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Loss of p27Kip1 leads to expansion of CD4+ effector memory T cells and accelerates colitis-associated colon cancer in mice with a T cell lineage restricted deletion of Smad4

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ABSTRACT

The cyclin-dependent kinase inhibitor $p27^{Kip1}$ is a tumor suppressor whose intrinsic activity in cancer cells correlates with tumor aggressiveness, invasiveness, and impaired tumor cell differentiation. Here we explore whether $p27^{Kip1}$ indirectly influences tumor progression by restricting expansion and survival of effector memory T cell (T_{EM}) populations in a preclinical model of spontaneous colitis-associated colorectal cancer (CAC). We show mRNA and protein expression of $p27^{Kip1}$ to be significantly decreased in the colons of mice with a T cell-restricted deletion of the TGF-β intermediate, SMAD4 ($Smad4^{TKO}$). Loss of $p27^{Kip1}$ expression in T cells correlates with the onset of spontaneous CAC in $Smad4^{TKO}$ mice by 8 months of age. This phenotype is greatly accelerated by the introduction of a germline deletion of *CDKN1b* (the gene encoding $p27^{Kip1}$) in $Smad4^{TKO}$ mice ($Smad4^{TKO}/p27^{Kip1-/-}$, DKO). DKO mice display colon carcinoma by 3 months of age and increased mortality compared to $Smad4^{TKO}$. Importantly, the phenotype in DKO mice is associated with a significant increase in the frequency of effector CD4 T cells expressing abundant IFN-γ and with a concomitant decrease in $Foxp3^{+}$ regulatory T cells, both in the intestinal mucosa and in the periphery. In addition, induction of inflammatory mediators (IFN-γ, TNF-γ, IL-6, IL-1β, iNOS) and activation of Stat1, Stat3, and IκB is also observed in the colon as early as 1–2 months of age. Our data suggest that genomic alterations known to influence $p27^{Kip1}$ abundance in gastrointestinal cancers may indirectly promote epithelial malignancy by augmenting the production of inflammatory mediators from a spontaneously expanding pool of T_{EM} cells.

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths in western countries.[1] Genetic mutations involved in CRC influence cellular processes including proliferation, adhesion, apoptosis, and stem cell differentiation.[2–4] The majority of these gene mutations lead to activation of the Wnt pathway, with mutations in the adenomatous polyposis coli (*APC*) gene being the most common, including both germline mutations in familial CRC and acquired *APC* mutations that are found in at least two-thirds of sporadic cases of CRC. While sporadic mutations act principally in a tumor intrinsic manner, germline mutations leading to Wnt pathway activation could influence the proliferation and differentiated function of stromal cells in the tumor microenvironment (TME), and thereby act in a tumor extrinsic manner to promote tumor progression. Demonstrations of stromal APC haploinsufficiency support the notion that the consequences of Wnt pathway activation in stromal cells may be essential determinants of the cancer phenotype.[5]

An important molecular target of Wnt pathway activation in cancer cells is the cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$, a member of the Cip/Kip family of Cdk inhibitors.[6] Mitogen withdrawal, treatment of cells with TGF-β, and cadherin-mediated cell-cell contact each lead to increased $p27^{Kip1}$ binding to cyclin E/Cdk2 and cyclin A/Cdk2 complexes, and inhibition of G1/S progression *In vitro*.[7,8] The binding of $p27^{Kip1}$ to Cdk4 can also inhibit cyclin D/Cdk4 complex formation.[9,10] In human cancers, reduced $p27^{Kip1}$ expression is an unfavorable prognostic marker in tumors of the colon, stomach, breast, lung, prostate, and ovary.[11,12] Loss of $p27^{Kip1}$ within tumor cells is also correlated with increased tumor aggressiveness and depth of tumor cell invasion, as well as a poor state of differentiation.[13,14] In colon cancer, low $p27^{Kip1}$ expression is linked to a more advanced stage and to more poorly differentiated tumors.[15–18] Median five-year survival rates are also lower for CRC patients with reduced $p27^{Kip1}$ expression.[19,20] Relevant to the current study, low $p27^{Kip1}$ expression has also been reported in colitis-associated CRC (CAC); however, mechanisms underlying this loss of $p27^{Kip1}$ and its contribution to cancer development in CAC remain to be elucidated.[21]

These observations suggest the contribution of $p27^{Kip1}$ to the maintenance of the proliferation, differentiation, and function of stromal cells and mucosal immune cells is particularly important.[22] Abnormal CD4 T cell activation is associated with

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the development of colitis, which ultimately contributes to the pathogenesis of CAC. This aberrant effector $CD4^+$ T cell activity leads to tissue accumulation of pro-inflammatory cytokines that enhance the risk for mutations in oncogenes and tumor suppressor genes and contribute to genomic instability via various mechanisms.[23–25] Recent reports have demonstrated a central role for tissue-resident memory T cells (T_{RM} cells) as well as for intestinal memory T cell trafficking in the pathogenesis of inflammatory bowel disease (IBD) and CAC.[26,27] Additionally, a reduction in the number and function of regulatory T cells (Tregs) can contribute to the development of inflammatory disease and CAC progression in the gastrointestinal tract as a reduction in Treg frequency underlies the abnormal activation and expansion of pathogenic CD4 T cells.[24,28] Our group previously reported that $p27^{Kip1}$ deficiency reduces the requirement for CD28-mediated co-stimulation in naïve $CD8^+$ T lymphocytes and that $p27^{Kip1}$ acts in synergy with $p21^{Cip1}$ to alter the sensitivity of naïve T cells to TGF-β-mediated G1 arrest through modulation of IL-2 responsiveness.[29,30] Similarly, we have shown that a reduction in expression of the TGF-β-receptor-activated intermediate Smad3 cooperates with the loss of $p27^{Kip1}$ to promote spontaneous T-cell leukemogenesis in mice.[31] More recently, others have shown a critical role for $p27^{Kip1}$ as a negative regulator of the proliferation and expansion of effector/memory subsets of both $CD8^+$ and $CD4^+$ T cells.[22,32–34] The impact of $p27^{Kip1}$ expression on T cell fate has also been evidenced by the increased disease severity observed in $p27^{Kip1}$ knockout ($p27^{Kip1-/-}$ or $p27^{KO}$) mice in models of experimental arthritis, a phenotype attributed to a reduction in $Foxp3^+$ Tregs.[35]

Here we explore the potential that stromal cell expression of $p27^{Kip1}$ might influence disease progression in an established model of CAC in which a T cell-restricted loss of the *SMAD4* gene ($Smad4^{co/co;Lck\text{-}cre}$, $Smad4^{TKO}$) leads to spontaneous CAC.[36] $Smad4^{TKO}$ mice exhibit mucosal epithelial hyperplasia that is accompanied by increased expression of Cyclin D1, pRB, PCNA, and by a significant reduction in the expression of $p27^{Kip1}$. Introduction of the $Smad4^{TKO}$ conditional deletion onto a background with a germline deletion of *CDKN1b*, the gene encoding $p27^{Kip1}$ led to rapid acceleration and increased severity of the cancer phenotype in mice harboring both deletions ($Smad4^{TKO}/p27^{Kip1-/-}$ or DKO). Mechanisms involved in this process include not only enhanced mucosal epithelial proliferation, but also a dramatic expansion of pathogenic effector $CD4^+$ T cells and a reduction in the frequency of mucosal Treg cells. These data provide evidence that the common genomic alterations in CRC may impact cancer phenotype through a tumor extrinsic function in stromal cells, and particularly lymphocytes, within the TME.

Methods and materials

Antibodies

Anti-$p27^{Kip1}$ (C-19), anti-$p21^{Cip1}$ (N-20), anti-phospho-iκB (B-9) and anti-iNOS (M-19) were purchased from Santa-Cruz biotechnology. Anti-phospho-Akt (Ser473) (#4060), anti-phospho-Rb (Ser807/811) (D20B12), anti-cyclin D1 (DCS6) (#2926), anti-PCNA (D3H8P), anti-phospho-Foxo1/3, anti-phospho-Stat1 (Tyr701) (58D6), and anti-phospho-Stat3 (Thyr705) (D3A7) were purchased from Cell Signaling. Anti-CD3, anti-CD28, anti-CD44, anti-CD62L, anti-IFN-γ, and anti-TNF-α were purchased from BD Biosciences. Anti-Foxp3 antibody was purchased from eBioscience. Anti-Ki-67 was purchased from Abcam.

Animals

T cell-restricted deletion of the *SMAD4* gene ($Smad4^{co/co;Lck\text{-}cre}$, $Smad4^{TKO}$) in mice has been described previously.[36,37] The model characterized by germline deletion of $p27^{Kip1}$ ($p27^{Kip1-/-}$, $p27^{KO}$) was kindly provided by Dr. Koff (Memorial Sloan-Kettering, New York, NY).[38] The $p27^{KO}$ mice express a truncated 20-kDa protein that is devoid of any cyclin/Cdk inhibitory activity. To generate mice deficient for both $p27^{Kip1}$ germline and for Smad4 in the T cell lineage only, $p27^{KO}$ males ($p27^{KO}$ females are infertile) were crossed with $Smad4^{TKO}$ females. The resulting F1 heterozygotes were then bred to generate all genotypes. Mice were housed in a pathogen-free facility. All animal experiments were performed in accordance with institutional guidelines and with approval of the Institutional Animal Care and Use Committee at Case Western Reserve University.

Assessment of neoplasia and colitis

The colon was excised from the ileocecal junction to the anal verge, flushed with phosphate-buffered saline (Gibco), and opened longitudinally. Gross examination was performed to measure colon length and colon weight and to evaluate tumor size and number. The thickening of the intestinal mucosa was assessed by measurement of the colon length to colon weight ratio. The incidence (defined as the number of mice with tumors/total mice in the group), the mean number of tumors/mouse ± standard deviation, and the mean tumor size ± standard deviation were calculated for each group. Tumor size was determined by image analysis using imaging software (ImageJ). Images were taken with a scale bar and lengths were measured in pixels and correlated to the known distance in scale bars. Colonic tissues as well as colon tumors were processed for histopathological evaluation and further biochemical analyses.

Nitrite assay

Serum Nitric oxide (NO) levels were measured by photometric analysis by using a nitrite/nitrate assay kit (Cayman Chemical) according to the manufacturer's instructions.

Quantitative RT-PCR analysis

Colon mucosa was obtained from scrapings of full-length colon and total RNA was isolated using Trizol reagent (Invitrogen). For reverse transcription-PCR (RT-PCR), cDNA was synthesized using a High Capacity cDNA synthesis kit (Applied Biosystems). Quantitative RT-PCR was performed using a BioRad CFX96 Real-Time System C1000 Thermal Cycler. The expression of target genes was normalized to the

expression of housekeeping gene β-actin. The relative gene level was expressed as $2^{-\Delta\Delta Ct}$, in which ΔΔCt equals ΔCt of the experimental sample ($p27^{KO}$, $Smad4^{TKO}$, or DKO mouse sample) minus ΔCt of the control sample (WT mouse sample).

Western blotting

For Western blot, colon mucosa was obtained from scrapings of full-length colon and lysed by incubation in lysis buffer (150 mM NaCl, 20 mM Tris-Cl, pH 7.5, 1 mM PMSF, 1 mM Na3VO4, 25 mM NaF, 1% aprotinin, 10 μg/ml leupeptin) on ice for 30 min. About 20 μg aliquots of proteins were separated by electrophoresis in 10% SDS/PAGE minigels and transferred to nitrocellulose membrane (Invitrogen). Following blocking, membranes were incubated in a buffer containing the primary antibody, followed by washing and incubation for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies. Immunostaining was visualized by ECL.

Histology

For hematoxylin and eosin staining (H&E), excised colons were washed with PBS and fixed in 10% formalin. Samples were embedded in paraffin wax, sectioned, stained with H&E, and examined by light microscopy. For immunohistochemistry (IHC), slides were deparaffinized, and rehydrated and heat-induced epitope retrieval was performed prior to blocking with Peroxidazed 1 (BioCare, PX968) and Rodent Block M (BioCare, RBM961). Slides were incubated with primary antibodies, Ki-67, $p27^{Kip1}$, and CD3 for 1 hour at room temperature. Antibodies were detected using Rabbit-on-Rodent HRP polymer (BioCare, RMR622) and visualized using the Betazoid DAB chromogen kit (BioCare, BDB2004). The percentage of Ki67 positive cells were assessed on six randomly selected field using a digital eyepiece.

FACS analysis

Cell suspensions were prepared from spleens or colon lamina propria by filtering through a nylon mesh (40-μm diameter). Erythrocytes were lysed using ACK lysis buffer (BioWhittaker) and cells were washed twice in RPMI 1640 supplemented with 10% heat-inactivated FBS, 50 μM 2-ME, penicillin, and streptomycin (GIBCO/Life Sciences). Viable cells were counted using trypan blue exclusion and a hemocytometer. All Antibodies used in FACS analyses were purchased from BD Pharmingen. Pan T cells were purified from the spleen and lymph node using a Pan T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions (purity greater than 95%).

Regulatory T cell induction and intracellular cytokine staining

Splenocytes from each genotype were activated *in vitro* by plate-bound anti-CD3 and anti-CD28 antibodies in 24-well plates, in the absence or presence of TGF-β for 72 h. The cells were harvested, washed with PBS, stained with anti-CD4 and CD8 antibodies, and stained for intracellular Foxp3 using a Foxp3 staining Kit (eBioscience) according to the manufacturer's instructions. For intracellular staining for cytokines, lymphocytes from spleen and colon lamina propria were activated with plate-bound anti-CD3 and anti-CD28 antibodies for 48 h and re-stimulated with PMA and ionomycin for the last 5 h in the presence of Golgi stop solution prior to washing and staining with antibodies for CD4 and CD8. Intracellular staining for IFN-γ and TNF-α were performed using an intracellular staining kit (BD Biosciences) according to the manufacturer's instructions.

Statistical evaluation

Data are expressed as means ± SE. Statistical significance was determined by 1-way ANOVA with Tukey–Kramer Multiple Comparisons Test. The Fisher's Exact Probability test was used for comparison of the incidence of lesions between the two groups. Statistical significance was accepted to be a p-value less than or equal to 0.05, with $^{*}p< .05$, $^{**}p< .01$, $^{***}p< .001$.

Results

Proliferation of intestinal epithelial cells is increased while $p27^{Kip1}$ expression is suppressed in the mucosal epithelial cells and T cells in the colon of $Smad4^{TKO}$ mice

We previously established a novel murine model of colitis-associated colorectal cancer (CAC) through the creation of a T cell-specific deletion of the Smad4 gene in mice.[36] In this model, selective loss of Smad4-dependent signaling in T cells ($Smad4^{co/co;Lck\text{-}cre}$, Smad4TKO) leads to spontaneous intestinal inflammation-induced cancer throughout the gastrointestinal tract. Smad4TKO mice invariably develop CAC after 8 months of age, with inflammatory cell infiltration of the mucosa, loss of body weight, and bloody diarrhea. To examine the status of cell proliferation in mice exhibiting CAC symptoms, we evaluated Ki-67 expression by immunohistochemistry (IHC) analysis of colon tissue. Ki-67 positive cells in the intestinal crypts of $Smad4^{TKO}$ mice (85%) were significantly greater than that of wild type (WT) mice (40%) at 6 months of age (Figure 1a). Increased proliferation of colon epithelial cells in Smad4TKO mice correlated with an increase in expression of the proliferating cell nuclear antigen (PCNA) in the colonic mucosa of Smad4TKO mice as determined by Western blot analysis (Figure 1b). Increased mucosal epithelial cell proliferation is also associated with a consistent, significant increase in the expression of Cyclin D1 and an abundance of the phosphorylated retinoblastoma protein (pRb) (Figure 1b). Synthesis of cyclin D is initiated during the G1 phase of the cell cycle and drives the G1/S phase transition, a step tightly regulated by the Rb protein. These data indicate that epithelial cell proliferation in the colon of Smad4TKO mice is increased relative to that of WT mice and is associated with changes in expression of cell-cycle regulatory proteins that act to enhance G1-S phase transition.

The CIP/KIP family proteins $p21^{Cip1}$ and $p27^{Kip1}$ have essential roles regulating the proliferation, differentiation, and viability of mucosal epithelial cells.[39–41] Interestingly, while we found no change in the abundance of $p21^{Cip1}$ expression in the colonic mucosa of $Smad4^{TKO}$ mice relative to WT control mice, there was a significant reduction in the level of $p27^{Kip1}$

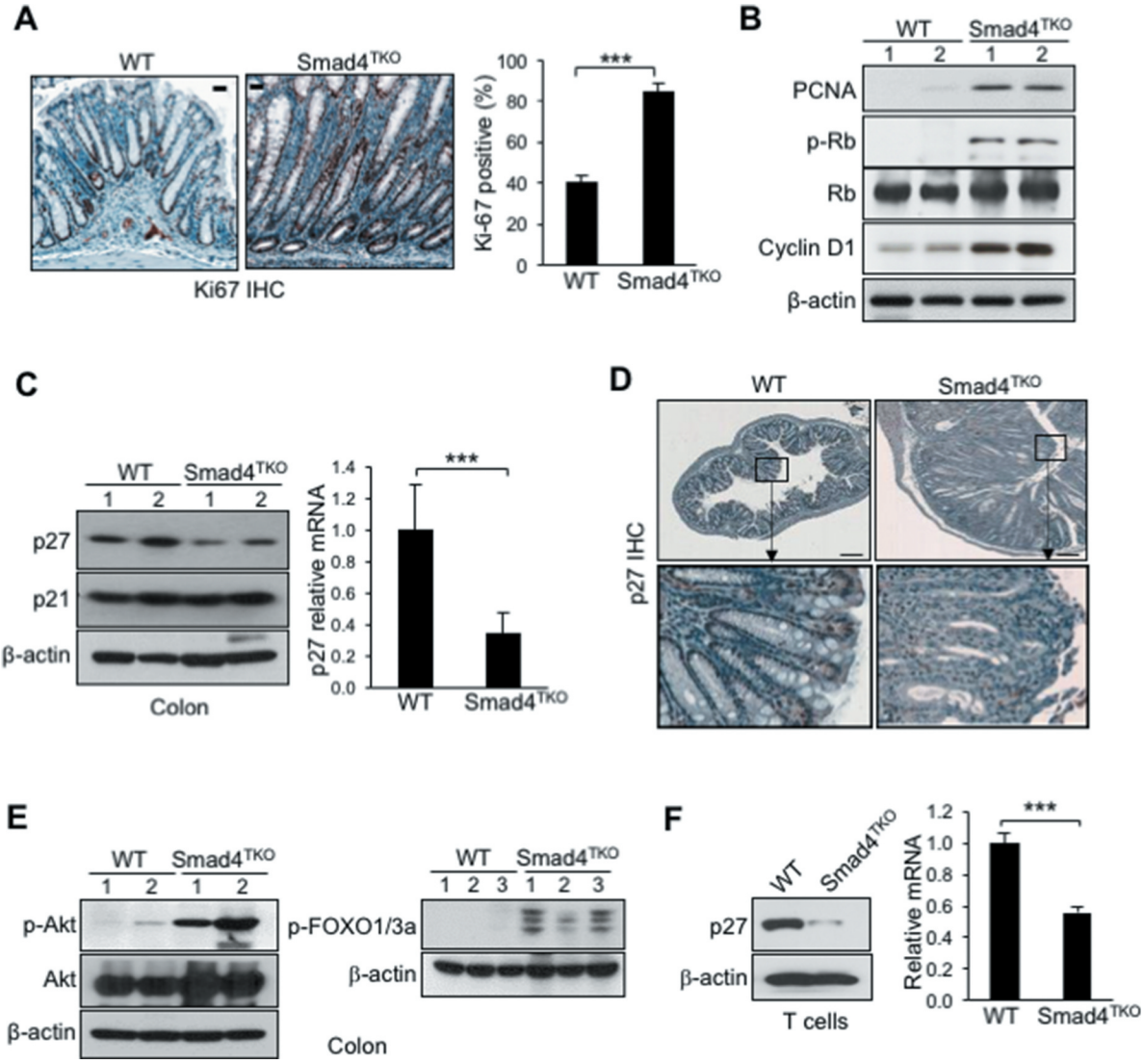

Figure 1. Proliferation of mucosal epithelial cells is increased and $p27^{kip1}$ expression is decreased in the colon and T cells of $Smad4^{TKO}$. A) Immunohistochemistry (IHC) analysis for Ki-67 in the colon of wild type (WT) and $Smad4^{TKO}$ mice. The percentage of Ki-67 positive cells among all colon epithelial cells was determined in the colon of WT and $Smad4^{TKO}$ mice at 6 months of age. Data represent the average ± S.E. (n = 9). Scale bar = 50 μm. B) Expression of PCNA, phospho-Rb, Rb, Cyclin D1, and β-actin was measured by Western blot. The results shown are representative of four separate experiments. C) Protein expression of $p27^{kip1}$ and $p21^{cip1}$ was examined in the colon of WT and $Smad4^{TKO}$ mice at 6 months of age by Western blot analysis. $p27^{kip1}$ mRNA expression was examined in the colon of WT and $Smad4^{TKO}$ mice at 6 months of age by real-time PCR analysis. D) Localization of $p27^{kip1}$ protein in the colon of WT and $Smad4^{TKO}$ mice at 6 months of age were examined by IHC analysis. Scale bar = 200 μm. E) Phospho-Akt and Phospho-FOXO1/3a were measured in the colon of WT and $Smad4^{TKO}$ mice by Western blot analysis. β-actin was used as internal control. F) Protein expression of $p27^{kip1}$ as examined in the isolated T-cell from the spleen of WT and $Smad4^{TKO}$ mice at 6 months of age by Western blot analysis. $p27^{kip1}$ mRNA expression was examined in T cells isolated from the spleen of WT and $Smad4^{TKO}$ mice at 6 months of age by real-time PCR analysis. Results are representative of three independent experiments using pooled spleens from four mice per genotype. ***P < .001.

protein expression when compared to colonic mucosa from WT mice, as observed by Western blot (Figure 1c). A similar reduction in $p27^{Kip1}$ mRNA was also observed in the colon of $Smad4^{TKO}$ mice by real-time PCR (Figure 1c). To examine whether suppressed $p27^{Kip1}$ in the $Smad4^{TKO}$ mouse is localized to either epithelial cells or infiltrating lymphocytes, colon cross-sections were analyzed for intracellular $p27^{Kip1}$ expression by IHC. Normal colon tissue from WT mice demonstrated nuclear $p27^{Kip1}$ protein expression in terminally differentiated epithelial cells in the uppermost one-third of crypts as well as in the infiltrating lymphocytes in the lamina propria. These phenomena are markedly reduced in $Smad4^{TKO}$ mice (Figure 1d). Previous reports have shown that $p27^{Kip1}$ expression is transcriptionally regulated by the family of forkhead transcription factors (FOXO) which play an essential role in cellular proliferation, apoptosis, and differentiation.[42] PI3 kinase (PI3K) activates Akt through phosphorylation and activated Akt, in turn, phosphorylates FOXOs directly, which results in their nuclear exclusion and inhibition of FOXO mediated gene expression.[43] To investigate the mechanism underlying the decreased expression of $p27^{Kip1}$ in $Smad4^{TKO}$ mice, we examined the status of Akt and FOXO activation in the colon of $Smad4^{TKO}$ mice. Western blotting analysis shows high phosphorylation of Akt in the colons of $Smad4^{TKO}$ mice while there was no change in the total Akt protein expression, indicating activation of Akt (Figure 1e). Phosphorylation of FOXO1/3

was also observed in the colons of Smad4^{TKO} mice, but not in WT mice, indicating a mechanistic role for the PI3K-Akt pathway in suppression of p27^{Kip1} expression (Figure 1e).

This reduction in the expression of p27^{Kip1} was closely associated with the accumulation of mucosal T lymphocytes, as enumerated by CD3 immunoreactivity. The accumulation of CD3^{+} T cells in the mucosa of Smad4^{TKO} mice relative to WT mice indicates a link between the abundance of p27^{Kip1} expression in T cells and the development of the CAC phenotype observed in Smad4^{TKO} mice (Supplementary Figure 1). We investigated this relationship further by examining the expression of p27^{Kip1} in splenic T cells by Western blot and real-time PCR analysis. Protein expression of p27^{Kip1} was significantly decreased in T cells of Smad4^{TKO} spleen compared to that of WT mice, and decreased mRNA expression of p27^{Kip1} of Smad4^{TKO} was confirmed by quantitative RT-PCR (figure 1f). These results indicate that the reduction of p27^{Kip1} expression in T cells is an important event that contributes to the accumulation of mucosal T lymphocytes and that this reduction in p27^{Kip1} is associated with the pathogenesis of CAC in Smad4^{TKO} mice.

Germ line deficiency of p27^{Kip1} accelerates colitis-associated colorectal tumorigenesis in Smad4^{TKO} mice

Based on the data described above, we hypothesized that the loss of T cell expression of p27^{Kip1} undermines the maintenance of both intestinal epithelial and mucosal immune homeostasis. Therefore, to directly demonstrate the contribution of p27^{Kip1} in the pathogenesis of inflammation-driven colon cancer in Smad4^{TKO} mice, we introduced the lineage-restricted *SMAD4* deletion (Smad4^{TKO}) onto a background with a germline *CDKN1b* deletion (p27^{KO}) to generate a 'double knockout' (Smad4^{TKO}/p27^{KO}, DKO) model. DKO mice harboring the T cell-restricted deletion of the tumor suppressor *SMAD4* and a germline deletion of cell-cycle regulator *CDKN1b* developed CAC and inflammatory infiltration of the mucosa as early as 3 months of age, at which point the mortality rate of DKO mice began to increase. The survival rate of DKO was only 40% at the age of 5 months, compared to 98% for Smad4^{TKO} mice (Figure 2a). Clinical features of systemic illness (lethargy, hunched posture, disheveled fur) became evident by 3 months of age and necropsy studies at this age revealed significant gastrointestinal pathology as a cause of clinical symptoms. The colon (blue arrow) and duodenum (red arrow) of DKO mice were thicker than those of either the Smad4^{TKO} or p27^{KO} mice (Figure 2b) and the significant increase in colon thickness, as measured by colon weight-to-length ratio, was evident in DKO mice at 3 months of age (Figure 2c). Histological analysis by hematoxylin and eosin (H&E) staining of intestinal sections from DKO mice showed a clear difference in histopathology including the disrupted villus architecture with regions of epithelial atypia, as well as adenomas and invasive carcinomas compared to the colon histology of either the WT, p27^{KO}, or Smad4^{TKO} mice (Figure 2d). Necropsy and mucosal histology showed that DKO mice developed tumors in the colon at 3 months of age (Figure 2e), whereas the other genotypes had no lesions and displayed normal gastrointestinal villus architecture. At this age, tumor incidence in DKO mice was more than 80% (figure 2f). The average tumor size was 0.5 cm in DKO mice (Figure 2g) and tumor multiplicity was more than 6 tumors/mouse in DKO mice (Figure 2h). The phenotypic evaluation of DKO mice showed a similar but accelerated disease presentation as the Smad4^{TKO}, presenting at the earlier age of 3 months versus 8 months of age in Smad4^{TKO} mice, and clearly demonstrating that germline p27^{Kip1} deletion accelerates CAC development and tumorigenesis in the Smad4^{TKO} mice.

To evaluate the status of epithelial cell proliferation in symptomatic DKO mice, we examined intracellular Ki-67 staining by IHC. The percentage of Ki-67 positive cells in the intestinal crypts of DKO (74%) was significantly greater than that of wild type (WT) (32%), p27^{KO} (41%), and Smad4^{TKO} mice (35%) at 3 months of age (Figure 3a). We also found significantly elevated expression levels of PCNA protein in the colonic mucosa of DKO mice compared to those of WT, p27^{KO}, or Smad4^{TKO} mice at 3 months of age (Figure 3b). There was also significant phosphorylation of Rb protein in the colons of DKO mice at this young age (Figure 3b). As expected, we did not observe increased PCNA expression or Rb phosphorylation in Smad4TKO mice compared to WT mice. Our observations indicate that in DKO mice, the germline p27^{Kip1} deletion accelerates tumorigenesis in Smad4^{TKO} mice, in part, by increasing colonic epithelial cell proliferation.

Effector memory CD4 T cell population is increased by p27^{Kip1} deficiency in Smad4^{TKO} mice

While the data above confirm a known role for p27^{Kip1} regulating epithelial proliferation, the accelerated tumorigenesis in the DKO model may also be linked to a more rapid expansion of tissue-resident memory T cells, which are known to require TGF-β for their differentiation and accumulate in the mucosa of patients with IBD.[26,44] The function of CD4^{+} effector memory T (T_{EM}) cells in cancer is complex, and is often dysregulated within the TME.[24,25,45] Abnormal CD4 T cell activation is associated with the development of colitis, which ultimately contributes to the pathogenesis of CAC. Although p27^{Kip1} markedly limits the abundance of memory CD4 T cells, the relevance of p27^{Kip1} activity in T cells and the progression of CAC has not been explored. Thus, we examined the role of p27^{Kip1} in T cell differentiation and function in the Smad4^{TKO} mouse model of spontaneous CAC. We observed that the number of total spleen cells of DKO mice was significantly increased, compared with that of other genotypes (Figure 4a). We also observed a significant increase in both the percentage and number of total CD4^{+} T cells in the spleen of both p27^{KO} and DKO mice at 4 months of age, when compared with that of either WT or Smad4^{TKO} mice (Figure 4a). Populations of CD4^{+} T_{EM} cells expressing CD44^{High} and CD62L^{Low} were examined in the spleen from each genotype at 4 months of age by FACS analysis (Supplementary Figure 2a, b). The proportion of CD4^{+} T_{EM} cells in p27^{KO} mice was diminished when compared with that of WT mice, while the absolute number of memory CD4^{+} T cells was greater. Notably, both the proportion and the absolute number of CD4^{+} T_{EM} cells were greatly increased in the spleen of DKO mice, compared with that of either WT, p27^{KO}, or Smad4^{TKO} mice (Figure 4b). The population of

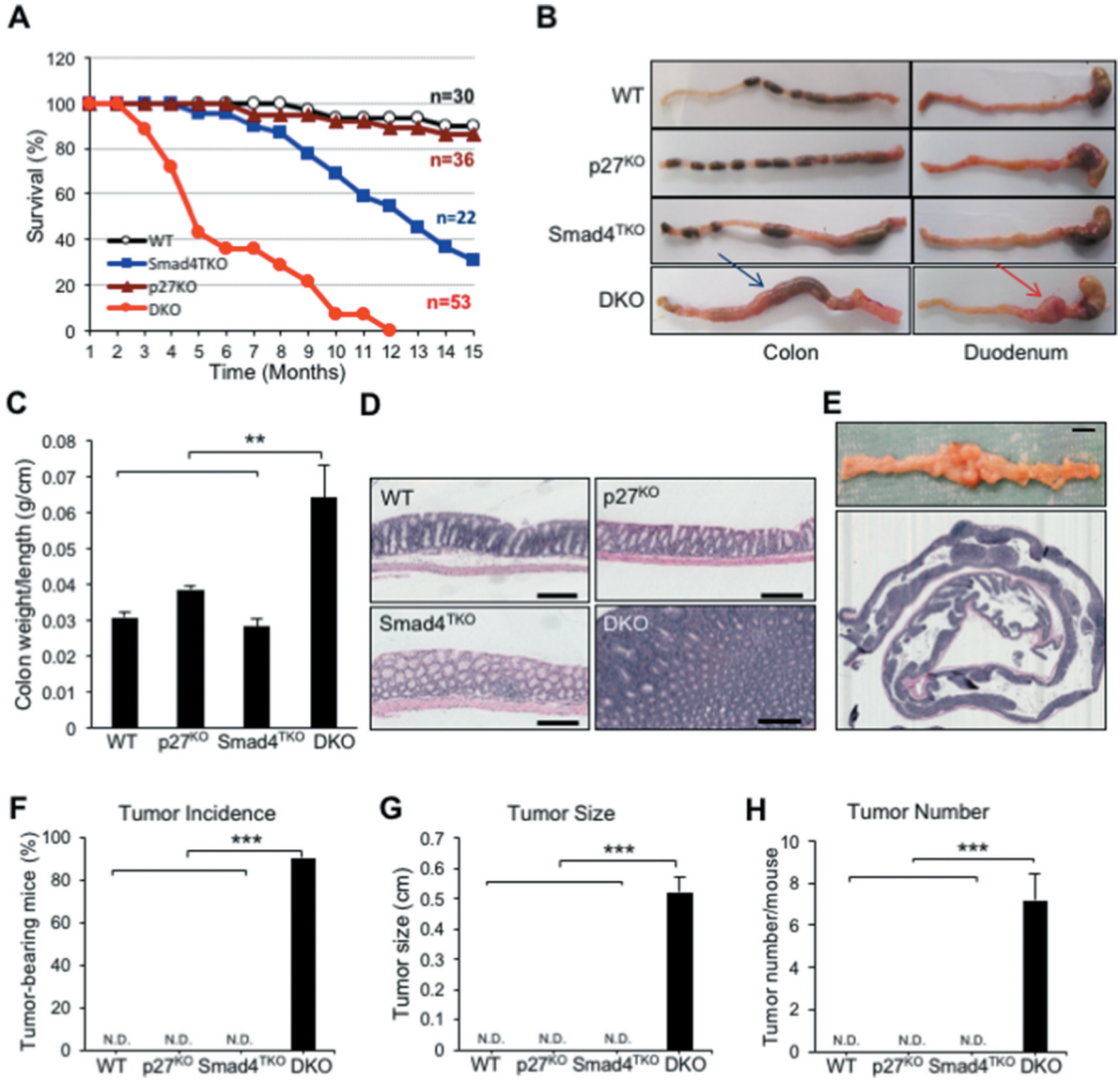

Figure 2. Deletion of p27^{kip1} in Smad4^{TKO} mice accelerates colitis-associated colon cancer. A) Survival curves of WT, Smad4^{TKO}, p27^{KO} and Smad4^{TKO}/p27$^{Kip1-/-}$ (DKO) mice. B) Photographs of colon, stomach and duodenum from each genotype at 3 months of age. C) Colon weight per length (g/cm) (n = 9). D) Hematoxylin and eosin (H&E) staining of the colon of each genotype at 3 months of age. Scale bar = 100 μm. E) Photograph and mucosal histology of the colon from DKO mice at 3 months of age. Paraffin-embedded sections were stained with H&E. Scale bar = 1 cm. F) Percentage of tumor-bearing mice (n = 10). G) Tumor size (n = 7). H) Tumor numbers per mouse at 3 months of age were determined using a digital eyepiece and an imageJ (n = 7). Error bars indicate S.E.; ***P < .001, **P < .01 compared with each genotype such as WT, p27^{KO} and Smad4^{TKO}.

CD4$^+$ T$_{EM}$ cells was not significantly increased in the spleen of Smad4^{TKO} at 6 months of age (Supplementary Figure 3a).

We next analyzed effector CD4$^+$ T cells producing pro-inflammatory cytokines known to promote genomic instability via various mechanisms. The number of effector T cells producing IFN-γ was increased in p27^{KO} and Smad4^{TKO} mice, compared with that of the WT group, while the proportion was not altered. Whereas in DKO mice, both the proportion as well as the number of effector T cells producing IFN-γ were greatly increased, compared with those of either WT, p27^{KO}, or Smad4^{TKO} mice at 4 months of age (Figure 4c, Supplementary Figure 2c). In addition, the population of TNF-α producing T cells was also slightly increased in the spleen of the DKO mice (Supplementary Figure 2c).

To specifically investigate the CD4$^+$ T$_{EM}$ cell population in the target organ of each genotypes, we further performed FACS analysis on colonic lamina propria from WT, p27^{KO}, Smad4^{TKO}, and DKO mice. CD3 immunohistochemistry analysis confirmed the substantial accumulation of T cells in the lamina propria and within the TME of the colons of DKO mice (Figure 4d). In addition, we also observed that the population of total CD4$^+$ T cells was significantly increased in the colonic mucosa of DKO mice, compared with that of other genotypes, while the percentage was increased in p27^{KO} and DKO mice (Supplementary Figure 2d). The proportion of CD4$^+$ T$_{EM}$ cells was greatly increased in the colonic mucosa of both the Smad4^{TKO} and DKO mice, compared with that of either WT or p27^{KO} mice (Figure 4e). The absolute number of the CD4$^+$ T$_{EM}$ cells was also greatly increased in DKO mice, compared with that of all other genotypes, including p27^{KO} mice, which similarly showed a significant increase in effector memory CD4$^+$ T cells when compared with WT mice. Taken together, these data suggest that p27^{Kip1} deficiency accelerates the expansion of a population of pathogenic

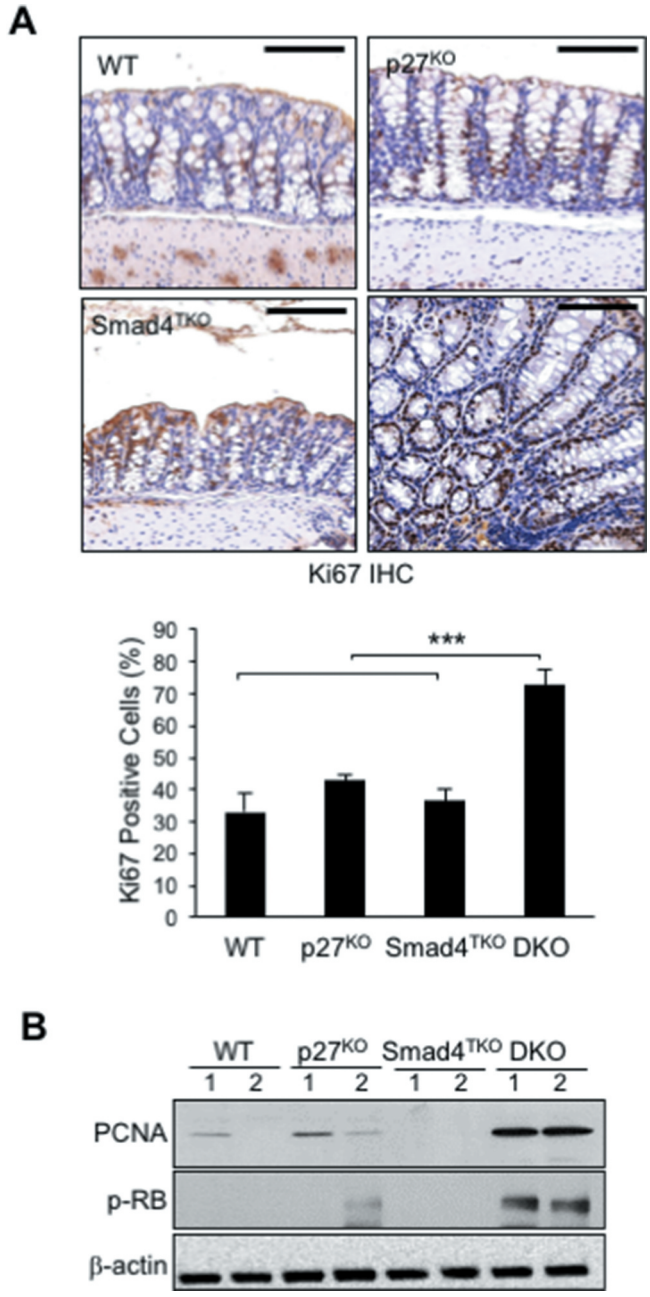

Figure 3. Proliferation of mucosal epithelial cells is increased in the colon of DKO mice. A) Immunohistochemistry (IHC) analysis for Ki-67 in the colon of WT, $p27^{KO}$, $Smad4^{TKO}$ and DKO mice. The percentage of Ki-67 positive cells among all colon epithelial cells was determined in the colon at 3 months of age using a digital eyepiece. Data represent average ± S.E. (n = 4). B) Expression of PCNA and, *p*-Rb, and β-actin was measured by Western blot. Results shown are representative of 3 separate experiments. ***P < .001. Scale bar = 100 μm.

Smad4-deficient $CD4^+$ T_{EM} cells, whose production of Th1 pro-inflammatory cytokines, such as IFN-γ and TNF-α, contributes to the pathogenesis of CAC in this model.

The population of Foxp3$^+$ regulatory T cells is decreased in DKO mice

Tregs play a key role in the maintenance of mucosal immune homeostasis. A deficiency of Tregs leads to inflammation in the gastrointestinal tract and is associated with the pathogenesis of CAC.[24] Therefore, we next investigated the profile of natural $Foxp3^+$ Treg (nTreg) in the spleen and colonic mucosa of each genotype. The proportion of $Foxp3^+$ nTregs was decreased in the spleen and colon of DKO mice at 4 months of age, when compared with that of all other genotypes (Figure 5a, Supplemental Figure 3c). We also observed that $Foxp3^+$ nTregs were significantly decreased in the colon of $Smad4^{TKO}$ mice at 6 months (Supplemental Figure 3b) and in the spleen of aged $p27^{KO}$ mice (15 months; data not shown), which is consistent with previous reports.[35,46] In an inducible Treg (iTreg) assay, exogenous recombinant TGF-β induced the expression of Foxp3 in naïve $CD4^+$ T cells of spleen from either WT or $p27^{KO}$ mice. However, TGF-β failed to induce Foxp3 expression in naïve $CD4^+$ T cells isolated from either $Smad4^{TKO}$ or DKO mice (Figure 5b), indicating that a decrease in the nTreg population and a deficiency of iTreg induction are observed only in the DKO mouse model. In addition, while TGF-β-induced $CD4^+CD25^+$ Treg from WT mice suppressed naïve T cell proliferation in the Treg suppression assay, $CD4^+CD25^+$ Treg isolated from $Smad4^{TKO}$ mouse did not (Figure 3c). These data imply that $p27^{Kip}$ deficiency may also enhance inflammatory conditions by predisposing to a decline in the mucosal Treg population in $Smad4^{TKO}$ mice.

Mucosal inflammation in Smad4^{TKO} mice is intensified by disruption of p27^{Kip1} expression

Cytokines including IFN-γ, TNF-γ, IL-6 and IL-1β, and many chemokines are known to promote inflammation and CAC development through the mechanisms that include deregulation of mucosal immune homeostasis and increased epithelial proliferation as well as through the induction of mutations in oncogenes and tumor suppressor genes.[23,47–49] In order to elucidate the mechanism underlying the pathogenesis of CAC promoted by $p27^{Kip1}$ deficiency in $Smad4^{TKO}$ mice, we examined expression levels of inflammatory mediators in the colon of WT, $p27^{KO}$, $Smad4^{TKO}$, and DKO mice. The mRNA levels of pro-inflammatory cytokines, such as IFN-γ, TNF-γ, and IL-1β were significantly elevated in the colons of DKO mice at 3 months of age, compared with those of either WT, $p27^{KO}$, or $Smad4^{TKO}$ mice (Figure 6a). We observed a correlation between the expression of these inflammatory cytokines and the activation of the intracellular mediators of their response, including NF-κB, Stat1, and Stat3 in intestinal mucosa in DKO mice, as assessed by Western blot analysis (Figure 6b). The phosphorylation of Stat1 is greatly increased in the colons of DKO mice at 3 months of age, whereas it is barely detectable in either WT, $p27^{KO}$, or $Smad4^{TKO}$ mice (Figure 6b). Stat3 and IκB phosphorylation was also significantly increased in colonic mucosal scrapings of DKO mice compared with those of other genotypes. Highly increased expression of iNOS is a common phenomenon during chronic inflammation. Inducible nitric oxide synthase (iNOS) mRNA expression in colonic mucosa and nitric oxide concentration in serum were also increased in DKO mice compared to other genotypes at this age (Figure 6a). While iNOS induction is not detected in the colon of WT, $p27^{KO}$, or $Smad4^{TKO}$ mice, it is significantly increased in DKO mice (Figure 6b). iNOS and phospho-Stat1 were significantly increased in the colonic mucosa of DKO mice even at 1 month of age (Supplementary Figure 4), and therefore may represent important early oncogenic events

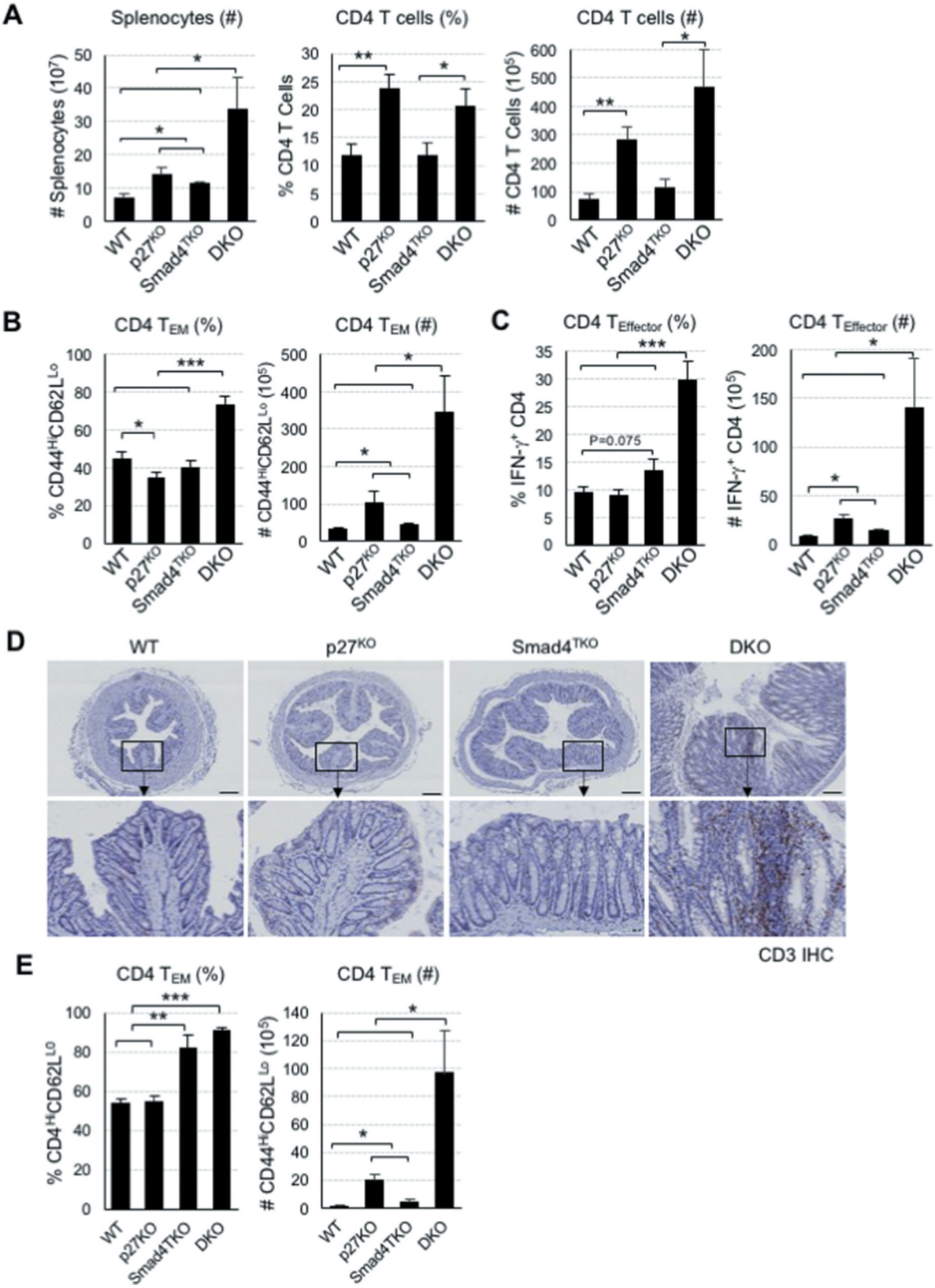

Figure 4. The population of effector memory CD4$^+$ cells in each genotype. A) Analysis of total spleen cells and CD4$^+$ T cells of each genotype at 4 months of age. Cell suspensions were prepared from spleens by filtering through nylon mesh. The cell numbers were counted by trypan blue exclusion assay. The population and number of CD4 T cells in each genotype. Splenocytes were stained with antibodies for CD4 and CD8. Events are gated on the live lymphocyte gate, based on forward and side light scatter (FSC X SSC) by FACS analysis. Bar graphs show the number of spleen cells and the proportion and absolute number of CD4 T cells, respectively. B) Analysis of memory markers (CD44^{High} and CD62L^{Low}) in CD4$^+$ T cell compartment of each genotype. The bar graphs show the proportion and absolute number of CD4 T_{EM} cells from the same experiment depicted in Supplementary Figure 2B. C) Analysis of effector CD4$^+$ T cells expressing pro-inflammatory cytokines of each genotype. Splenocytes per genotype were collected, stained on cell surface with antibody for CD4 and intracellularly with IFN-γ or TNF-γ antibody, and analyzed on CD4$^+$ T cells by FACS. The bar graphs are from the same experiment depicted in Supplementary Figure 2 C. D) IHC staining for CD3$^+$ T cells in colon of each genotype. E) Colon lamina propria per each genotype at 4 months of age were collected, stained with antibodies for CD4 and CD8, and analyzed for effector memory markers (CD44^{High} and CD62L^{Low}) in CD4$^+$ T cell compartment. Bar graphs show the proportion and absolute number of CD4 T_{EM} cells and effector CD4 T cells producing IFN-γ, respectively. ***P < .001, **P < .01, *P < .05. Scale bar = 200 μm.

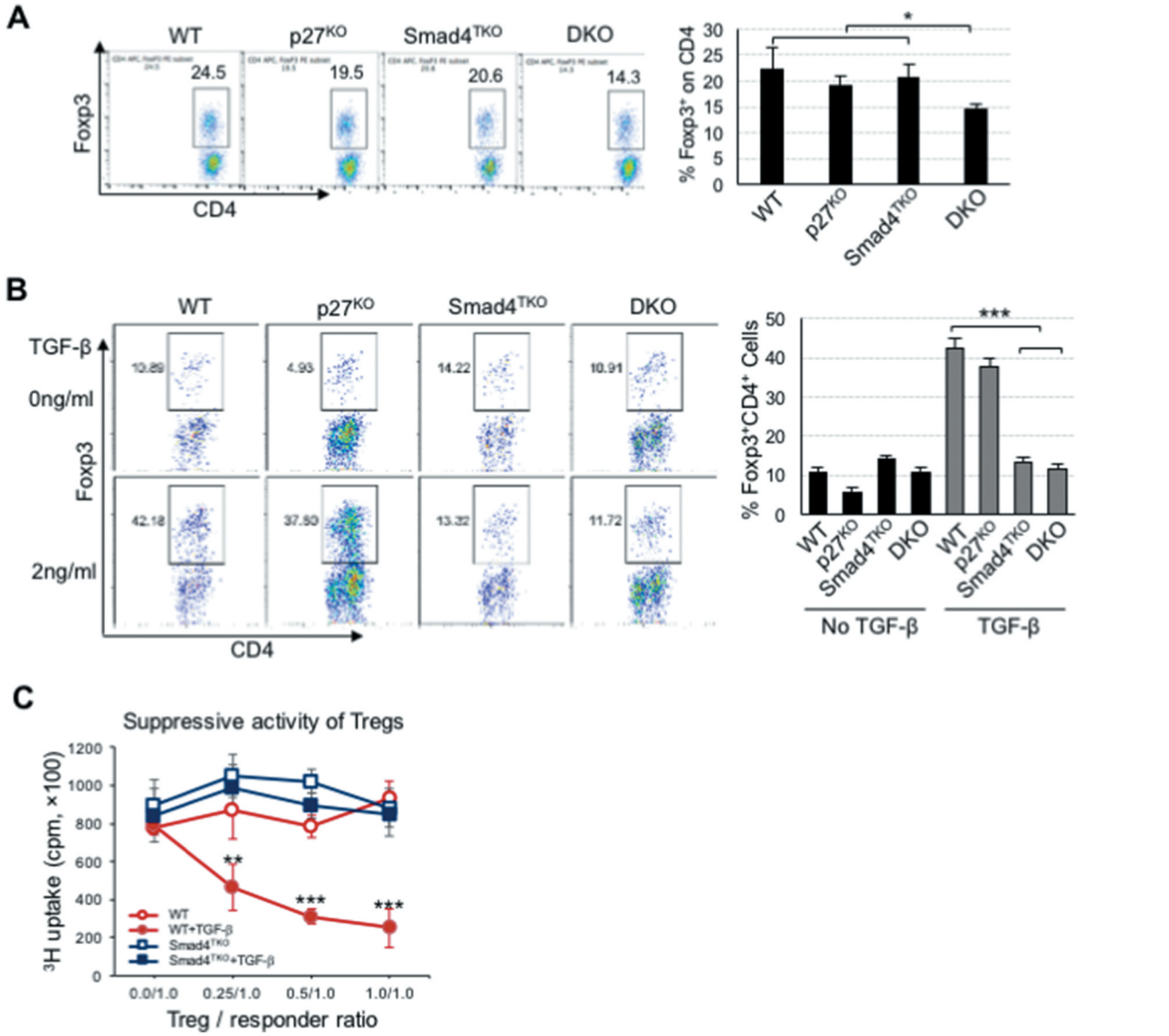

Figure 5. Decreased percentage of natural Foxp3$^+$ regulatory T cell (nTreg) and impaired TGF-β-induced Foxp3$^+$ Treg (iTreg) in DKO mice. A) Analysis of nTreg population in CD4$^+$ T cell compartment of each genotype mice. Splenocytes per genotype at 4 months of age were collected, stained on cell surface with antibody for CD4 and intracellularly with Foxp3 antibody, and analyzed on CD4$^+$ T cells by FACS. Events are gated on the live lymphocyte gate, based on forward and side light scatter (FSC X SSC). Bar graph represents percent nTreg from each genotype at 4 months. B) Analysis of iTreg population in CD4$^+$ T cell compartment of each genotype. Cells from Spleen per genotype at 2 months of age were collected and incubated with anti-CD3/CD28 for 72 hrs with (gray bar) or without (black bar) TGF-β (2 ng/ml) and intracellularly stained with Foxp3 antibody. Results are representative of three independent experiments using pooled spleens from two mice per genotype. Bar graph of percent iTreg from each group. C) Analysis of suppressive capacity of iTreg from CD4$^+$ T cell compartment of WT and Smad4^{TKO}. CD4$^+$CD25$^+$ iTregs from panel B were co-incubated with naïve CD4 T cells with 1 μg/ml of Con A for 3 days and the proliferation of naïve CD4 T cells was measured by H^3 thymidine incorporation for the final 16 hrs of culture. ***P < .001, **P < .01, *P < .05.

linked to the inflammatory process in DKO mice. Considering the highly expressed IFN-γ in DKO colon mucosa as well as increased IFN-γ secretion observed in CD4$^+$ effector T cells (Figure 4), we next investigated immune checkpoint molecules in the colons of each phenotype. IFN-γ is known to induce programmed death ligand-1 (PD-L1) expression on tumor cells and immune cells that are also abundant in the DKO colon. We found that PD-L1 is significantly increased in colon mucosa of DKO mice, compared with those of other groups (Figure 6c). Thus, these data suggest that p27^{Kip1} deficiency in T cells is associated with the induction of an inflammatory disease state and that may confer protection of transformed or malignant epithelial cells from host anti-tumor immune response through mechanisms that include induction of tumor PD-L1 expression. The significance of this observation is supported by data demonstrating elevated mucosal epithelial expression of PD-L1 in patients with IBD, and by the observation that preexisting IBD predicts risk for severe adverse events in cancer patients treated with immune checkpoint inhibitors.[50–52]

Discussion

In this study, we have demonstrated the distinct and essential contributions of both mucosal T cell and epithelial cell expression of p27^{Kip1} in the suppression of inflammation-related CRC. Our data indicate that a p27^{Kip1} deficiency accelerates gastrointestinal epithelial malignancy by increasing proliferation of epithelial cells

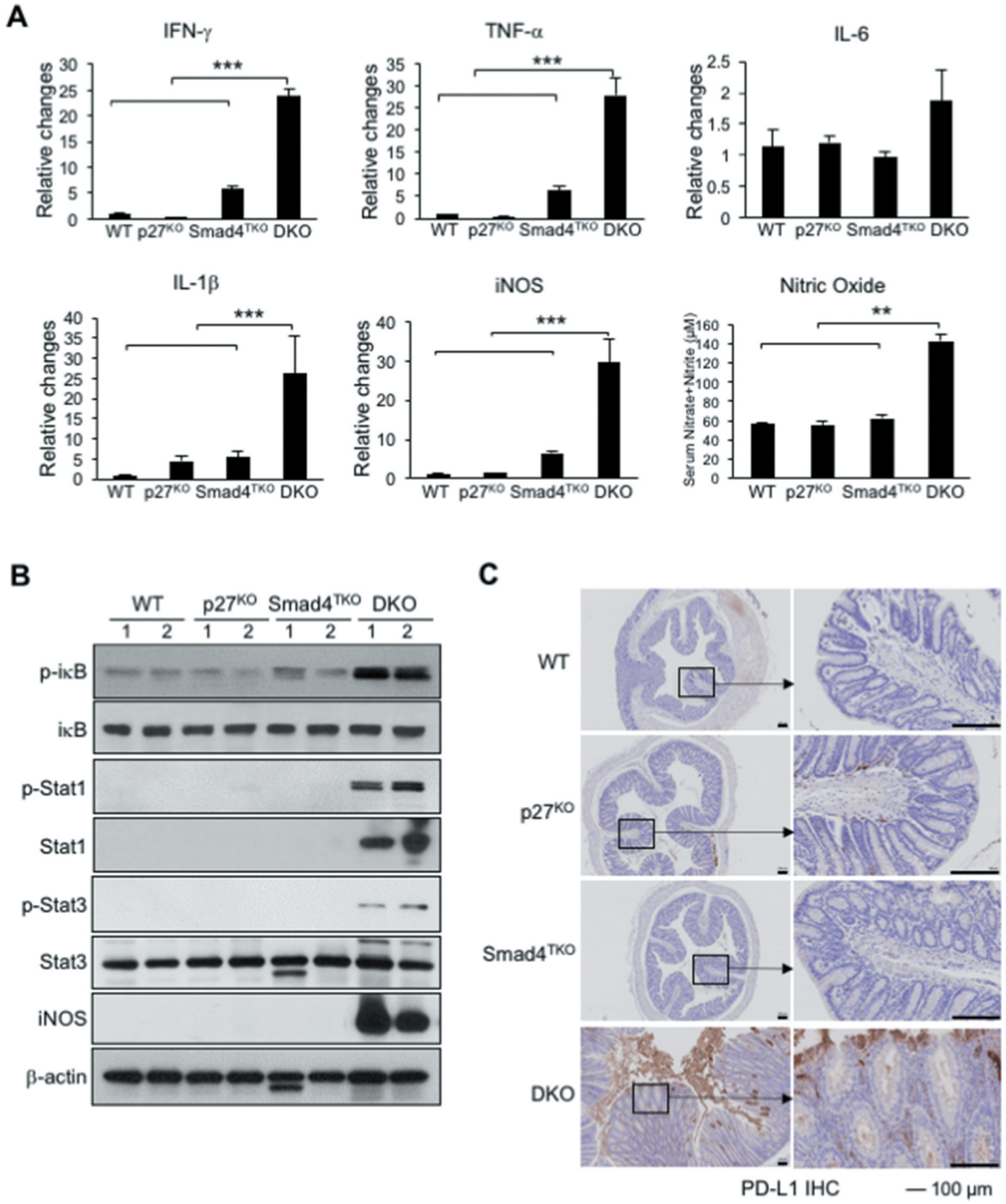

Figure 6. Increased inflammatory responses in the colon of DKO mice. A) IFN-γ, TNF-α, IL-6, IL-1β and iNOS were measured by real-time PCR in colon mucosa of WT, $p27^{KO}$, $Smad4^{TKO}$ and DKO mice at 3 month of age. Nitric oxide (nitrate + nitrite) concentration in sera of each genotype at 3 months of age. B) Expression of phospho-iκb, phospho-Stat1, phospho-Stat3, and iNOS was determined in colon epithelia of each genotype (3 month old) by Western blot analysis. β-actin was used as the loading control. C) Immunohistochemistry (IHC) staining for PD-L1 in colons of each genotype. Scale bar = 100 μm.

and by promoting epithelial cell transformation in a manner dependent on enhanced production of pro-inflammatory mediators by tissue-resident $CD4^+$ T_{EM} cells, whose expansion is accompanied by a reduction of mucosal Treg cells. Our observations in the CAC mouse model resulting from T cell-restricted loss of TGF-β-dependent Smad4 ($Smad4^{TKO}$) were validated by the introduction of the germline deletion of the *CDKN1b* ($p27^{Kip1}$) gene in $Smad4^{TKO}$ mice ($Smad4^{TKO}$/$p27^{KO}$, DKO). Utilizing the DKO mouse model, we discovered that the CAC phenotype in $Smad4^{TKO}$ mice is linked to loss of $p27^{Kip1}$ expression in lymphocytes, with the tremendous skewing of the mucosal $CD4^+$ T cell repertoire toward an activated, effector memory phenotype. Consequently, the colonic epithelium of DKO mice exhibited an inflammation-driven oncogenic signature that includes a significant elevation in the expression of iNOS, *p*-Stat1, and *p*-Stat3.

While the tumor suppressor function of $p27^{Kip1}$ is well recognized, the current study sheds new light on a unique mechanism through which a reduction in immune cell expression of $p27^{Kip1}$ promotes epithelial carcinogenesis. We discovered that, through direct effects on epithelial cell-cycle regulation, $p27^{Kip1}$ directly inhibits the initiation and progression of spontaneous epithelial carcinogenesis induced by inflammatory mediators, including iNOS, IFN-γ, TNF-α, and IL-6. The epithelial-intrinsic mechanisms underlying $p27^{Kip1}$ suppression of CAC include the regulation of the abundance of p-Rb and of cyclin D1 and an inhibition of Akt-mediated FOXO phosphorylation, which also inhibits $p27^{Kip1}$ expression.[53,54] Our observations are consistent with prior studies that have indicated that $p27^{Kip1}$ inhibits most cyclin/Cdk complexes and acts as a putative tumor suppressor for human cancer.[6,11–14] There is a known inverse correlation between $p27^{Kip1}$ protein levels and poor prognosis in CRC. Loda *et al*. first reported a low expression of $p27^{Kip1}$ in tumor samples obtained from 149 patients who underwent surgery for CRC.[15] Nine out of 13 retrospective multivariate analyses of CRCs (n = -80–418) showed reduced $p27^{Kip1}$ is associated with a 1.43–11 fold increase in relative risk of disease recurrence or death.[12,15–20] Three out the four remaining studies showed a trend toward significance between low $p27^{Kip1}$ protein levels and poor disease outcome. Our data are consistent with evidence linking the pathogenesis of CAC to low expression of $p27^{Kip1}$.[21]

Our data also suggest that the expansion of a pathogenic effector $CD4^+$ T cell population is a direct consequence of a $p27^{Kip1}$ deficiency, pointing toward an important mechanism through which $p27^{Kip1}$ influences the progression of CAC. Specifically, this study demonstrates that $p27^{Kip1}$ indirectly inhibits the initiation and progression of CAC through suppression of $CD4^+$ T cell-mediated mucosal inflammation. Aberrant or dysregulated $CD4^+$ T cell memory is suspected to contribute to multiple chronic or recurring inflammatory and immune-mediated disorders and to the progression of neoplastic disease.[55,56] Here we show $p27^{Kip1}$ deficiency increases the proportion and number of activated/memory $CD44^+CD62L^-$ $CD4^+$ T cells and effector $CD4^+$ T cells producing IFN-γ in $Smad4^{TKO}$ mice. These results are consistent with a previous report that $p27^{Kip1}$ negatively regulates the magnitude and persistence of $CD4^+$ T cell memory by promoting apoptosis and contraction of effector $CD4^+$ T cells.[34] Even though ubiquitination is the principal mechanism regulating $p27^{Kip1}$ protein degradation, prior studies have shown that both IL-2 and IL-5 lead to inhibition of $p27^{Kip1}$ mRNA expression in lymphocytes. Our present findings support the concept that $p27^{Kip1}$ contributes to the maintenance of native, mucosal Tregs and acts to impair the expansion of pathogenic T cells. A recent report demonstrates that $p27^{KO}$ mice have more severe disease scores in preclinical models of arthritis, and that this is a consequence of a reduction in the abundance of $Foxp3^+$ Tregs relative to WT control mice.[35] The latter indicates that $p27^{Kip1}$ is involved in the maintenance of mucosal homeostasis of $Foxp3^+$ Tregs. Indeed, $p27^{Kip1}$ deficiency in aged C57BL/6 mice is linked to a decrease in the number and activity of Treg cells and is associated with the development of arthritis and mild lupus-like abnormalities.[35] Together, these observations point to $p27^{Kip1}$ as a critical regulator of Treg cell differentiation and function through the positive modulation of TGF-β signaling strength in T cells.

Prior studies have shown that $p27^{KO}$ mice develop hyperplasia in multiple organs, resulting in a mouse roughly 20–30% larger than their WT counterparts.[38,57,58] Surprisingly, $p27^{KO}$ mice are relatively free of malignancy, with the exception of pituitary and prostatic hyperplasia that becomes increasingly severe with age.[59,60] While these data indicate that $p27^{Kip1}$ is a weak tumor suppressor, our data provide new evidence that $p27^{Kip1}$ deficiency can greatly accelerate CAC by enabling rapid expansion of a population of tissue-resident, activated effector memory $CD4^+$ T cells in a setting where Smad4-dependent TGF-β signaling is abrogated only in T cells. Germline mutations in *SMAD4* are found in over 50% of the patients with familial juvenile polyposis (FJP), an autosomal dominant disorder characterized by a predisposition to hamartomatous polyps and gastrointestinal cancer.[61,62] The histopathology of the intestines of $Smad4^{TKO}$ mice is in many respects a phenocopy of the pathology found in FJP, with a prominent stromal component.[36] The data presented here suggest that a reduction in the expression of $p27^{Kip1}$ in both epithelial cells and lymphocytes may be a determinant of disease severity in FJP, including the number of polyps, recurrent polyps, polyp development at a young age, and the highly increased risk for malignancy. The relevance of this biology is further supported by the demonstration that PTEN deficiency, common in FJP, leads to a loss of $p27^{Kip1}$ as PTEN is an important post-translational regulator of $p27^{Kip1}$ protein stability.[55]

To our knowledge, this is the first report describing dual, tandem mechanisms for tumor suppression by $p27^{Kip1}$, which include constitutive restriction of epithelial cell proliferation and the concomitant suppression of the expansion of effector memory $CD4^+$ T cells within the TME. It is notable that recent whole exome sequencing studies have identified a pathogenic variant of *CDKN1b* ($p27^{Kip1}$) in familial colorectal cancer, [63] supporting the idea that the genetics and presentation of disease in the DKO mouse model are indeed relevant to CAC development in humans. Furthermore, we previously reported the value of the $Smad4^{TKO}$ mouse model for assessing the efficacy of potential cancer chemopreventive agents, [37] and our current data reveal accelerated disease progression in the colon of DKO mice, thereby improving the utility of this model for preclinical assessment of novel cancer chemopreventive agents. Accordingly, we have leveraged the DKO model to demonstrate potent suppression of carcinogenesis in DKO mice by a natural triterpenoid, celastrol.[64] Importantly, strategies designed to modulate the expression and activity of $p27^{Kip1}$ in both the epithelial and stromal compartment in the TME may serve to concomitantly support the maintenance of mucosal epithelial homeostasis and suppress the expansion of pathogenic, tissue-resident effector T cells producing inflammatory cytokines, thereby forming a unique and effective approach to cancer chemoprevention.

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Conflicts of interest

The authors declare no potential conflicts of interest.

Authors' contributions

SHC, JJL, and BGK designed the studies and developed the methodology; SHC, KJG, and BGK performed and assisted with experiments; SHC, ECB, JJL, and BGK all assisted in interpreting the data and in writing the manuscript; JJL and BGK were responsible for overall project coordination and are co-senior authors.

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