Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRC4

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Contributed by Richard A. Flavell, November 9, 2010 (sent for review September 10, 2010)

Chronic inflammation is a known risk factor for tumorigenesis, yet the precise mechanism of this association is currently unknown. The inflammasome, a multiprotein complex formed by NOD-like receptor (NLR) family members, has recently been shown to orchestrate multiple innate and adaptive immune responses, yet its potential role in inflammation-induced cancer has been little studied. Using the azoxymethane and dextran sodium sulfate colitis-associated colo-rectal cancer model, we show that caspase-1−deficient (Casp\textsuperscript{1−/−}) mice have enhanced tumor formation. Surprisingly, the role of caspase-1 in tumorigenesis was not through regulation of colonic inflammation, but rather through regulation of colonic epithelial cell proliferation and apoptosis. Consequently, caspase-1−deficient mice demonstrate increased colonic epithelial cell proliferation in early stages of injury-induced tumor formation and reduced apoptosis in advanced tumors. We suggest a model in which the NLRC4 inflammasome is central to colonic inflammation-induced tumor formation through regulation of epithelial cell response to injury.

colorectal cancer | inflammation-induced colorectal cancer | NLR family, pyrin domain containing 3

colorectal cancer is one of the most common forms of fatal cancer in the world, yet its underlying molecular pathogenesis is poorly understood (1). Chronic inflammation is a known risk factor for tumorigenesis, and epidemiological data suggest that up to 15% of human cancer incidence is associated with inflammation (2, 3). Inflammation-induced colorectal cancer develops in patients with chronic inflammatory bowel disease, with risk estimated to increase by 0.5 to 1% per year after 8 to 10 y of inflammatory bowel disease (4). The nature of this strong association is largely unknown, and suggested mechanisms include chronic formation of reactive oxygen species (5) and tumorgenesis induced by chronic epithelial exposure or inflammatory stimuli, such as IL-6 and TNF-α (6–9). The importance of inflammation is further highlighted by the dependence of tumor growth and progression on the activation of NF-κB by the classic inhibitor of nuclear factor kappa-B kinase-dependent pathway, which is crucial for tumor growth and progression (10).

Inflammasomes are multiprotein complexes formed by several members of the NOD-like receptor (NLR) family, procaspase-1, and the adaptor protein ASC, originally described by Tschopp and colleagues to be central mediators of the innate immune response (11). Certain NLR family members can mediate inflammasome assembly in response to diverse danger signals, including pathogen-derived factors, as well as molecules such as ATP, monosodium uric acid crystals, and aluminum hydroxide (11, 12). Upon activation, caspase-1 cleaves the proinflammatory cytokines IL-1β and IL-18, resulting in secretion of their mature forms (11). The NLRP3 (also known as CIAS1 and NALP3) inflammasome is the most thoroughly characterized inflammasome; it can be activated by a number of chemically and structurally diverse triggers (12–14) and has recently been shown to orchestrate multiple innate and adaptive immune responses (12, 15).

NLRC4 belongs to the NLR family with a characteristic N-terminal CARD domain, a central NACHT domain, and C-terminal leucine-rich repeats. The NLRC4 inflammasome has been demonstrated to be important in host defense against a number of Gram-negative bacterial pathogens, such as Pseudomonas, Salmonella, and Shigella (16–18). Specifically, the NLRC4 inflammasome is activated by flagellin and components of the Type III secretion system of pathogenic Gram-negative bacteria (18–20). Unlike other inflammasomes, NLRC4 can activate caspase-1 in an ASC-dependent or -independent manner (18, 21). Like other described inflammasomes, NLRC4 inflammasome formation results in secretion of IL-1β and IL-18, but can also induce caspase-1-mediated cell death (18, 22). Furthermore, NLRC4 has also been suggested to participate in apoptosis pathways downstream of p53, as NLRC4 gene expression induced by p53 contributes to p53-dependent apoptosis in several human cell types (23, 24).

In addition to orchestrating multiple innate and adaptive immune responses upon encountering pathogens, the NLRP3 inflammasome has also been demonstrated to take part in IL-1β-dependent adaptive immune responses against dying tumor cells (25). ATP released from chemotherapy-treated tumors activates the NLRP3 inflammasome in dendritic cells, resulting in an effective CD8⁺ T-cell response directed against the tumor (25). However, the potential role of inflammasome signaling in the initiation and progression of inflammation-induced cancer has not been previously studied. Furthermore, mice deficient in MyD88, a central component in the signaling pathways downstream of the majority of Toll-like receptors (TLR), exhibit reduced tumor loads in a model of colon cancer (26). An explanation for this observation is that reduced colonic inflammation because of lack of TLR signaling following bacterial exposure decreases tumor formation in the colon. However, because MyD88 is also an essential component of the signaling cascade downstream of the IL-1 receptor family, the phenotype could be solely or partly explained by reduction of IL-1β and IL-18 signaling in the absence of a contribu- tion of TLRs. We decided, therefore, to test this hypothesis in caspase-1−deficient mice that lack the ability to produce IL-1β and IL-18 and that should therefore have decreased inflammation-induced carcinogenesis. Support for the involvement of IL-18 came from a recent article in which IL-18−/− mice were shown to develop severe colitis and also increased tumorigenesis compared with WT, suggesting that enhanced tumorigenesis in TLR dysfunctional mice is linked to signaling through the IL-18 receptor (27). The role of inflammasome components in the pathogenesis of colonic autoinflammation remains controversial. Three recent reports suggested that deficiency in caspase-1, ASC, or NLRP3 in...


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl;doi:10.1073/pnas.1016814108;DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1016814108

PNAS | December 14, 2010 | vol. 107 | no. 50 | 21635–21640
Formation.

indistinguishable from those of WT mice, where all tumors were

thermore, we demonstrate that increased tumorigenesis is mediated

sis, rather than through regulation of colonic in

profound effects on colonic epithelial cell proliferation and apopto-

azoxymethane (AOM) and dextran sodium sulfate (DSS) colitis-

(Fig. 1

heavy tumor load in 20% of Casp1

−/− mice resulted in large bowel

appeared more aggressive with invasion (arrows) of tumor cells

below the muscularis mucosae (*) noted in 75% of Casp1

−/− mice, compared with 14% invasion in WT group in advanced stages of
disease (day 200) (Fig. 1D, II). No evidence of distal metastasis in the
lungs, liver, spleen, or bone marrow was noted in mice that were
killed on day 120 of the CAC regimen.

One potential mechanism contributing to the increased tu-
morigenesis in Casp1

−/− mice is increased colonic inflammation in these mice. To test this possibility, we used the acute DSS colitis
model that employs a single 7-d-long cycle of DSS exposure (10).
We did not observe any differences in mass loss (Fig. 2A), colo-

scopic inflammation severity score (Fig. 2B), and histopatho-

logical morphology (Fig. 2 C and D), between Casp1

−/− and WT mice at both early (6–8 d) and late stages of acute colitis (11–14 d), when substantial inflammation was notable both endoscopically and pathologically. Likewise, when chronic DSS colitis was in-
duced by three 5-d cycles of DSS after AOM injection, no sig-
nificant difference in chronic colonic inflammation were noted
between Casp1

−/− and WT mice (Fig. 2E). In addition, no sig-
nificant changes were noted in both mRNA and protein levels of
inflammatory cytokine in both acute and chronic DSS colitis (Fig.
S1). This surprising result prompted us to investigate whether the
dramatic enhancement in tumor formation observed in Casp1

−/− mice may be caused by intrinsic effects in the colonic epithelial
cells, rather than by indirect effects stemming from the degree
of surrounding inflammation, as both hematopoietic and non-
 hematopoietic cells in the colon express caspase-1 (32). To in-
vestigate which of these cellular compartments is mainly
responsible for the increased tumorigenesis in Casp1

−/− mice, we
created bone marrow-chimeric mice, which were induced with
AOM-DSS inflammation-induced colon cancer. We found that
both WT bone marrow transplanted Casp1

−/− mice and Casp1

−/− bone marrow transplanted WT mice did not recapitulate the

Results and Discussion

Caspase-1 Deficiency Enhances Inflammation-Induced Colorectal Tumor Formation. To study the effects of caspase-1 on inflammation-induced colorectal cancer, we used the AOM-DSS model, in which system-

eically administered AOM induces colon tumorigenesis in mice

induced with chronic DSS colitis. In the AOM-DSS inflammation-

induced colorectal tumor model (Fig. 1A), Casp1

−/− mice featured

a significant increase in tumor numbers and tumor load compared with WT mice (Fig. 1B). In advanced stages of disease (day 80), the heavy tumor load in 20% of Casp1

−/− mice resulted in large bowel

obstruction and prevented the passage of a murine endoscope
(Fig. 1C). Histopathologically, tumors from Casp1

−/− mice were indistinguishable from those of WT mice, where all tumors were

composed of crypts and glands consistent with a well-differentiated
adenocarcinoma (Fig. 1D, I). However, tumors of Casp1

−/− mice

appeared more aggressive with invasion (arrows) of tumor cells

below the muscularis mucosae (*) noted in 75% of Casp1

−/− mice.
WT and Casp1−/− mice had no increase in the number of Ki67+ cells (Fig. 3A) and a moderately decreased number of BrdU+ cells (Fig. 3B). Grafting variability between experiments and limits the interpretation of hands severely alters the microbiotic epithelial compartment are required for the increased tumorigenesis observed in Casp1−/− mice. Expression of caspase-1, NLRP3, and NLRC4 mRNAs in the epithelial compartment is reduced in colon tumors (day 65 and day 200) with antibodies against CD3, Myeloperoxidase (MOP) and NK1.1. No differences were noted between the WT and KO mice in regard to infiltration of the tumor by T cells and neutrophils (CD3 staining; WT tumors, 47.20 ± 2.666 CD3+ cells/field; Casp1−/− tumors, 51.88 ± 3.193 CD3+ cells/field; MOP-1 staining: WT tumors, 18.50 ± 1.190 MOP-1+ cells/field; Casp1−/− tumors, 19.42 ± 1.694 MOP-1+ cells/field; mice, n ≥ 6, day 200 of the CAC regimen) and very rare NK1.1+ cells were noted in all samples, which is in agreement with the lack of difference in overall inflammation at earlier time points.

**Increased Tumorigenesis in Casp1−/− Mice Is Mediated by the NLRC4 Inflammasome.** Increased tumor formation in Casp1−/− mice suggests that inflammasome activation participates in pathways controlling the development and growth of tumors. The two most widely studied inflammasomes are the NLRP3 and NLRC4 inflammasomes. Both have been shown to participate in innate immune response to exogenous and endogenous “danger signals,” such as ATP, crystals, and Gram-positive and -negative bacteria.

In additional, the NLRC4 inflammasome has been suggested to participate in antiapoptosis pathways downstream of p53 (23, 24). To investigate the possible involvement of these NLRs in inflammation-induced cancer formation, we first examined the expression of caspase-1, NLRP3, and NLRC4 mRNAs in the epithelial and hematopoietic compartments of naive WT colons (Fig. 4A). Caspase-1 and NLRC4 were found to be relatively highly expressed in both colonic epithelial cells and CD45+ hematopoietic cells in the colon as compared with hypoxanthine phosphoribosyltransferase (HPRT). In contrast, NLRP3 expression was measured in both colonic epithelial cells and CD45+ hematopoietic cells in the colon as compared with hypoxanthine phosphoribosyltransferase (HPRT).

**Enhanced Colon Epithelial and Tumor Cell Proliferation in Casp1−/− Mice During Inflammation-Induced Carcinogenesis.** One of the suggested mechanisms to explain the initiation of colonic injury-induced cancer is repetitive epithelial cell destruction and subsequent regeneration, resulting in enhanced mutation (33, 34). To investigate this possibility, we analyzed the proliferation and survival of intestinal epithelial cells at day 15 of the CAC regimen in Casp1−/− and WT mice. At this stage, when inflammation is at its peak, the majority of the crypts appear normal in WT and Casp1−/− mice (Fig. 3C); however, small foci of crypt hyperplasia/dysplasia (Fig. 3D) were observed in both strains of mice. Immunohistochemistry with Ki67 (Fig. 3E) and BrdU (Fig. 3C), both markers of cellular proliferation, revealed significant increases in colon crypt cell proliferation in Casp1−/− mice compared with WT mice. However, in foci of crypt hyperplasia/dysplasia (Fig. 3D), there was no increase in the number of Ki67+ cells (Fig. 3E) and a moderate increase in the number of BrdU+ cells (Fig. 3F) in Casp1−/− mice when compared with WT mice in advanced tumors. In advanced tumors at day 65 (Fig. 3G), the numbers of both Ki67+ cells (Fig. 3H) and BrdU+ cells (Fig. 3I) were higher in Casp1−/− mice compared with WT mice.
rather than inflammation-related changes, account for the differences in tumor formation in inflammasome-deficient mice. As NLRC4 is implicated in p53-dependent apoptosis, it may provide a link to the increased tumorigenesis we observed in Casp1−/− mice and the changes in proliferation and apoptosis noted in epithelial cells in these mice (23). The fact that the NLRC4 inflammasome is activated by Gram-negative bacteria may point to its involvement in modulation of the colonic tumor formation by events involving the gut commensal bacterial flora (35). Future studies should aim to elucidate the patterns of molecular interaction between NLRC4 and caspase-1 that may lead to enhanced tumorigenesis in both.

To further study the molecular mechanism of Casp1−/− epithelial tumor transformation, we studied caspase-1 mRNA expression levels in normal colon tissue and colon tumors from WT mice. We observed a significant reduction in caspase-1 mRNA expression level in the tumor compared with colon tissue, suggesting that lack of caspase-1 may play a role in tumor progression (Fig. 4F). Furthermore, isolated colonic epithelial cells from either distal or proximal colons of mice with AOM-DSS–induced tumors featured a significant reduction in caspase-1 mRNA expression levels compared with naive mice, suggesting that reduction in caspase-1 expression specifically in epithelial cells may play a role in tumorigenesis (Fig. 4G). Similar results in humans showed that caspase-1 is down-regulated in human colon adenocarcinomas both at the mRNA and protein level (32). Similarly to Casp1−/− mice, NLRC4−/− mice featured significantly enhanced proliferation in both steady state and the early phase of inflammation-induced tumor formation and reduced apoptosis in tumors (Fig. S5).
In conclusion, our results support a model in which caspase-1 is central in the prevention of colonic inflammation-induced tumor formation through regulation of the epithelial cell response to injury. These effects are mediated through the NLRC4 inflammasome. Lack of such activation results in intrinsic changes in epithelial gene-expression programming, including enhanced proliferation, reduced apoptosis in tumor tissue, and overall enhanced tendency for tumor formation. In agreement with our findings, inflammasome-deficient mice were recently suggested to feature enhanced tendency for inflammation-induced tumorigenesis, but in our studies we found no evidence for either enhanced DSS-induced inflammation in Casp1−/− mice, or major involvement of NLRP3 in inflammation-induced tumor for-###mation through regulation of the epithelial cell response to inflammation, which may enable the recognition of new therapeutic targets.

**Materials and Methods**

**Experimental Animals.** All mice were cared for in accordance with institutional animal care and use committee-approved protocols at the Yale University School of Medicine animal facility.

**Histopathology.** After processing, colons were embedded lengthwise in paraffin (Blue Ribbon; Surgipath Medical Industries). Blocks were sectioned to the level of the lumen and then the next 5-μm section was stained with H&E, followed by placement of coverslips by routine methods. Sections were evaluated and were assigned scores by investigators blinded to experimental manipulation (37). A detailed histopathology procedure is described in SI Materials and Methods.
Isolation of Epithelial Cells and Hematopoietic Cells from the Intestine. Epithelial cells and hematopoietic cells were isolated from the freshly dissected colon by HBSS/EDTA shake and centrifugation. Detailed isolation procedures are described in SI Materials and Methods.

Immunohistochemistry. Paraffin-embedded sections were dehydrated and incubated with the following antibodies: anti-BrdU (1:4,000; Sigma), anti-Ki67 (1:100, Thermo; Lab Vision), anti-CD3 (1:400; Biocare Medical), anti-myeloperoxidase (1:100, Thermo; Lab Vision), anti-NK1.1 (1:100, Novus Biologicals). DAKO Envision System was used for detection. All sections were counterstained with hematoxylin. BrdU, TUNEL assay, and quantifications are detailed in SI Materials and Methods.

Colon Culture and ELISA. Sections of 1 cm of the proximal colon were cut, removed of feces, washed with PBS, and then cut half longitudinally. The colon sections were placed into culture in RPMI-1640 media (Sigma) with high concentration of penicillin and streptomycin and cultured at 37 °C with 5% CO2. Supernatants were harvested after 24 or 36 h, and the concentration of cytokine was determined by ELISA. Antibody pairs were purchased from BD Pharmingen (IFN-γ) and SouthernBiotech (IL-17), and ELISA was performed according to the manufacturer’s protocols.

RNA Analysis. Total RNA was extracted from colon tissue, tumors, and purified cells using TRIzol Reagent. SuperScript II Reverse Transcriptase (Invitrogen) was used for synthesis of cDNA. Real-time PCR analysis using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems) was performed on a 7500 Fast Real-time PCR system machine (Applied Biosystems). Expression data were normalized to HPRT. The primers are listed in SI Materials and Methods.

Statistical Analysis. Data are expressed as mean ± SEM. Differences were analyzed by Student’s t test and one way ANOVA and post hoc analysis for multiple group comparison. P values < 0.05 were considered significant.

ACKNOWLEDGMENTS. We thank Lauren Zenzewicz, Adam Williams, Tony Ferrandino, Jon Alderman, Elizabeth Eynon, Anthony Rongvaux, William O’Connor, Rachavendan Rathinam, Yasushi Kobayashi, Jorge Henao Mejia, Paula Licona Limon, and all members of the R.A.F. laboratory. Namiko Hoshi for technical help and scientific discussion; Fran Manzo for manuscript preparation; Ruslan Medzhitov, David Schatz, and Patrick Sung for scientific discussion; and the Yale Research Histology Laboratory in the Department of Pathology and the Yale Mouse Research Histopathology in the Section of Comparative Medicine for histology and immunohistochemistry help. This study was funded in part by a Cancer Research Institute postdoctoral fellowship, a supplemental grant by the United States-Israel Educational Foundation (to E.E.), Deutsche Forschungsgemeinschaft Hu1714/1-1 and the James Hudson Brown-Alexander Brown Postdoctoral Fellowships (to S.H.), National Institutes of Health Grant T32HL007974 (to S.C.E.), and the Howard Hughes Medical Institute (R.A.F.).
Colonoscopy was performed at indicated time points to monitor for severity of colitis and tumorigenesis. According to the murine endoscopic index of colitis severity system (1), colitis was scored on five parameters: granularity of mucosal surface, stool consistency, vascular pattern, translucency of the colon, and fibrin visible (score between 0 and 3 for each parameter). Tumor sizes were graded from 1 to 5 as follows: Grade 1 (very small but detectable tumor), Grade 2 (tumor covering up to one-eighth of colonic circumference), Grade 3 (tumor covering up to one-fourth of the colonic circumference), Grade 4 (tumor covering up to half of the colonic circumference), and Grade 5 (tumor covering more than half of the colonic circumference) (1). Tumors observed during endoscopy were counted to obtain the total number of tumors per animal. The size of each tumor in every mouse was recorded, and the sum of all tumor sizes per mouse was calculated as the tumor score.

Histopathology. After processing, colons were embedded lengthwise in paraffin (Blue RibBon; Surgipath Medical Industries). Blocks were sectioned to the level of the lumen and then the next 5-μm section was stained with H&E, followed by placement of coverslips by routine methods. Colons were evaluated and were assigned scores by investigators blinded to experimental manipulation. Each section was evaluated for pathological changes in the mucosa, submucosa, muscularis externa, and serosa, including inflammation, edema, mucosal changes of ulceration, hyperplasia, and attenuation. Digital light microscopic images were recorded with a Zeiss Axios Cam MRc5 camera, and AxioVision 4.7.1 imaging software (Carl Zeiss Microimaging).

Immunohistochemistry. Paraffin-embedded sections were dehydrated and incubated with the following antibodies: anti-BrdU (1:4,000; Sigma), anti-Ki67 (1:100, Thermo; Lab Vision), anti-CD3 (1:400, Biocare Medical), and anti-myeloperoxidase (MOP) (1:100, Thermo; Lab Vision), anti-Nk1.1 (1:100, Novus Biologicals). DAKO EnVision System was used for detection. All sections were counterstained with hematoxylin. For BrdU proliferation assay, mice were injected intraperitoneally with 1 ml BrdU (Sigma) solution, and killed 24 h after injection. TUNEL staining was performed using ApoAlert DNA Fragmentation Detection Kit (Millipore) according to the manufacturer’s instructions. Percentage of TUNEL-positive cells in tumor tissues were counted on slides from five sections of each slide and divided by the total cell number of each field at the magnification 10 × 40. Ki67+ cells were quantitated from 30 crypts of each mouse at day 15 of the colitis-associated colorectal cancer (CAC) model regimen. BrdU+ cells were quantitated from ≥ 25 crypts of each mouse at day 15. The percentage of Ki67+ and BrdU+ cells in tumor tissues were counted from six sections of each slide and divided by the total cell number of each field at the magnification 10 × 100. The CD3+ and MOP-1+ cells in tumor tissues were counted from greater than or equal to six fields of tumor tissue from each slide at the magnification of 10 × 100.

Isolation of Epithelial Cells and Hematopoietic Cells from the Intestine. Epithelial cells and hematopoietic cells were isolated from the freshly dissected colon. After removal of the Payer’s patches and the adventitial fat, the colon was cut longitudinally and washed with PBS. For disruption of the epithelial cells, the colon was incubated in HBSS/EDTA in a 37 °C water bath, manually shaking for 20 min. The supernatant was collected and further separated in CD45+ (IEL) and negative cells using MACS. The remaining colon was cut into small pieces and digested using Collagenase/DNase incubation in a 37 °C water bath, manually shaking, and after filtered through a 100-μm cell strainer. CD45+ cells (LPL) were purified using MACS. In a second step CD45+ IEL and LPL cells were further purified using a MoFlo cell sorter. The purity of CD45+ cells was > 95%, epithelial cells were > 99% CD45−.

TaqMan Gene Expression Assays’ Catalog Numbers. Caspase-1: Mn00438023_m1; NLRC4: Mn01223150_m1; NLRP3: Mn00840904_m1; IL-17a: Mn00439618_m1; IFN-γ: Mn00801778_m1; L-18: Mn00434225_m1; IL-11: Mn00434162_m1; IL-1β: Mn01336189_m1; TNF-α: Mn00443258_m1; IL-22: Mn00444241_m1; IL-6: Mn00446190_m1.

Fig. S1. Cytokine quantification in WT and Casp1−/− mice in the acute and chronic phases of colitis. TNF-α, IFN-γ, IL-1α, IL-18, IL-6, IL-22, IL-11, and IL-17A mRNA expression in WT and Casp1−/− colons was measured at day 15 of the CAC regimen (A) or 10 d following the cessation of the last cycle of dextran sodium sulfate (DSS) treatment (B). For protein measurement, 1-cm halved colon explants were incubated for 24 h and ELISA was performed on the supernatant during acute and chronic phases of colitis (C and D, respectively).

Fig. S2. Bone-marrow chimera experiments. WT or Casp1−/− bone marrow was transferred to WT or Casp1−/− mice following sublethal irradiation (two doses of 550 rad). Azoxymethane (AOM)-DSS CAC regimen was induced 8 wk later. Tumor load and tumor numbers/mouse were determined by colonoscopy on day 65 of the CAC regimen.
**Fig. S3.** Enhanced colon epithelial cell proliferation during the acute inflammatory phase and tumor cell proliferation in Casp1−/− mice. BrdU+ cells were quantitated from 30 crypts of each mouse at baseline (A) and day 15 of the CAC regimen (B, Left). BrdU+ cells were quantitated from ≥25 crypts of each mouse at day 15 of the CAC regimen (B, Right). The percentage of Ki67+ and BrdU+ cells (C) in tumor tissues were counted from six sections of each slide and divided by the total cell number of each field at the magnification 10 × 100. P values < 0.05 were considered statistically significant.

**Fig. S4.** NLRC4−/− mice develop aggressive locally invasive tumors. Representative histopathologic sections of colon adenocarcinomas and foci of tumor invasion from WT and NLRC4−/− mice. Invasive tumor foci (arrows) were not found in WT mice, but they were present in 66% of NLRC4−/− mice, where tumor foci were surrounded by abundant amounts of pale blue mucin. H&E staining; *muscularis mucosae; m, muscularis externa. (Scale bars, 200 μm.)
Fig. 55. Enhanced colon epithelial cell proliferation during the acute inflammatory phase and tumor cell proliferation in NLRC4−/− mice. BrdU+ cells were quantified from ≥25 crypts of each mouse at steady-state conditions (A); Ki67+ and BrdU+ cells were quantitated from ≥25 crypts of each mouse at day 15 of the CAC regimen (B). TUNEL-positive cells in tumor tissues were counted from greater than or equal to six fields of each slide at the magnification 10×40 (C). P values < 0.05 were considered statistically significant.