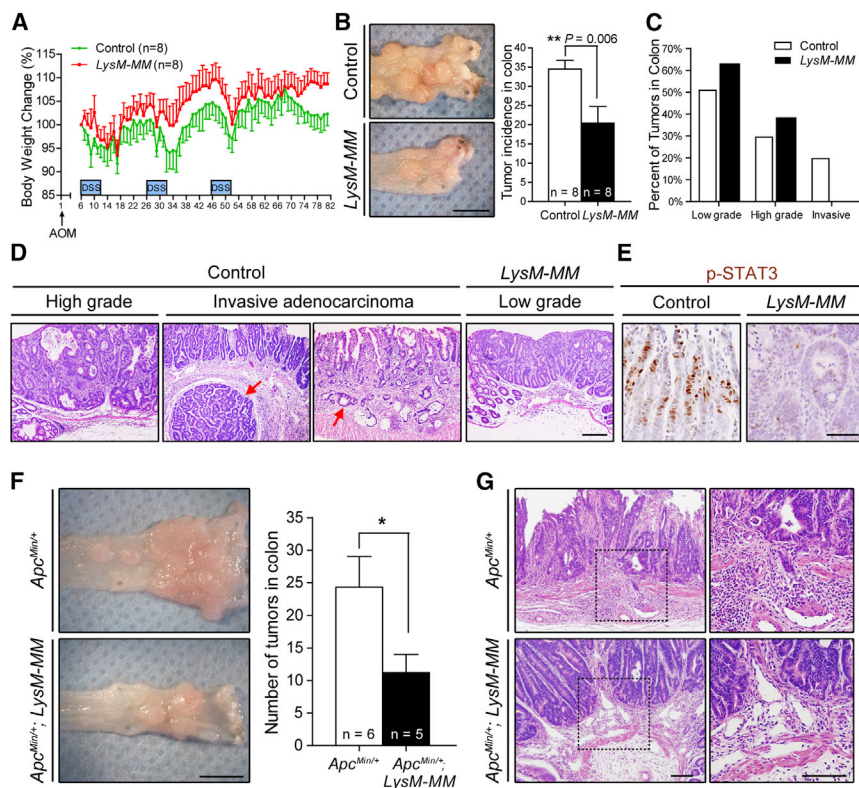See also Figure S1.
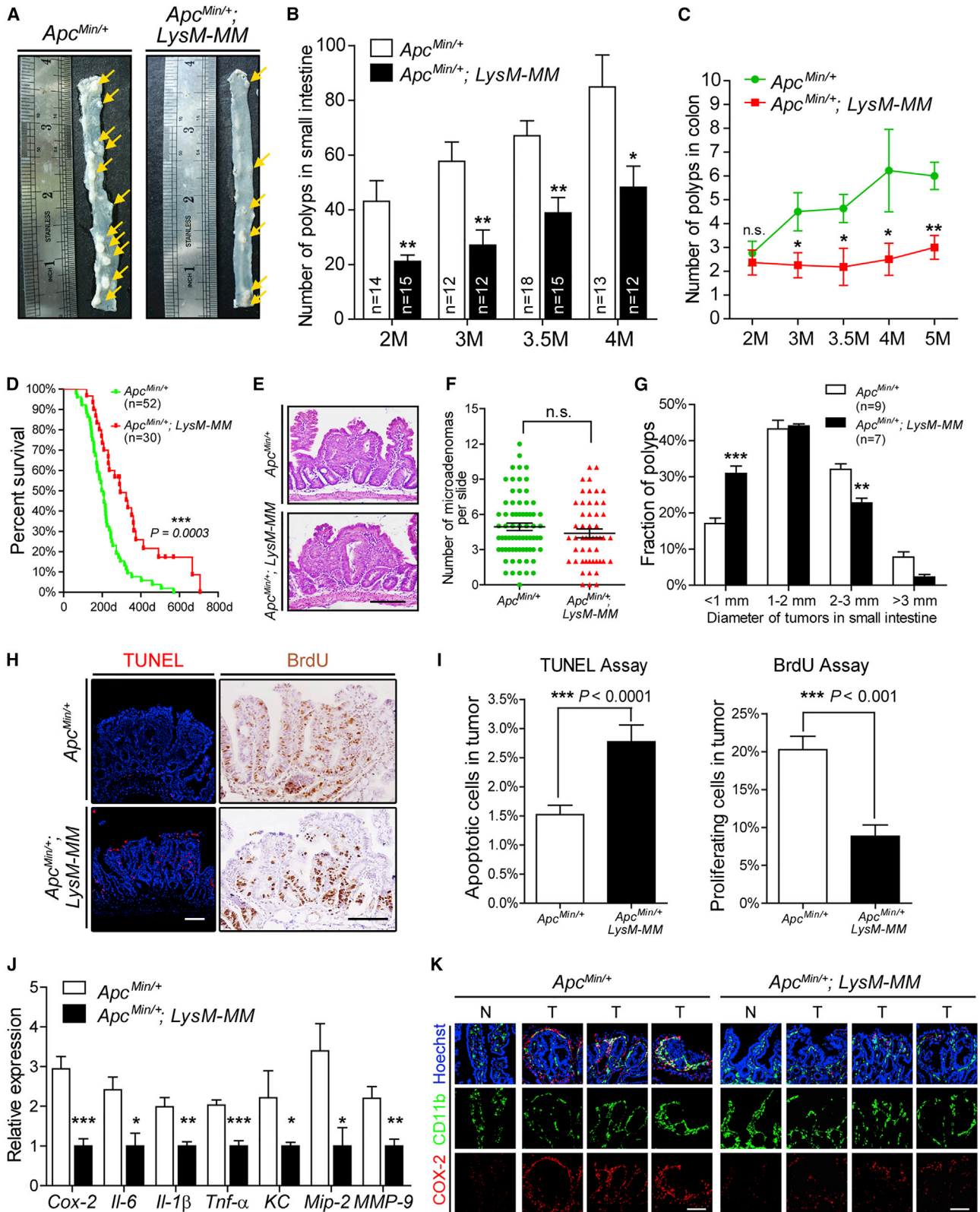
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Figure 3. *LysM-MM* Mice Were More Resistant to Colitis-Associated Colorectal Tumorigenesis

(A) Daily body weight of control and *LysM-MM* mice during AOM/DSS treatment.

(B) Macroscopic view of distal colons and total colorectal tumor incidence at the end of AOM/DSS treatment (day 80). Scale bar, 5 mm. Values are means ± SEMs, p = 0.006 (t test).

(C) Grading of tumors in each genotype. Note a lack of invasive adenocarcinomas in *LysM-MM* mice.

(D) Representative images of graded colorectal tumors of control and *LysM-MM* mice. Arrows indicated invasive adenocarcinomas. Scale bar, 200 μm.

(E) Immunostaining of phspho-STAT3 on the AOM/DSS-induced tumors in control and *LysM-MM* mice. Scale bar, 50 μm.

(F) Macroscopic view and tumor incidence in colons of DSS-treated $Apc^{Min/+}$ control and $Apc^{Min/+}$; *LysM-MM* mice. Scale bar, 5 mm. Values are means ± SEMs, p = 0.0247 (t test).

(G) Images of colon adenocarcinomas (left) and their submucosa (right, boxed area in left). Scale bar, 100 μm.

See also Figure S4.

LysM-Cre; Mdm2^{FM/+}; Mdm4^{+/−} Mice Were More Resistant to Colitis-Associated Colorectal Tumor Invasion

Inflammatory stimulus was a strong promoter following the initiating genetic alterations in colon carcinogenesis. We induced the CAC model by injecting a single dose of the procarcinogen azoxymethane (AOM), followed by repeated administrations of the irritant DSS on 6- to 8-week-old control and *LysM-MM* mice (Figure S4A) as previously reported (Greten et al., 2004). Repeated DSS administration promotes tissue damage and chronic inflammation, which greatly increases the incidence of AOM-induced tumors, thereby mimicking inflammatory bowel disease (IBD). Changes in body weight were monitored daily and colorectal tumor burdens were determined 80 days after the initial AOM treatment. Throughout the treatment, *LysM-MM* mice exhibited less body

weight loss relative to control mice (Figure 3A). By day 80, *LysM-MM* mice showed a lower frequency of colorectal tumors (a 40% decrease), accompanied with less enlarged spleens and mesenteric lymph nodes, as opposed to control mice (Figures 3B and S4C).

Histological examination and grading of tumors were performed over the full length of the H&E-stained colons (Figure S4B) according to the standards reported previously (Cooper et al., 2001). Among 71 tumors analyzed from control mice, 21 of them (29.6%) were determined as high-grade dysplasia, and 14 tumors (19.7%) were classified as invasive adenocarcinomas (Figures 3C and 3D, red arrows). In contrast, among 45 tumors in the colon of *LysM-MM* mice, although 17 (37.8%) were determined as high-grade dysplasia, none were found to be invasive (Figures 3C and 3D). Furthermore, pTyr-STAT3 staining, as an indicator of inflammation, was virtually absent from *LysM-MM*

Figure 2. Attenuated Tumor Progression in $Apc^{Min/+}$; *LysM-MM* Mice

(A) Representative small intestinal segments of $Apc^{Min/+}$ control and $Apc^{Min/+}$; *LysM-MM* mice at 3 months of age. Arrows indicated visible polyps.

(B and C) Quantifications of polyps on longitudinal sections throughout small intestine (B) and colon (C). Values are means ± SEMs. *p < 0.05; **p < 0.01 (t test).

(D) Long-term survival of the $Apc^{Min/+}$ control (n = 52) and $Apc^{Min/+}$; *LysM-MM* (n = 30) mice (p = 0.0003, Mantel-Cox/log-rank test).

(E and F) Representative morphology (E) and quantification (F) of microadenomas from 4- to 5-week-old mice. Scale bar, 100 μm.

(G) Size distribution of tumors from 3- to 4-month-old mice. Values are means ± SEMs. **p < 0.01; ***p < 0.001 (two-way ANOVA).

(H) TUNEL (left, red) and BrdU labeling (right) of tumors from 2- to 3-month-old $Apc^{Min/+}$ control and $Apc^{Min/+}$; *LysM-MM* mice. Scale bar, 100 μm.

(I) Apoptotic and proliferative indices in tumors measured by TUNEL and BrdU staining shown in (H), respectively. Values are means ± SEMs. ***p < 0.001 (t test).

(J) Relative mRNA expressions of inflammatory-related genes normalized to *Hprt* in size-matched polyps of $Apc^{Min/+}$; control and $Apc^{Min/+}$; *LysM-MM* mice (n ≥ 5) at 100 days of age. Values are means ± SEMs. *p < 0.05; **p < 0.01; ***p < 0.001 (t test).

(K) Representative immunofluorescence staining for CD11b (green) and COX-2 (red) in size-matched polyps (T) and normal intestinal epithelium (N) from 3-month-old $Apc^{Min/+}$ control and $Apc^{Min/+}$; *LysM-MM* mice. Scale bar, 100 μm.
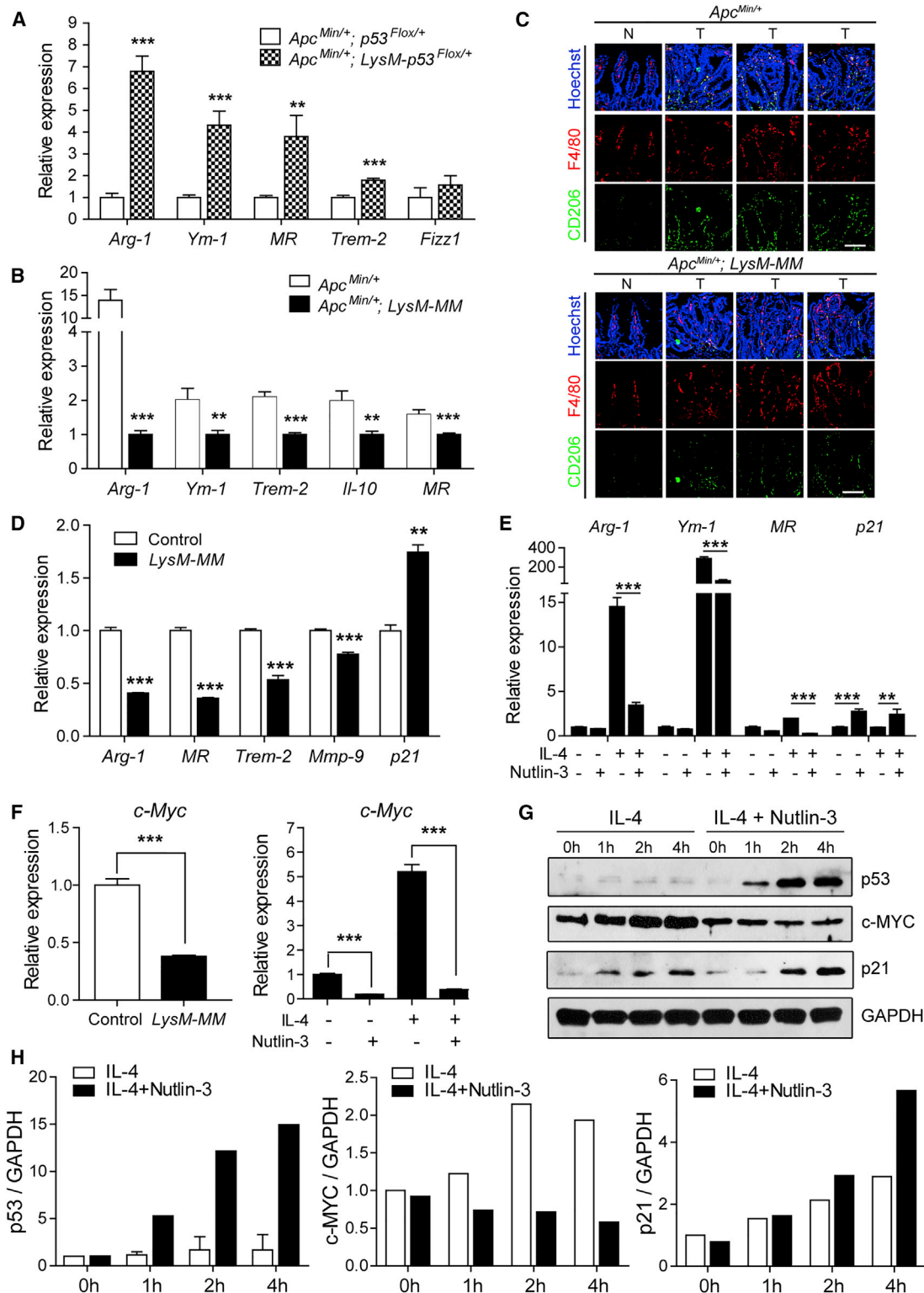
See also Figures S2 and S3.

Figure 4. p53 Status Influenced Alternative Activation of Macrophages

(A and B) Expression of M2-related genes in polyps was assessed by qRT-PCR. Size-matched polyps were from at least six mice in each group.

(C) Representative immunofluorescence staining for CD206 (green) and F4/80 (red) in size-matched polyps (T) and normal intestinal epithelium (N) from 3-month-old $Apc^{Min/+}$ control and $Apc^{Min/+}$; $LysM$-MM mice. Scale bar, 100 μm.

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tumors, in sharp contrast to the tumors of the control mice (Figure 3E).

Although *Apc^Min/+* mice develop benign tumors mainly in the small intestine, strong inflammatory stimuli such as DSS treatment promote the development and growth of colorectal neoplasms (Tanaka et al., 2006). Reminiscent of our findings in the AOM/DSS model, after 5 weeks of low-dose DSS treatment, a lower frequency of colorectal tumors appeared in DSS-treated *Apc^Min/+; LysM-MM* mice as compared to *Apc^Min/+* control mice (Figures S4D and 3F). Although invasive adenocarcinomas were found in four of six *Apc^Min/+* control mice examined, none of the tumors were found to be invasive in five *Apc^Min/+; LysM-MM* mice (Figure 3G). Tumor invasion was also not observed in the *Apc^Min/+; LysM-MM* mice under another treatment scheme of DSS (Cooper et al., 2001) (Figures S4E–S4G).

Collectively, these observations indicated that increased p53 signaling in myeloid lineage suppressed inflammation-promoted tumor progression and invasion.

Alternative Macrophages Polarization in Tumors Was Influenced by p53 Status

Besides the tumor promoting roles of the pro-inflammatory cytokines, it is also well known that some TAMs exhibited features of an alternatively activated (M2) phenotype, which is associated with tumor angiogenesis, progression, invasion, and evading immune surveillance (Erreni et al., 2011). We therefore examined whether p53 status in myeloid lineage might also modulate the M2 phenotypes of TAMs in the intestinal tumors. Indeed, when compared with the size-matched *Apc^Min/+; p53^Flox/+* tumors, genes associated with M2 phenotype including *Arg-1, Ym-1, MR,* and *Trem2* were significantly upregulated in tumors from *Apc^Min/+; LysM-p53^Flox/+* mice (Figure 4A). Conversely, the expressions of M2-related genes were reduced in *Apc^Min/+; LysM-MM* tumors (Figure 4B). The distribution of macrophages with M2 marker in *Apc^Min/+* polyps was further detected by immunostaining. F4/80 and CD206 double-positive macrophages were scarcely detected in the stroma of *Apc^Min/+; LysM-MM* polyps but infiltrated significantly into the stroma of *Apc^Min/+* control polyps (Figure 4C). Moreover, only minor staining of CD206 could be detected in the normal intestinal epithelium (Figure 4C).

To examine whether p53 activation in macrophages would directly retard an M2 phenotype in vitro, we stimulated PECs under M2-polarizing conditions. Induction of M2-associated genes was substantially downregulated in *LysM-MM* PECs after 24 hr of IL-4 treatment (Figure 4D). With the treatment of p53 small-molecule activator Nutlin-3, IL-4-induced mRNA expression of M2 markers was significantly suppressed (Figure 4E). c-MYC was recently recognized as a key regulator in alternative macrophage activation. c-MYC blockade in macrophages showed an inhibitory effect on the IL-4-dependent induction of about 45% of all M2 genes (Pello et al., 2012). Although strong induction

of *c-Myc* was observed after exposure to IL-4 in peritoneal macrophages, significant downregulation of *c-Myc* were found both in PECs from *LysM-MM* mice and in PECs administrated with IL-4 and Nutlin-3 (Figure 4F). Western blot analysis on RAW 264.7 cells confirmed a lower expression level of c-MYC protein accompanied with p53 activation (Figures 4G and 4H).

DISCUSSION

Tumor microenvironment contributes significantly to the development of tumors by affecting tumor initiation, progression, or metastasis, depending on specific contexts. The most obvious tumor promoting role is from macrophages, which have been manifested in inflammation-associated cancers. In this study, we report a functional role of p53 in tumor suppression in a non-cell-autonomous manner through modulating myeloid lineage cells associated with the tumor inflammatory microenvironment. Activation of p53 also downregulates c-MYC and suppresses alternative macrophage activation. Our genetic studies clearly demonstrate that p53 exerts important functions in the myeloid lineage, which is critical for protection against intestinal tumorigenesis.

Growing evidence supports that the pro-inflammatory cytokines are essential mediators of the origin cause of many cancers through the interactions between pre-neoplastic epithelium and surrounding activated immune cells (Bollrath and Greten, 2009). Breakdown of the protective intestinal barriers and chronic intestinal inflammation is intimately associated with tumorigenesis in the GI tract (Grivennikov et al., 2012). Persistent chronic inflammation could induce DNA damage and oncogenic mutations through intermediates such as reactive oxygen and nitrogen species (ROS and RNI) (Hussain et al., 2003). Another mechanism through which inflammation may trigger adenoma initiation is from elevated NF-κB signaling, which enhances Wnt activation and induces transformation and expansion of crypt stem cells that acquire tumor-initiating capacity (Schwitalla et al., 2013a). Of note, p53 mutations are enriched in inflamed colonic tissue well before neoplastic lesions become detectable (Hussain et al., 2000). Altogether, these inflammation-associated processes contribute to enhanced mutagenesis required for adenoma initiation. Previous studies have shown that high amounts of Nos2 (or iNOS) led to earlier tumor onset in mice with intestinal epithelial-cell-specific deletion of *Apc* (Shaked et al., 2012). Conversely, inhibition of Nos2 can attenuate formation of adenomas in *Apc^Min/+* mice (Ahn and Ohshima, 2001). Interestingly, p53 is known to inhibit Nos2 expression (Forrester et al., 1996). Here, we found much higher frequencies of microadenoma initiation at a very early stage in *Apc^Min/+; LysM-p53^Flox/+* mice. High expression of *Nos2* and the pro-inflammatory cytokines in naive PECs from *LysM-p53^Flox/Flox* mice as well as in tumors from *Apc^Min/+; LysM-p53^Flox/+* mice possibly contributed to the

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(D) mRNA expressions in PECs from control and *LysM-MM* mice under the treatment of IL-4 (20 ng/ml) for 24 hr.

(E) mRNA expressions of PECs from C57/BL6 mice under the treatment of Nutlin-3 (10 μM) and/or IL-4 (20 ng/ml) for 24 hr.

(F) *c-Myc* expression in peritoneal macrophages after exposure to IL-4 for 24 hr.

(G) Western blot analysis on RAW 264.7 cells under the treatment of IL-4 (20 ng/ml) with or without Nutlin-3 (10 μM).

(H) Quantifications of protein expression levels in (F) normalized against GAPDH.

Values are means ± SEMs, *p < 0.05; **p < 0.01; ***p < 0.001 (t test).

increased adenoma initiation. In addition, the antioxidant function of p53 (Sablina et al., 2005) may also help to maintain a redox balance in the microenvironment during early microadenoma initiation.

Aside from tumor initiation, chronic inflammation present during CAC development can influence all proposed stages of tumor progression and metastatic dissemination (Terzić et al., 2010). A great deal of evidence implicates p53 as playing an instrumental role in suppressing CAC. p53 mutations occur as an early genetic change in IBD-associated cancers (Brentnall et al., 1994). When exposed to DSS, mice harboring a germline p53 mutation develop severe chronic inflammation and persistent tissue damage and are highly prone to inflammation-associated colon cancer, with augmented NF-κB activation found in tumors (Cooks et al., 2013). Loss of p53 in intestinal epithelium also resulted in the formation of an NF-κB-dependent inflammatory microenvironment, induction of epithelial-mesenchymal transition (EMT) and further lymph node metastasis in a colorectal cancer mouse model (Schwitalla et al., 2013b). EMT can be influenced by NF-κB and/or STAT3 signaling and be regulated by various pro-inflammatory cytokines (Terzić et al., 2010). Therefore, NF-κB and STAT3 are instrumental in immune cells for the production of pro-inflammatory cytokines, which enforce a feedforward loop to activate STAT3 and NF-κB in invading cancer cells to stimulate their proliferation, survival, and metastatic spread (Bollrath and Greten, 2009). In our mouse models, activation of p53 in tumor microenvironment attenuated tumor proliferation and invasion in a non-cell-autonomous manner. While AOM/DSS-treated mice and DSS-treated $Apc^{Min/+}$ mice exhibited obvious tumor invasions, no tumor invasion was found in mice with activated p53 in myeloid lineage under the same treatments, which was accompanied with dampened activation of STAT3 pathways. Collectively, while the mutual interactions between tumor cells and their microenvironment seem to form "vicious cycles" to promote tumorigenesis, p53 may critically act in both cell compartments to attenuate the inflammatory response and alleviate such exacerbating effects.

Under certain circumstances, macrophages would be "re-educated" to alternative activated (M2) TAMs by tumor cells after infiltrating into the tumors (Hagemann et al., 2008) and further promote tumor progression in a number of experimental models. Here, consistent with a most recent report demonstrating the role of p53 in inhibiting the M2 phenotype (Li et al., 2015), we further demonstrated that the alternative polarization of macrophages in $Apc^{Min/+}$ tumors was also influenced by p53 status and might be another mechanism for p53 to modulate the tumor microenvironment. Therefore, the abilities of p53 to repress the expressions of cytokines and inflammatory-related molecules that may belong to either M1 or M2 categories suggested that p53 may play an important role in attenuating the inflammatory response and polarization of macrophages during tumorigenesis. Given the highly plastic, dynamic, or even mixed phenotypes of macrophages during tumorigenesis (Biswas and Mantovani, 2010; Martinez and Gordon, 2014), these results underscored the various inducers and the resulting complex phenotypes of the macrophages existed in the tumors and the impacts of p53 on these diverse set of macrophages. Recently,

clinical relevant tumor therapies are under development aiming to target both the pro-inflammatory-related molecules including TNF-α, IL-6, and IL-4-related M2 factors (Ruffell and Coussens, 2015). In this regard, p53 activation in macrophages may help to control the expressions of both types of genes and serve as a therapeutic option.

Taken together, our results suggest that p53 activation within cells of tumor microenvironment is able to inhibit tumor growth and malignant progression. Activation of stromal p53 by chemotherapy or radiotherapy might be part of the mechanisms by which these treatments cause cancer regression (Bar et al., 2010). Although additional studies are needed for more mechanistic insights on how p53 might influence the state and response in the tumor inflammatory microenvironment in more details, our findings here highlight a non-cell-autonomous tumor-suppressive function of p53. Importantly, our study suggested a possible development of therapeutic strategies in inflammation-associated cancer patients through pharmacological activation of p53 in tumor microenvironment.

EXPERIMENTAL PROCEDURES

Mice

Mice were bred and maintained under specific pathogen-free conditions, and experiments were conducted in accordance with the institutional animal care and use committee at Model Animal Research Center of Nanjing University. C57BL/6J-LysM-cre mice (Clausen et al., 1999) obtained from Jackson Laboratory were crossed to $Mdm4^{+/-}$; $Mdm2^{FM/FM}$ mice (Grier et al., 2006; Xiong et al., 2006) and $p53^{Flox/Flox}$ mice (Jonkers et al., 2001) from MMHCC. C57BL/6J-$Apc^{Min/+}$ mice (Moser et al., 1990) from Jackson Laboratory were crossed to LysM-cre; MM mice or LysM-cre; $p53^{Flox/Flox}$ mice. $p53^{Flox/Flox}$ mice were backcrossed to C57BL/6 background for at least five generations. $Mdm4^{+/-}$; $Mdm2^{FM/FM}$ mice were backcrossed to C57BL/6 background for four generations. To examine AOM/DSS-induced tumorigenesis, mice had been backcrossed to a FVB/NJ background for five generations. In all experiments, littermate controls were used.

Immunohistochemistry and Immunofluorescent Analyses

Paraffin-embedded slides were de-paraffinized and rehydrated. Antigen unmasking was performed by steaming slides in 10 mM sodium citrate buffer (pH 6.0) for 20 min. For phospho-Stat3 monoclonal antibody (Cell Signaling Technology), antigen retrieval was performed in Tris-EDTA buffer (pH 9.0). Frozen tissue sections were incubated with the following antibodies: anti-β-catenin (Cell Signaling Technology), anti-COX-2 (Maixin), anti-CD11b (eBioscience), anti-F4/80 (eBioscience), anti-Ki-67 (Dako), and anti-phospho-Ser-15-p53 (Cell Signaling Technology) overnight at 4°C. After washing the sections, secondary antibodies (Jackson ImmunoResearch) were used. Nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich). Fluorescence was evaluated using a Leica TCS SP2 confocal laser scanning microscope, or Olympus FV 1000 confocal microscope.

Statistical Analysis

Data are expressed as mean ± SEM of at least three independent experiments. Statistical analyses were carried out using GraphPad Prism 5 and 6 software. Specific tests used were unpaired t test, log-rank analysis, two-way ANOVA, and one-way ANOVA with Dunnett's multiple comparisons. All p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.045.

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