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Helicobacter Infection Is Required for Inflammation and Colon Cancer in Smad3-Deficient Mice

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Abstract

Accumulating evidence suggests that intestinal microbial organisms may play an important role in triggering and sustaining inflammation in individuals afflicted with inflammatory bowel disease (IBD). Moreover, individuals with IBD are at increased risk for developing colorectal cancer, suggesting that chronic inflammation may initiate genetic or epigenetic changes associated with cancer development. We tested the hypothesis that bacteria may contribute to the development of colon cancer by synergizing with defective transforming growth factor-β (TGF-β) signaling, a pathway commonly mutated in human colon cancer. Although others have reported that mice deficient in the TGF-β signaling molecule SMAD3 develop colon cancer, we found that SMAD3-deficient mice maintained free of the Gram-negative enterohepatic bacteria *Helicobacter* spp. for up to 9 months do not develop colon cancer. Furthermore, infection of $SMAD3^{-/-}$ mice with *Helicobacter* triggers colon cancer in 50% to 66% of the animals. Using real-time PCR, we found that *Helicobacter* organisms concentrate in the cecum, the preferred site of tumor development. Mucinous adenocarcinomas develop 5 to 30 weeks after infection and are preceded by an early inflammatory phase, consisting of increased proliferation of epithelial cells; increased numbers of cyclooxygenase-2–positive cells, $CD4^+$ T cells, macrophages; and increased MHC class II expression. Colonic tissue revealed increased transcripts for the oncogene c-*myc* and the proinflammatory cytokines interleukin-1α (IL-1α), IL-1β, IL-6, IFN-γ, and tumor necrosis factor-α, some of which have been implicated in colon cancer. These results suggest that bacteria may be important in triggering colorectal cancer, notably in the context of gene mutations in the TGF-β signaling pathway, one of the most commonly affected cellular pathways in colorectal cancer in humans.

Introduction

Recent studies suggest that the presence of certain bacteria may contribute to both the induction of inflammation in inflammatory bowel disease (IBD) and the progression of

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inflammation to neoplasia (1–3). However, the mechanisms of bacteria-induced carcinogenesis remain unclear, in part due to the lack of an animal model that accurately reflects the pathophysiology of bacteria-induced cancer in humans. *Helicobacter* organisms have been linked to IBD (4, 5), as well as liver (6) and colon cancer in mice (3, 7, 8), and gastric cancers in humans (9), suggesting that they may be a useful “tool” to study this phenomenon. We therefore sought to develop a mouse model of bacteria-induced colon cancer that more directly mimics the changes associated with colon cancer in humans using *Helicobacter* as the “trigger” organism.

The evolution of colon cancer seems to follow a predictable pattern of histologic changes and concurrent genetic and epigenetic changes, which ultimately provide a growth advantage resulting in the clonal expansion of transformed cells. At least three forms of genomic instability contribute to colon cancer, including microsatellite instability, chromosome instability, and chromosomal translocations (10). Chromosome instability results primarily from deregulation of DNA replication checkpoints and mitotic spindle checkpoints, whereas microsatellite instability results primarily from inactivation of mutation mismatch repair genes. The most commonly mutated pathway in colon cancer exhibiting microsatellite instability is the transforming growth factor-β (TGF-β) pathway.

TGF-β is a multifactorial cytokine with cytostatic and apoptotic functions, which help restrain the growth of epithelial and immune cells (11). TGF-β signals through the heteromeric complex of the type I and type II receptors and activates several members of the SMAD family of transcription factors, including SMAD2 and SMAD3 (12, 13). Activated SMAD2 and SMAD3 then associate with SMAD4 and travel to the nucleus, where they stimulate the expression of an incompletely defined set of genes. TGF-β dysregulation has been associated with Crohn’s disease, ulcerative colitis (14), and colorectal cancer (15, 16). Mutations in the *TGFBR2* gene are found in up to 90% of all microsatellite instability–positive cancer patients with hereditary nonpolyposis colorectal cancer, and up to 30% of all colon cancers, whereas mutations in SMAD2 or SMAD4 are found in 5% to 10 % of sporadic colon cancers (17, 18). Hence, alterations in the TGF-β pathway contribute significantly to the initiation and/or progression of colon cancer.

To directly test the hypothesis that bacteria play a critical and required role in the development of colon cancer in the context of dysregulation of the TGF-β pathway, we infected *Helicobacter*-free, SMAD3-deficient (SMAD3$^{-/-}$) and *Apc*-deficient (*Apc*$^{Min/+}$) mice with *Helicobacter* species. SMAD3$^{-/-}$ mice (*Smad3*$^{exo2/exo2}$) were originally reported to develop colorectal cancer by 6 months of age (19). However, two additional lines of SMAD3-deficient mice (*Smad3*$^{exo8/exo8}$ and *Smad3*$^{exo1/exo1}$) were independently generated and were reported to have impaired TGF-β function but lacked the florid colonic adenocarcinoma phenotype (20, 21). Here, we report that SMAD3$^{-/-}$ (*Smad3*$^{exo2/exo2}$) mice, when maintained in a *Helicobacter*-free environment for up to 9 months, do not manifest the previously reported colon cancer phenotype, whereas infection of the same mice with *Helicobacter* triggers colon cancer in 50% to 66% of the animals. These results suggest that bacterial triggers may be important in human colorectal cancer, notably in the context of genetic loss of anti-inflammatory and antiproliferative signals, such as those provided by TGF-β.

Materials and Methods

Mice

Breeder pairs of *Helicobacter*-free 129-*Smad3*$^{tm/Par}$/J (referred to as SMAD3$^{-/-}$) mice were generously donated by The Jackson Laboratory (Bar Harbor, ME). Homozygous SMAD3$^{-/-}$ mice were generated either by breeding homozygous males to heterozygous female mice or breeding heterozygous animals with genotypes verified by PCR. Four to 9-week-old male and female, homozygous, heterozygous, and L129/J (wild type) SMAD3 mice were used in five infection studies, and 9- to 10-month-old animals were evaluated in one *Helicobacter*-free aging study (Table 1). Additionally, 7- to 8-week-old female and male *Helicobacter*-free C57BL/6J-*Apc*^{Min}/J mice (The Jackson Laboratory) were used in one infection study. All mice were specific pathogen free (SPF) and certified free of *Helicobacter* species by the vendor and retested on site by fecal PCR. Animals were housed in an SPF facility in polycarbonate microisolator or ventilated cages and fed either irradiated Picolab rodent diet 20 5053 (PMI Nutrition International, Brentwood, MO; studies 1 and 6) or autoclaved rodent chow (Animal Specialities, Portland, OR; studies 2, 3, 4, and 5), autoclaved, acidified water, and monitored as previously described (2). Mice were euthanized by CO_2 in accordance with the AVMA Panel on Euthanasia when they developed severe diarrhea, 20% body weight loss, significant anemia, or signs of illness, and tissue samples were taken. All animal procedures were approved by the University of Washington Animal Care and Use Committee.

Helicobacter isolates and infection

The sources of *Helicobacter bilis* and *Helicobacter hepaticus* were previously described (2). A novel *Helicobacter* species originally obtained from a diabetes-prone (BBDP) rat colony (22) was also used. Organisms were cultured, and mice infected and infection confirmed as previously reported (2). In an attempt to optimize a *Helicobacter* infection regimen that yielded the highest frequency of tumor occurrence, *Helicobacter* spp. and timing of infection differed slightly among the studies and are summarized in Table 1. Briefly, studies 1, 2a and 2b, 3, 4, and 6 involved *Helicobacter* infection of SMAD3$^{-/-}$ mice. Study 5 involved aging of *Helicobacter*-free SMAD3$^{-/-}$ mice, and study 7 involved *Helicobacter* infection of *Apc*$^{Min/+}$ mice.

Pathology

Necropsy, tissue sampling, processing, and histologic examination was done as described (2). Samples for colonic tissue analyses for epithelial preparations (23) and reverse transcription-PCR (RT-PCR) and real-time PCR were harvested from grossly visible tumors and normal tissue from the proximal colon. The remaining colon, tumor, and all major organs were processed routinely for histologic assessment. Tumors were noted and classified phenotypically as mucinous adenocarcinoma (24). Inflammation scores were graded on a 0 to 16 scale (2) based upon proximal colon only. Mesenteric and any grossly enlarged lymph nodes were examined for presence of metastases by H&E and periodic acid Schiff staining and anti-cytokeratin-18 immunohistochemistry. For the *Apc*$^{Min/+}$ mice, the small and large intestines were fixed as Swiss rolls (25), and the cecum was processed separately. Histologic examination of H&E-stained sections was done by pathologists (H.B.O. and P.T.) blinded to

infection status. The number and location of the adenomas was noted, and any evidence of colitis or enteritis was scored.

Immunohistochemistry

Analysis of antigen expression was done on tissues immunolabeled using methods previously described (2). Colonic expression of cyclooxygenase-2 (COX-2), β-catenin, $CD4^+$ T cells, macrophages (F4/80), MHC class II, and the Ki67 antigen was assessed in two to four animals in each experimental group using the following primary antibodies: anti-COX-2 (Cayman Chemical, Ann Arbor, MI), anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA), F4/80 for macrophages, M5/114 for MHC class II, anti-CD4 (BD Biosciences, San Diego, CA), and anti-Ki67 (Vector, Burlingame, CA).

Flow cytometric analysis and proliferation assays

Cells were isolated from bone marrow, spleen, and thymus; stained for surface markers; and analyzed by flow cytometry as previously described (26). For mitogen stimulations, erythrocyte-depleted splenocytes or thymocytes were stimulated as previously described (27) using the indicated concentrations of 2C11 (PharMingen, San Diego, CA), F(ab′) anti-μ (Pierce, Rockford, IL) in triplicate, or *Helicobacter* sonicates (28). Stimulations were done with or without TGF-β at 2.5 ng/mL (Promega, Madison, WI). For *Helicobacter* antigen stimulations, total splenocytes were labeled with CFSE dye (27) and stimulated with either 100 μg/mL of whole-cell lysates from *H. bilis* or *H. hepaticus*, or lipopolysaccharide (10 μg/mL), or media only for 72 hours. T-cell and B-cell division were determined by flow cytometry using phycoerythrin-conjugated anti-B220 and biotinylated anti-CD3ε and gating on B220 or CD3 positive cells followed by CFSE analysis.

Colonic tissue preparation, multiplex PCR, and real-time PCR analyses

Colon epithelial cells were prepared as described (2) using a modification of Ogawa et al. (23) and further purified using negative selection with antibody-coated magnetic beads (2). RNA was extracted from purified epithelial cells and whole colonic tissue and assayed for *c-myc, p15, p21* by real-time PCR and cytokine mRNA levels by multiplex RT-PCR or real-time PCR (2).

Real-time PCR for *Helicobacter*

DNA from small intestine, cecum, proximal colon, distal colon, and their contents, tissue samples and their contents were collected from euthanized mice using 3 cm of mid-small intestine, total cecum, 3 cm of proximal colon, 3 cm of distal colon for tissue samples, and fecal material from each portion (2). Real-time PCR was done as previously described (4).

Results

Helicobacter-infected but not *Helicobacter*-free $SMAD3^{-/-}$ mice develop colitis and colorectal cancer

To test whether *Helicobacter* may play a role in the induction of colon cancer in $SMAD3^{-/-}$ mice, we obtained *Helicobacter*-free mice from The Jackson Laboratory and examined the

incidence of colon cancer. We found that *Helicobacter*-free SMAD3$^{-/-}$, SMAD3$^{+/-}$, and wild-type mice, maintained in a *Helicobacter*-free environment for up to 9 months in our SPF facility, remained free of colitis, colonic dysplasia, or neoplasia as judged by normal health exams and normal gross and histopathology exams (Fig. 1*A*; data not shown). To more directly examine the role of bacteria in colon cancer, *Helicobacter*-free SMAD3$^{-/-}$, SMAD3$^{+/-}$, or wild-type mice were orally challenged with combinations of *H. bilis, H. hepaticus*, a novel *Helicobacter* species, or broth only. Uninfected broth SMAD3$^{-/-}$ mice did not develop colon cancer up to 32 weeks after "infection" (Fig. 1*A*). However, in five separate studies, we found 50% to 66% of the SMAD3$^{-/-}$ mice infected with *Helicobacter* developed colonic adenocarcinomas, whereas none of the *Helicobacter*-infected SMAD3$^{+/-}$ or SMAD3$^{+/+}$ mice developed cancer (data not shown). Colonic tumors occurred at the cecocolic junction (Fig. 1*B*–*C*) and were classified as mucinous adenocarcinomas with tumors transmurally effacing the normal mucosal and submucosal architecture and large pools of tumor-produced mucin forming within serosal lymphatics and surrounding stroma (Fig. 1*D*). Distant thoracic lymph nodes contained mucin lakes with admixed isolated cells expanding the sinuses, which is highly suggestive of metastases (Fig. 1*E*). Serial cryostat sections of enlarged thoracic and mesenteric lymph nodes were examined for micrometastasis by immunohistochemistry for the epithelial cell marker cytokertain-18 and the macrophage marker F4/80. Although neoplastic epithelial cells were not definitively identified in serial sections of lymph nodes with mucin-filled sinuses, epithelioid cells positive for cytokeratin-18 and negative for the macrophage marker F4/80 were present within the affected sinuses. Collectively, the presence of mucin lakes and cytokeratin-positive cells within lymph nodes are indicative of micrometastasis to the lymph nodes (29).

Helicobacter-infected SMAD3$^{-/-}$ mice develop diarrhea and inflammation before development of colon tumors

To examine whether neoplastic transformation was associated with increased inflammation, we measured the amount of inflammation in *Helicobacter*-infected and uninfected SMAD3$^{-/-}$ mice. Diarrhea routinely developed in *Helicobacter*-infected mice 1 to 6 weeks after *Helicobacter* infection, which later resolved and did not result in body weight loss. In a cohort of animals necropsied relatively early after infection, six of six *Helicobacter*-infected SMAD3$^{-/-}$ mice had high inflammation scores (of 13–14) in the proximal colon at 2 to 5 weeks after infection (Fig. 2*A*) characterized by hypertrophic, thickened colonic proliferative bowel mucosa, and intense leukocyte infiltrates in lamina propria (Fig. 2*B*, H&E, *right*). In contrast, 0 of 10 broth-gavaged SMAD3$^{-/-}$ mice manifested inflammation (inflammation scores = 0), and four of eight *Helicobacter*-infected SMAD3$^{-/-}$ mice had high inflammation scores (of 13–14) that persisted to 12 to 18 weeks after infection (Fig. 2*A*). One *Helicobacter*-infected SMAD3$^{-/-}$ animal, which lacked inflammation in the proximal colon, had inflammation and carcinoma *in situ* in the mid-colon and rectum. To further characterize the type of inflammatory infiltrates, immunohistochemistry was done with antibodies against selected inflammation-induced antigens and immune cells. In uninfected SMAD3$^{-/-}$ mice, low levels of MHC class II antigen were expressed on lamina propria and intestinal epithelial cells, whereas *Helicobacter*-infected SMAD3$^{-/-}$ mice showed dramatic increases in all areas of colonic epithelium (Fig. 2*B*, MHC II, *right*). This was accompanied

by increased numbers of F4/80$^+$ macrophages (Fig. 2*B*, F4/80, *right*) and CD4$^+$ T cells (Fig. 2*B*, CD4, *right*).

Colonic tissue from *Helicobacter*-infected SMAD3$^{-/-}$ mice also had increased numbers of Ki67$^+$ cells (Fig. 2*B*, Ki67, *right*), indicating increased proliferation of epithelial cells. Whereas uninfected SMAD3$^{-/-}$ mice were devoid of inflammation (Fig. 3*A*, *left*) and had only rare, individually scattered COX-2$^+$ cells in the colonic mucosa (Fig. 3*A*, *right*), infected SMAD3$^{-/-}$ mice had clusters of COX-2$^+$ cells (Fig. 3*B*, *right*) associated with areas of irregularly branched glands and intense inflammation (Fig. 3*B*, *left*). Uninfected SMAD3$^{-/-}$ mice had occasional isolated COX-2$^+$ cells (Fig. 3*A*, *right*) and no inflammation (Fig. 3*A*, *left*).

Splenic B and T cells from *Helicobacter*-free SMAD3$^{-/-}$ mice are relatively resistant to TGF-β–mediated inhibition of cell proliferation

We examined the ability of lymphocytes from *Helicobacter*-free and *Helicobacter*-infected SMAD3$^{-/-}$ mice to proliferate in response to antigen receptor stimulation by directly cross-linking the T-cell receptor or B-cell receptor using antibodies, or by adding *Helicobacter* sonicates *in vitro*, and then measuring proliferative potential via ^{3}H-thymidine uptake or CFSE dilution, with or without TGF-β in the media. Thymocytes and splenocytes from *Helicobacter*-free SPF SMAD3$^{-/-}$ mice proliferate equivalently compared with cells derived from normal wild-type control mice in response to anti-CD3ε or anti-IgM stimulation (Fig. 4*A*–*C*). However, cells from SPF SMAD3$^{-/-}$ mice were relatively resistant to inhibition by TGF-β, as measured by a reduction of ^{3}H incorporation of wild-type cells relative to SMAD3$^{-/-}$ cells following *in vitro* culture for 72 hours with TGF-β. These results are consistent with studies by others, where the pathogen status of the mice was unknown (20, 30). However, in our hands, B cells from SMAD3$^{-/-}$ were not completely resistant to TGF-β inhibition, suggesting that other intracellular signaling molecules may partially compensate for the loss of SMAD3 in B lymphocytes. We noted no significant differences in lymphocyte subsets in thymus or peripheral lymphoid tissue from uninfected and infected SMAD3$^{-/-}$ and wild-type mice (Supplementary Figs. 1 and 2).

Helicobacter sonicates contain a B-cell mitogen

To examine whether lymphocytes in *Helicobacter*-infected mice may be primed to proliferate in response to *Helicobacter* antigens, splenocytes and mesenteric lymph nodes cells from *Helicobacter*-infected and uninfected mice were stimulated with *Helicobacter* sonicates *in vitro*, and their ability to proliferate was assessed using CFSE dye or ^{3}H-thymidine incorporation. We found that total splenocytes and lymph nodes cells from SMAD3$^{+/+}$, SMAD3$^{+/-}$, and SMAD3$^{-/-}$ mice proliferate equivalently in response to *Helicobacter* sonicates relative to media only (data not shown). However, flow cytometric analysis of the stimulated cells, using CFSE dye in conjunction with antibodies against the T-cell marker CD3ε and the B-cell marker B220, revealed that the splenocytes and lymph node cells, which were stimulated to divide by *H. bilis* antigens, were predominantly B cells (Fig. 4*D*). B cells from *Helicobacter*-infected and uninfected SMAD3$^{-/-}$ mice divided equally well in response to *Helicobacter* sonicates *in vitro*, indicating that the B-cell response is nonspecific and does not require immunologic memory. These results suggest

that *H. bilis* contains a B-cell specific mitogen, which could contribute to the inflammatory response observed in *Helicobacter*-infected SMAD3$^{-/-}$ mice. In contrast, we did not observe any significant T-cell response to whole *Helicobacter* sonicates.

Helicobacter organisms preferentially colonize the cecum and proximal colon

Gross and histologic analyses of the gastrointestinal tracts of *Helicobacter*-infected SMAD3$^{-/-}$ mice indicated that tumors develop preferentially at the cecocolic junction. Because murine enterohepatic helicobacters preferentially colonize the cecum and colon (31), we wanted to determine the relative concentration of *Helicobacter* organisms residing in those locations in *Helicobacter*-infected SMAD3$^{-/-}$ mice, possibly contributing to the focal nature of the carcinogenesis. We compared the relative amount of *Helicobacter* organisms in cecal, proximal colic, and distal colic tissue, and respective luminal contents, relative to the small intestine by real-time PCR using oligonucleotides specific for *Helicobacter* DNA (2) or β-actin (as the loading control). In samples taken at 7 and 12 weeks after infection, we found that *Helicobacter* genomes were much more concentrated in cecal tissue (Fig. 5, *left, top* and *bottom*) and cecal luminal contents (Fig. 5, *right, top* and *bottom*) relative to the proximal or distal colon tissue and contents, which correlates with the predominant location of tumor development.

SMAD3$^{-/-}$ mice have increased expression of proinflammatory cytokines

Studies by us and others suggest that *Helicobacter* can induce chronic inflammation, during which reactive oxygen species (ROS) may be generated, ultimately resulting in DNA damage. Because inflammation arises in part by the production of proinflammatory cytokines, we sought to characterize the types of cytokines produced in colonic tissue in response to *Helicobacter* infection. Using whole colonic tissue preparations (containing epithelial cells and associated immune cells), we found that both uninfected and *Helicobacter*-infected SMAD3$^{-/-}$ mice express increased transcripts for interleukin-1α (IL-1α) and IL-1β mRNA by multiplex PCR compared with wild-type mice (Fig. 6*A*). Further analysis by real-time PCR confirmed that colonic tissue from SMAD3$^{-/-}$ mice are in a “proinflammatory” state relative to wild-type mice, even in the absence of *Helicobacter* infection. Broth-only gavaged SMAD3$^{-/-}$ mice have increased IL-6, tumor necrosis factor-α (TNF-α), IFN-γ, and IL-4 mRNA levels (2- to 5-fold; Fig. 6*B*), relative to broth-gavaged SMAD3$^{+/+}$ mice. After *Helicobacter* infection, there were increases in IL-1β, IL-6, and TNF-α (2- to 6-fold) and dramatic increases in IFN-γ mRNA expression (30-fold; Fig. 6*C*), whereas expression of Th2 cytokines (IL-4 and IL-10) and IL-13 tended to decrease. No differences were noted in the expression of IL12p40 mRNA and TGF-β (data not shown). These results suggest that uninfected SMAD3$^{-/-}$ mice are in a proinflammatory state, which is exacerbated by *Helicobacter* infection, with further increases in potent inflammatory mediators, such as IL-1β, TNF-α, and IFN-γ, which persist over the course of infection. The shift towards Th1-type cells is consistent with a recent study, whereby blockade of TGF-β signaling in natural killer cells caused the accumulation of IFN-γ–producing cells (32).

Colonic epithelial cells from SMAD3$^{-/-}$ mice express increased levels of the oncogene c-*myc*

TGF-β inhibits cell cycle progression primarily by activating cytostatic gene responses, which target the G_1-S phase transition. For example, in epithelial cells, TGF-β–mediated activation of SMAD3-SMAD4 complexes results in binding to the c-*myc* promoter and rapid downregulation of c-*myc* expression. c-*myc* is also known to tether with the zinc-finger protein Miz-1, which together bind the promoter regions of the cell cycle inhibitors $p21^{Waf1}$ and $p15^{ink4b}$, resulting in inhibition of expression. Hence, one model for TGF-β–mediated inhibition of cell cycle progression involves downregulation of the growth promoter c-*myc*, which results in up-regulation of cell cycle inhibitors *p21* and *p15*. To determine if this model is supported in intestinal epithelial cells from SMAD3$^{+/+}$ and SMAD3$^{-/-}$ mice, we examined the relative expression of *p15, p21*, and c-*myc* in purified colonic epithelial cells from uninfected SMAD3$^{-/-}$ and SMAD3$^{+/+}$ mice. Using real-time PCR, we found that purified epithelial cells from uninfected SMAD3$^{-/-}$ mice express 2- to 4-fold more c-*myc* relative to SMAD3$^{+/+}$ mice (Fig. 7), whereas expression of *p15* and *p21* did not differ significantly.

Helicobacter infection does not accelerate epithelial cell transformation in *Apc* heterozygous mice

To determine if *Helicobacter* infections altered intestinal tumor frequency and phenotype in mice having lost one allele of the *APC* tumor suppressor gene, we infected a group of Apc^{Min} ($Apc^{Min/J}$) heterozygous mice with *H. bilis* and *H. hepaticus* and followed tumor formation and clinical disease, as manifested by anemia, diarrhea, and weight loss. There were no differences noted between the numbers or location of the adenomas as the infected mice averaged 42 intestinal adenomas with a range of 14 to 62 and the uninfected averaged 38 with a range of 19 to 57. Additionally, *Helicobacter*-infected $Apc^{Min/+}$ mice did not exhibit hyperplastic colitis, enteritis (except for minimal mucosal inflammation), erosion, or ulceration in the largest adenomas, nor were invasive adenocarcinomas observed (data not shown).

Discussion

The notion that the intestinal microflora interplay in the development of some types of colon cancer was originally proposed >25 years ago for chemically induced animal cancer models (33), and more recently in transgenic and knockout mouse lines, including RAG2$^{-/-}$ and IL-10$^{-/-}$ mice (1, 3, 7, 8, 34, 35). However, because deficiencies in RAG2 or IL-10 are not part of the known genetic alterations or pathways that contribute to colorectal cancer in humans, it is unclear how *Helicobacter* spp. or other bacterial organisms interact with the epigenetic or genetic changes observed in the induction of colon cancer in humans. Here, we report an association between enterohepatic helicobacters and TGF-β dysregulation in the development of colon cancer in SMAD3$^{-/-}$ mice. Mutations in genes involved in TGF-β signaling are found in human colorectal cancer exhibiting chromosome instability and microsatellite instability, and TGF-β dysregulation has been associated with inflammatory bowel disease (Crohn's disease and ulcerative colitis; ref. 14), disorders conferring an increased risk for colon cancer. These observations, along with studies reported here,

suggest that induction of colon cancer in SMAD3$^{-/-}$ mice with *Helicobacter* infection has particular relevance as a model to investigate the role of microorganisms and inflammation in colorectal cancer in humans exhibiting disrupted TGF-β signaling. The fact that colon cancer only occurs in the presence of *Helicobacter* in SMAD3$^{-/-}$ mice further suggests that the inflammatory response to microorganisms or other stimuli may be critical in the pathogenesis of colorectal cancer. Although SMAD3$^{-/-}$ mice (*Smad3*$^{exo2/exo2}$) were originally reported to develop colorectal cancer by 6 months of age (19), two additional lines of SMAD3-deficient mice (*Smad3*$^{exo8/exo8}$ and *Smad3*$^{exo1/exo1}$) were independently generated and were reported to have impaired TGF-β function but not cancer (20, 21). Our results suggest that the original *Smad3*$^{exo2/exo2}$ mice may have been infected with *Helicobacter* spp. or other colitis-inducing organisms, whereas the *Smad3*$^{exo8/exo8}$ and *Smad3*$^{exo1/exo1}$ mouse lines were *Helicobacter*-free or lacked other gastrointestinal organisms capable of triggering inflammation. In addition, our results are also consistent with other studies showing that TGF-β$^{-/-}$ RAG2$^{-/-}$ mice only develop colon cancer when *H. hepaticus* is present in the mouse colony (8), and mice expressing dominant-negative TGF-β type I/II receptors and infected with *H. pylori* develop gastric adenocarcinomas (36).

Infection with *Helicobacter* spp. restored the colon cancer phenotype in SMAD3$^{-/-}$ mice but had no effect on another rodent intestinal cancer model, the *Apc*$^{Min/+}$ mouse. Mutations in the *APC* gene are also found in 80% of sporadic colon cancers in humans, resulting in the accumulation of another protein, β-catenin, a nuclear protein that is mutated in sporadic colon cancers. We saw no changes in β-catenin expression or localization by immunohistochemistry in colonic tissue from *Helicobacter*-infected SMAD3$^{-/-}$ mice (data not shown), and Zhu et al. found no loss of *Apc* in the original report of SMAD3$^{-/-}$ mice (19). These results suggest that the *Apc* pathway is not likely to be involved in the induction of colon cancer by *Helicobacter* spp. It is noteworthy that we did not find hyperplastic inflammatory colitis in *Helicobacter*-infected *Apc*$^{Min/+}$ mice, whereas *Helicobacter*-infected SMAD3$^{-/-}$ mice showed florid IBD before tumor development. In addition, the *Apc*^{min} mutation is not known to affect immune cells. These results suggest that inflammation may be critical in the induction of colon cancer by microorganisms, and that the induction of colon cancer in SMAD3$^{-/-}$ mice by *Helicobacter* may reflect defects in both the immune system and epithelial cells themselves. Our findings are in contrast to studies reported by Newman et al. (34) using *Apc*$^{Min/+}$ mice where bacterial infection with *Citrobacter rodentium* resulted in an increase in adenoma formation with an associated hyperplastic inflammatory colitis. This suggests that there may be differences in the pathogenesis of colitis caused by different enteric pathogens.

The association between chronic inflammation and cancer has been noted in human patients with Crohn's disease, ulcerative colitis (37), pancreatitis (38), and hepatitis (39, 40), strongly supporting the notion that inflammation contributes to the induction of some types of cancer. *H. pylori*–induced gastritis in humans also leads to gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. Our observation that not all SMAD3$^{-/-}$ mice infected with *Helicobacter* develop large bowel inflammation could also account for the <100% tumor incidence we observe in *Helicobacter*-infected SMAD3$^{-/-}$ mice. Although we did not note any significant differences in lymphocyte subsets in thymus or peripheral lymphoid tissue from uninfected and infected SMAD3$^{-/-}$ and wild-type mice (see

Supplementary Figs. 1 and 2), we did observe that thymocytes and splenic B and T cells from SMAD3$^{-/-}$ mice were relatively resistant to inhibition by TGF-β. Hence, the relative increase in inflammation in colonic tissue from SMAD3$^{-/-}$ mice might be due in part to hyperactivation of SMAD3$^{-/-}$ lymphoid cells and further stimulation and recruitment of innate immune responses. Consistent with this notion, we found increased expression of MHC class II on intestinal epithelial cells and increased infiltration of macrophages, CD4^{+} T cells, and COX-2–expressing cells in colonic tissue from SMAD3$^{-/-}$ mice following infection with *Helicobacter*. Macrophages in particular are known to produce a variety of factors that promote mutations, growth and survival of tumor cells, and angiogenesis (41). Using an inflammation-associated model of epithelial cell cancer in skin, Marotta et al. (40) recently reported that B cells can also promote malignant transformation by producing soluble factors that assist in recruiting myeloid cells, such as macrophages, which result in increased inflammation (42). Our observation that *Helicobacter* organisms produce a B-cell mitogen resulting in B-cell activation also suggests that B cells may play a role in *Helicobacter*-induced inflammation and colon cancer. In addition, defective TGF-β signaling in T cells infiltrating colorectal tumors could contribute to the transformation of epithelial cells (43). Studies in B-cell or T-cell/SMAD3 double-deficient mice will be required to address the specific roles of lymphocytes in *Helicobacter*-induced colon carcinogenesis.

Although TGF-β–resistant effector T and B cells may have some role in the hyper-inflammatory response in the colon of SMAD3$^{-/-}$ mice, it is also possible that defective regulatory T cells have a significant role in bacteria-induced colon cancer. Regulatory T cells (T_R) were originally identified by their ability to suppress autoimmune disease (44) and are thought to control inflammation in some models of IBD (45) and colon cancer (3, 7, 46). T_R cells modulate autoimmune disease induced by both T cells and innate immune cells (47), in an IL-10– and TGF-β– dependent manner (48). Specifically, TGF-β is required for the expression of the forkhead winged-helix transcription factor Foxp3 within T_R cells, and Foxp3 expression is essential for T_R cell development and function (49, 50). Although it is not known whether SMAD3 also controls T_R development and function, T and B lymphocytes from SMAD3$^{-/-}$ mice do not respond normally to TGF-β suppression *in vitro*, which suggests that SMAD3$^{-/-}$ mice may lack or have defective T_R cells, or that their T_R cells are unable to suppress effector T cells that have defective TGF-β signaling. Studies done in dominant-negative TGF-β receptor II mice recently reported by Fahlen et al. (50) are consistent with the last possibility in which the lack of TGF-β signaling on pathogenic CD4^{+} T cells mediating colitis makes them refractory to control by T_R cells.

Our studies suggest that the synergy between host genetic predisposition and bacterial infections in the induction of colon cancer is likely multifactorial and complex. For example, in the present study, we found that intestinal epithelial cells of unmanipulated SMAD3$^{-/-}$ mice are in a “cancer-predisposed” state, with increased expression of the nuclear oncogene c-*myc*, increased cell division, and increased expression of proinflammatory cytokines, including IFN-γ, TNF-α, IL-6, and IL-1. In humans, c-*myc* is overexpressed in most colon cancers, and coexpression of c-*myc* and β-catenin are associated with markedly reduced patient survival (51). Genetic polymorphisms in both IL-1 and TNF-α are associated with increased risk of *Helicobacter*-induced gastric cancer (52), and IL-1 increases tumor invasiveness and metastasis by increasing expression of adhesion molecules, cytokines, and

proangiogenic molecules. Levels of IL-6 are increased in the serum of patients with colon cancer, and increasing levels of IL-6 correlates with tumor size. IL-6 has also been shown to promote the growth of colon cancer cells *in vitro* (53), and anti-IL6 antibody inhibits colon carcinogenesis in mice (54). These results suggest that impairment of TGF-β signaling alone is sufficient to induce many alterations in cellular behavior, which favor tumor growth and metastasis. However, as shown in SMAD3$^{-/-}$ mice, we found no histologic evidence of inflammation or cancer in the absence of a "bacterial trigger," which in the present study was *Helicobacter* spp. Infection with *Helicobacter* induces inflammation characterized by further increases in IL-1β, TNF-α, and IFN-γ, all of which are potent drivers of the innate immune response and are gene targets of the nuclear factor-κB transcriptional regulator. Indeed, proinflammatory cytokines have been shown to result in enhanced epithelial cell survival, whereas inflammation results in the elaboration of DNA-damaging ROS, potentially leading to genomic instability and transformation. *Helicobacter* infections increase ROS, which could damage DNA (55). *H. hepaticus*–infected *Gpx* double knockout mice, which lack the two isoforms of *Gpx* expressed in the intestinal tract and therefore deficient in their ability to scavenge hydrogen peroxides, have a significantly higher incidence of intestinal cancer compared with *H. hepaticus*–free *Gpx* double knockout mice (1).

In instances of bacteria-induced carcinogenesis, the pathogenesis likely involves a combination of the genetic susceptibility of the host and chronic inflammation induced by the bacteria, which together regulate the survival and growth of the involved cells. In this study, genetic susceptibility of the host is modeled in part by loss of SMAD3, which we find alters the sensitivity of epithelial cells and inflammatory cells in the surrounding stroma to *Helicobacter* infection. Induction of colon cancer in SMAD3$^{-/-}$ mice with *Helicobacter* infection provides a useful model to study the role of bacteria and inflammation in the evolution of certain types of colon cancer in humans, particularly cancers exhibiting microsatellite instability or chromosome instability, which can exhibit alterations in TGF-β signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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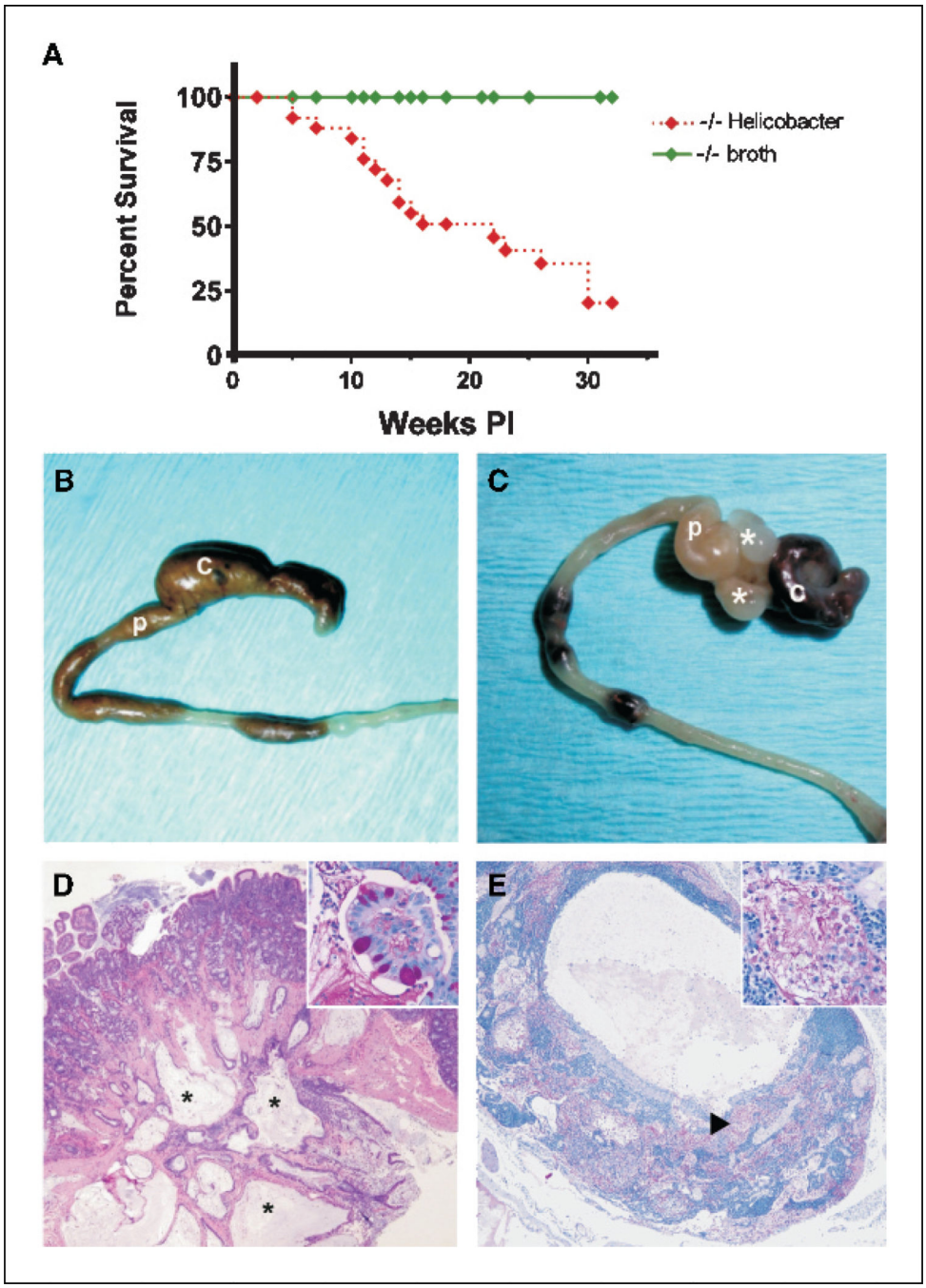

Figure 1.

Colon cancer in SMAD3$^{-/-}$ mice requires *Helicobacter* infection. *A,* Kaplan-Meier plot of results from studies 1, 2, and 3. Uninfected broth SMAD3$^{-/-}$ mice remained tumor free, whereas only *Helicobacter*-infected SMAD3$^{-/-}$ mice developed tumors. The earliest colonic tumors developed in *Helicobacter*-infected SMAD3$^{-/-}$ animals at 5 weeks post infection (*PI*) with <25% of infected mice being tumor free by 32 weeks post infection when the study was terminated ($P < 0.0001$, Mantel-Haenszel test). No colorectal lesions were noted in any of the uninfected broth SMAD3$^{-/-}$ animals or in any infected or broth heterozygote or wild-

type animals (data not shown). Only *Helicobacter*-infected SMAD3$^{-/-}$ mice developed colonic adenocarcinoma. *B,* relatively normal cecum (*c*) and proximal colon (*p*) from an uninfected SMAD3$^{-/-}$ mouse. *C,* grossly visible colonic tumors at the cecal-colic junction. Notice the pale multilobulated mass at the cecocolic junction (*). *D,* histologically, the majority of tumors were infiltrative mucinous adenocarcinoma with neoplastic glands penetrating through the wall of the colon and proliferating within the serosa and mesentery. Note the mucin lakes and neoplastic epithelial cells within the muscularis and serosa (*). H&E staining. Original magnification, ×4. *Inset,* periodic acid Schiff–stained section of primary tumor serosal extension from the same animal as (*D*) with intense staining of globet cells and tumor-produced mucin. Original magnification, ×20. *E,* periodic acid Schiff–stained section of mediastinal lymph node. The architecture is effaced by expanded and cystic sinuses containing mucin (*arrowhead*). Original magnification, ×4. *Inset,* notice the periodic acid Schiff–positive mucin and individualized cells within an expanded sinus of (*E*). Original magnification, ×20.

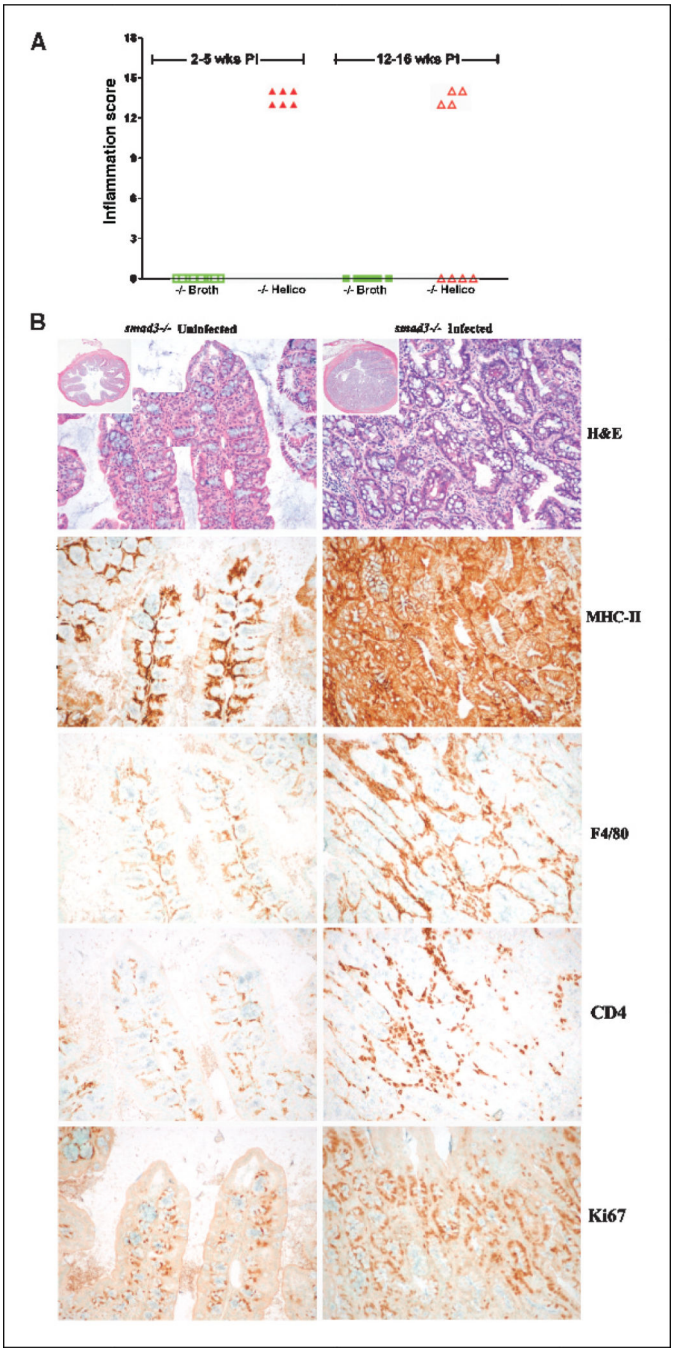

Figure 2.

Inflammation is only noted in *Helicobacter*-infected SMAD3$^{-/-}$ mice and is characterized by mucosal hyperplasia and chronic inflammatory infiltrates with increased expression of MHC II, infiltration of F4/80^{+} macrophages, CD4^{+} T cells, and epithelial cell proliferation. *A,* inflammation was noted (inflammation scores = 13–14) in the proximal colon in 6 of 6 *Helicobacter*-infected SMAD3$^{-/-}$ mice at 2 to 5 weeks post infection (*PI*; $P < 0.001$, Bonferroni's multiple comparison test). At 12 to 16 weeks post infection, inflammation was still present in 4 of 8 *Helicobacter*-infected SMAD3$^{-/-}$ mice ($P < 0.01$, Bonferroni's

multiple comparison test). No inflammation was noted in uninfected broth SMAD3$^{-/-}$ mice in the absence of *helicobacter. B,* cryosections of ornithine carbamyltransferase–embedded proximal colon from SMAD3$^{-/-}$ mice. *Left,* uninfected; *right, Helicobacter*-infected. H&E. The mucosa of the uninfected SMAD3$^{-/-}$ mouse is relatively normal with the prominent fronds of the proximal colon evident and even mucosa with minimal inflammatory cells. In contrast, the mucosa of the infected SMAD3$^{-/-}$ mouse has loss of normal architecture with irregularly branching and bunched glands and moderate chronic inflammatory infiltrates in lamina propria. Original magnification, ×20 and ×4 (*inset*). MHC II. Immunohistochemical staining for MHC II antigen (*brown staining*). The MHC II signal is markedly and diffusely increased in the epithelial cells of infected SMAD3$^{-/-}$ mice compared with uninfected SMAD3$^{-/-}$ mice, where the signal is primarily restricted to mononuclear cells in the lamina propria. Methyl green counterstain. Original magnification, ×20. F4/80. Immunohistochemical staining for F4/80 antigen (*brown staining*) highlighting macrophages. Note increased signal in the lamina propria of the infected mucosa compared with the lamina propria of the uninfected mucosa. Methyl green counterstain. CD4. Immunohistochemical staining for CD4 antigen (*brown staining*). Notice the increase of CD4^{+} cells within the lamina propria compared with uninfected SMAD3$^{-/-}$ mice. Ki67. Immunohistochemical staining for the Ki67 antigen, a marker of proliferation. Notice the increased signal (*brown staining*) of epithelial cells in the infected SMAD3$^{-/-}$ mouse. Original magnification for all images, ×20 except where noted.

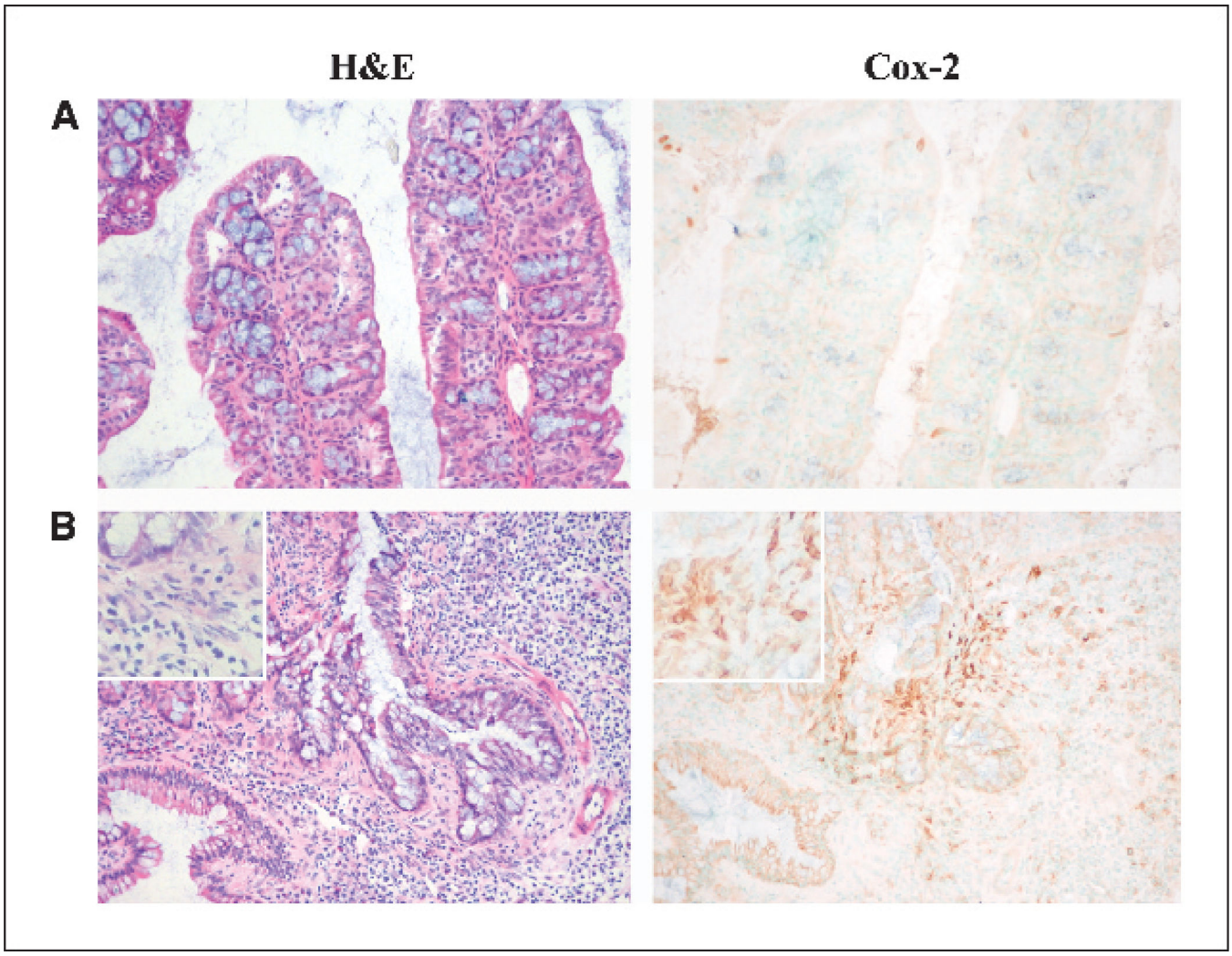

Figure 3.

COX-2 signal is increased in *Helicobacter*-infected SMAD3$^{-/-}$ mice. Serial cryosections of ornithine carbamyltransferase–embedded proximal colon from SMAD3$^{-/-}$ mice stained with H&E for orientation and immunohistochemically for COX-2 antigen. *A,* uninfected SMAD3$^{-/-}$ mouse. The lamina propria has relatively low cellularity; notice the occasional isolated positive cell (*brown staining*) within the mucosa and background staining of luminal contents. *B,* infected SMAD3$^{-/-}$ mouse. The lamina propria contains diffuse and dense infiltrates of inflammatory cells, principally macrophages, and CD4^{+} T cells as determined by immunohistochemistry (see Fig. 2). COX-2^{+} inflammatory cells (*dark brown staining*) are present in clusters within the lamina propria associated with an irregularly branching gland. Original magnification for all images, ×20 and reduced or cropped (*inset*) to fit.

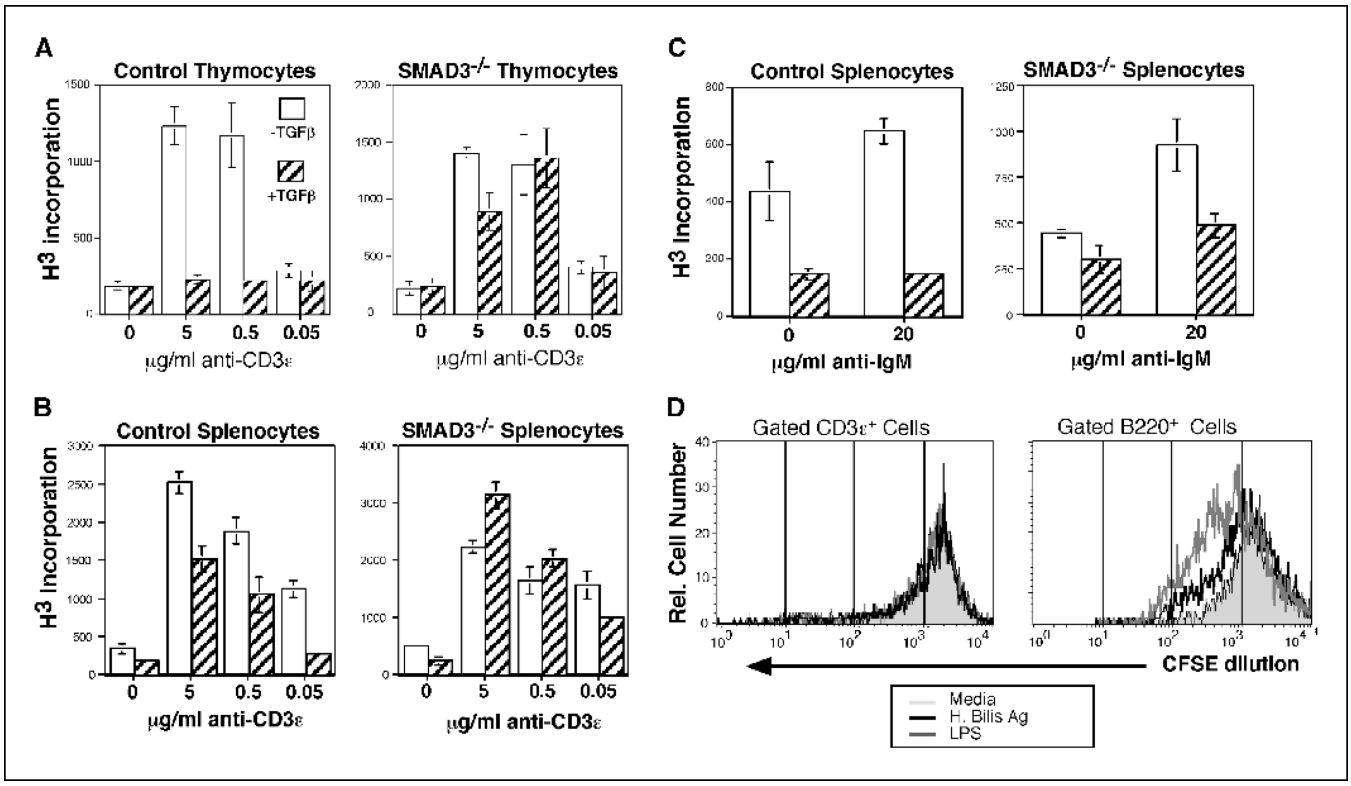

Figure 4.

Lymphocytes from SMAD3$^{-/-}$ mice are relatively resistant to TGF-β suppression. *A,* thymocytes from SMAD3$^{-/-}$ and wild-type mice were stimulated with the indicated concentrations of anti-CD3ε for 72 hours in the presence or absence of TGF-β (2.5 ng/mL). ^{3}H was added during the last 12 to 16 hours. *B,* splenocytes from the same mice were cultured as in (*A*). Both thymocytes and splenic T cells from SMAD3$^{-/-}$ mice are resistant to growth inhibition by TGF-β. *C,* splenocytes from the indicated mice were stimulated with anti-μ at the indicated concentrations, using the protocol described in (*A*). Splenic B cells are partially resistant to growth inhibition by TGF-β. *D,* splenocytes from mice of the indicated genotypes were labeled with CFSE dye and cultured for 72 hours in the presence or absence of 100 g/mL of whole *Helicobacter* sonicates, or lipopolysaccharide (*LPS*, 10 g/mL). After 72 hours, cells were harvested and stained for flow cytometry with biotinylated anti-CD3 and phycoerythrin-conjugated anti-B220 followed by streptavidin tricolor. T cells do not divide in the presence of lipopolysaccharide or *Helicobacter* sonicates, whereas B cells divide in response to both lipopolysaccharide and *H. bilis* antigens to a lesser extent.

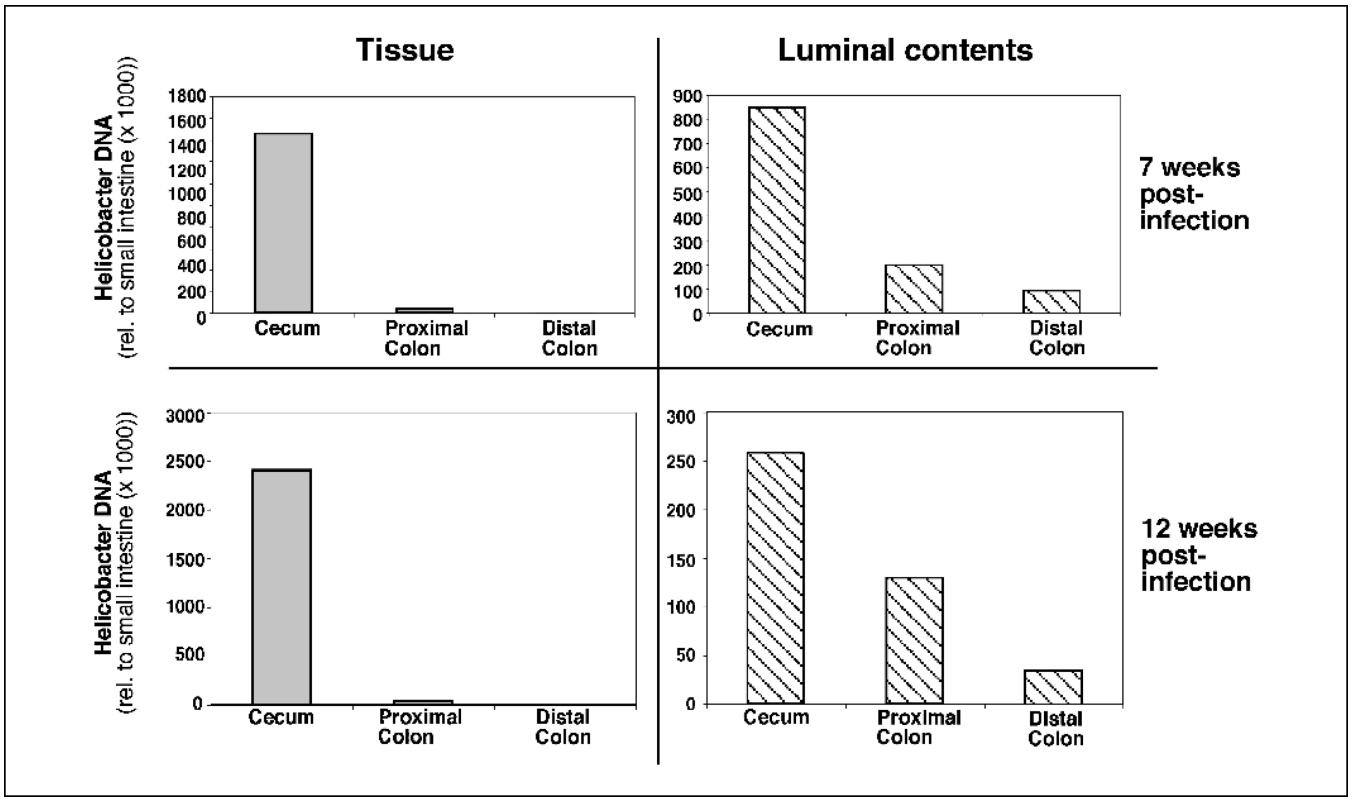

Figure 5.

Helicobacter preferentially colonizes the cecum, where adenocarcinomas eventually arise. 3-cm sections of small intestine, cecum, proximal colon, and distal colon were isolated, and intestinal contents were extracted from *Helicobacter*-infected SMAD3$^{-/-}$ mice at 7 weeks (*top*) and 12 weeks (*bottom*) after infection. DNA was made from the tissue and contents (2). Real-time PCR for *H. bilis* was done as previously described using β-actin as a loading control. Fold level of *Helicobacter* DNA relative to small intestine. *Helicobacter* DNA is 1,000× more concentrated in cecal tissue (*solid columns, left*), relative to proximal and distal colon. In addition, cecal contents (*hatched columns, right*) also contain much more *Helicobacter* DNA.

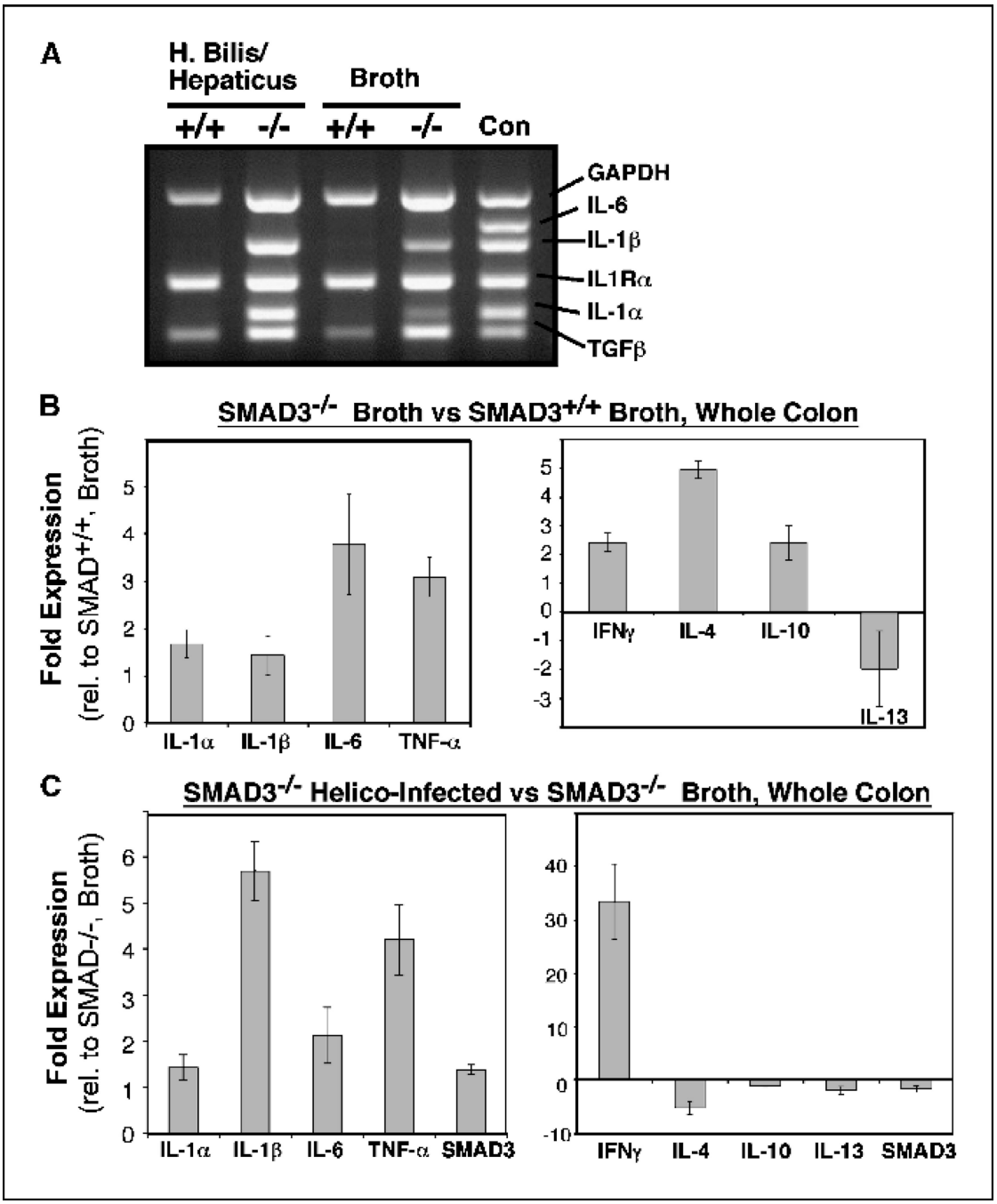

Figure 6.

Increased expression of proinflammatory cytokines in SMAD3$^{-/-}$ mice. Whole colonic tissue was isolated from SMAD3$^{-/-}$ and SMAD3$^{+/+}$ mice, total RNA was isolated, and cDNA was generated. *A,* shown is a multiplex PCR showing that both broth and *H. bilis/H. hepaticus*–infected SMAD3$^{-/-}$ mice express increased IL-1α and IL-1β message. *B,* real-time PCR showing uninfected broth SMAD3$^{-/-}$ mice express increased IL-6, TNF-α, IFN-γ, IL-4, and IL-10 message relative to SMAD3$^{+/+}$ mice. *C,* real-time PCR showing *Helicobacter* infection of SMAD3$^{-/-}$ mice leads to further increases in IL-1β, IL-6, TNF-α,

and dramatic increases in IFN-γ expression, relative to uninfected broth SMAD3$^{-/-}$ mice. *Columns,* mean of triplicate samples from a representative experiment; *bars,* SE. *A,* from animals at 12 to 18 weeks after infection. *C,* from animals at 4 weeks after infection. Note differences in scale. β-Actin message was used as a loading control.

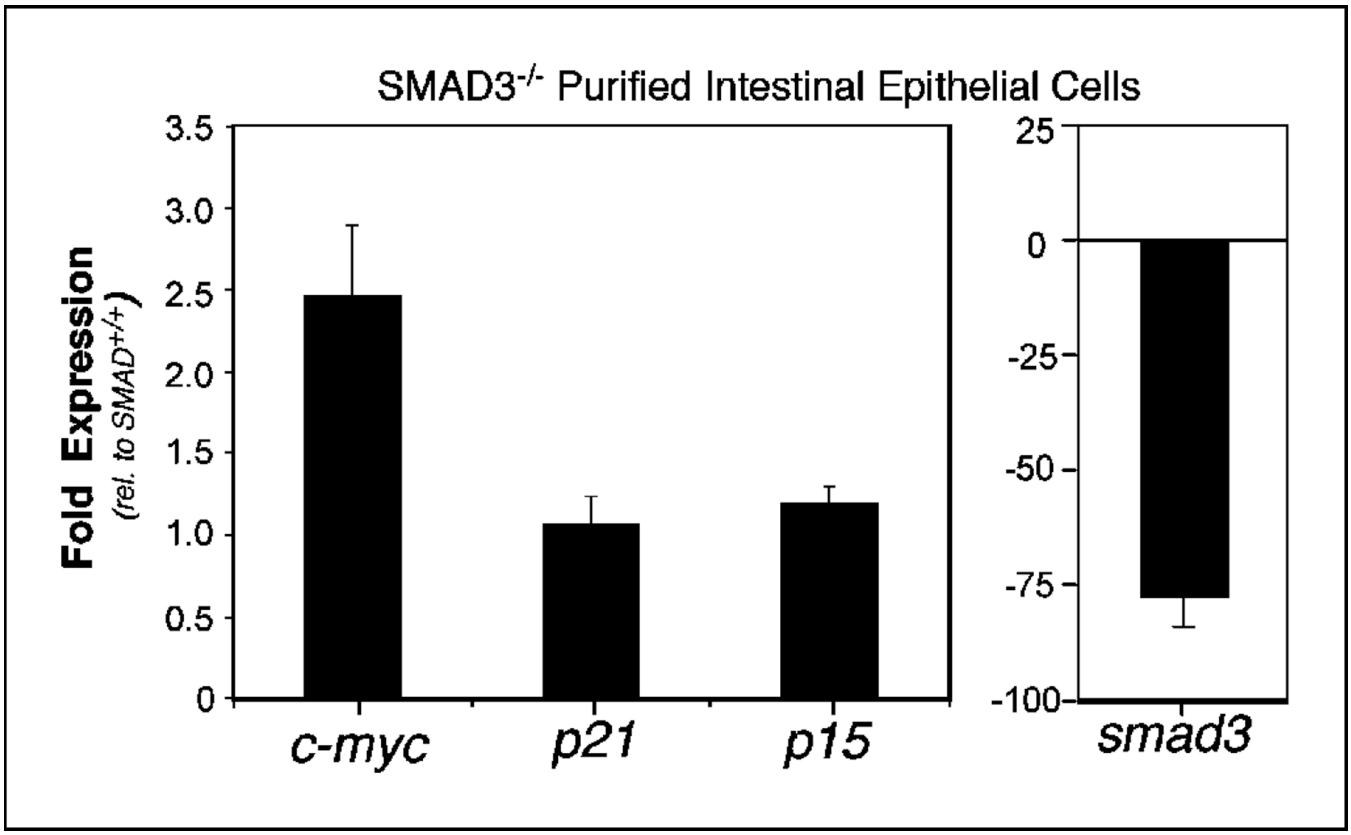

Figure 7.

Increased expression of the c-*myc* oncogene in purified epithelial cells from SMAD3$^{-/-}$ mice. Intestinal epithelial cells were purified from broth-gavaged SMAD3$^{-/-}$ and SMAD3$^{+/+}$ mice using standard procedures, and immune cells were removed by negative selection using magnetic beads. Total RNA was isolated from the purified cells, and cDNA was synthesized. Epithelial cells from SMAD3$^{-/-}$ mice express increased c-*myc* message relative to SMAD3$^{+/+}$ mice. *Columns,* mean of triplicate samples from a representative experiment; *bars,* SE.

Table 1

Tumor incidence in the *Helicobacter*-infected and uninfected broth SMAD3$^{-/-}$ mice

Study	SMAD3$^{-/-}$ plus broth	% Tumor incidence	SMAD3$^{-/-}$ plus *Helicobacter*	Bacteria*	% Tumor incidence	Time to tumor development
1	0/8	0	5/9	Hb; Hh	56	10–22 wk
2a	0/6	0	4/6	Hb; Hb	66	7–26 wk
2b	—	0	4/6	Hb; Hh	66	23–30 wk
3	0/10	0	5/10	Hb; Hn	50†	5–14 wk
4	0/9	0	2/9	Hb	22†	8–12 wk
5	NA	0	NA	none	0	Aging
6 Set 1	0/5	0	0/5	Hb; Hh	0	Inflammation evaluation
6 Set 2	0/5	0	3/5	Hb; Hh	60†	12–14 wk

NOTE: Studies 1 to 4 contained 11 to 19 SMAD3$^{+/-}$ and SMAD3$^{+/+}$ mice; no tumors were noted in *Helicobacter*-infected and broth SMAD3$^{+/-}$ and SMAD3$^{+/+}$ mice. Study 5 contained 11 SMAD3$^{-/-}$, 19 SMAD3$^{+/-}$, and 11 SMAD3$^{+/+}$ mice. Study 6 contained 30 SMAD3$^{-/-}$, 31 SMAD3$^{+/-}$, and 7 SMAD3$^{+/+}$ mice. Study 7 contained 6 broth *Apc*$^{Min/+}$ mice and 6 Hb/Hh-infected *Apc*$^{Min/+}$ mice (data not shown).

Abbreviations: Hb, *H. bilis*; Hh, *H. hepaticus*; Hn, novel *Helicobacter* spp.; NA, not available.

*Mice were gavaged with 2×10^7 organisms twice within 1 week (study 4) and again 1 week later (studies 1, 3, and 6), and the infection regimen was repeated again 1 month later (studies 2a and 2b).

†Does not reflect true incidence because animals were terminated at early time points to obtain tissues.