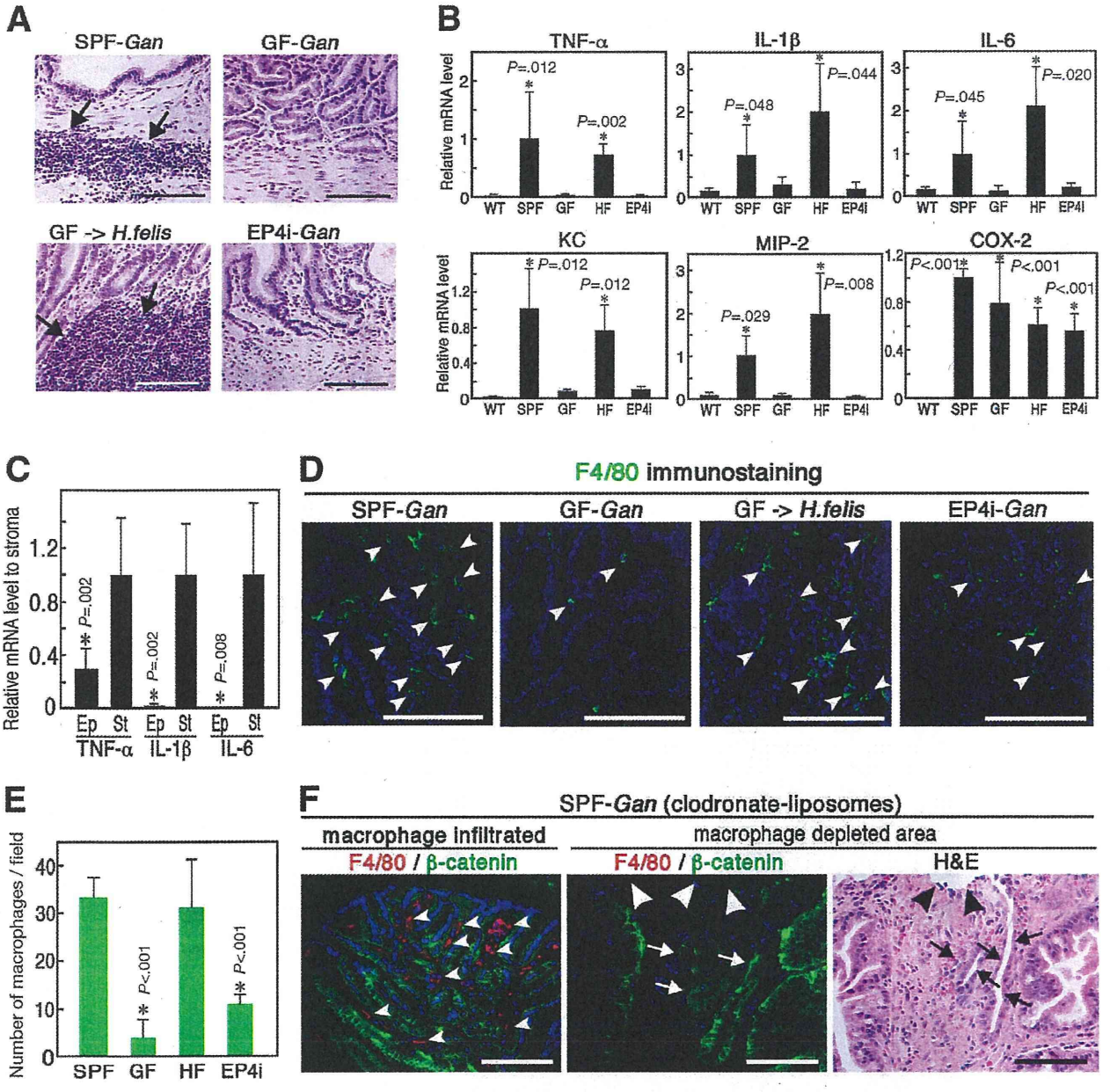

Figure 3. Macrophage recruitment in the *Gan* mouse tumors. (*A*) H&E staining of the SPF-*Gan*, GF-*Gan*, *H felis*-infected GF-*Gan* (GF->*H felis*), and EP4 inhibitor-treated *Gan* (EP4i-*Gan*) mouse stomachs. *Arrows* indicate lymphocyte infiltration. *Scale bars*, 100 μm. (*B*) Relative messenger RNA (mRNA) levels of inflammatory cytokines, chemokines, and COX-2 in wild-type mouse stomach (*WT*) and SPF-*Gan* (*SPF*), GF-*Gan* (*GF*), *H felis*-infected GF-*Gan* (*HF*), and EP4i-*Gan* (*EP4i*) mouse gastric tumors (mean ± standard deviation [SD]). *$P < .05$ vs wild-type level. (*C*) The mRNA level of inflammatory cytokines in the microdissected tumor epithelial cells (*Ep*) relative to the level in the tumor stroma (*St*). *$P < .01$. (*D*) Immunostaining of F4/80 (*green*) with 4′,6-diamidino-2-phenylindole staining (*blue*) in gastric tumors of the indicated groups. *Arrowheads* indicate F4/80-positive macrophages. *Scale bars*, 100 μm. (*E*) The mean number of F4/80-positive macrophages per microscopic field (mean ± SD). *$P < .001$ vs SPF level. (*F*) Immunostaining of F4/80 (*red*) and β-catenin (*green*) in gastric tumors of a control *Gan* mouse (*left*) and clodronate liposome-treated *Gan* mouse (*center*). H&E staining of serial section of a clodronate liposome-treated *Gan* mouse tumor (*right*). *Scale bars*, 100 μm. *Arrowheads* (*left*) indicate macrophages. *Arrowheads* and *arrows* (*center* and *right*) indicate the mucosal surface of the tumor and tumor cells, respectively, in the macrophage-depleted area.

an M2 marker, mannose receptor, was detected by immunohistochemistry in the SPF-*Gan* mouse tumors (Supplementary Figure 5*B*). It has been reported that CD4+ T cells regulate the M2 properties of macrophages.[24] CD4+ T cells infiltrated into the SPF-*Gan* mouse tumors (Supplementary Figure 5*C*). However, M2 macrophages were also found in SPF-*Rag2*$^{-/-}$ *K19-C2mE* mouse stomachs (Supplementary Figure 5*D*), suggesting

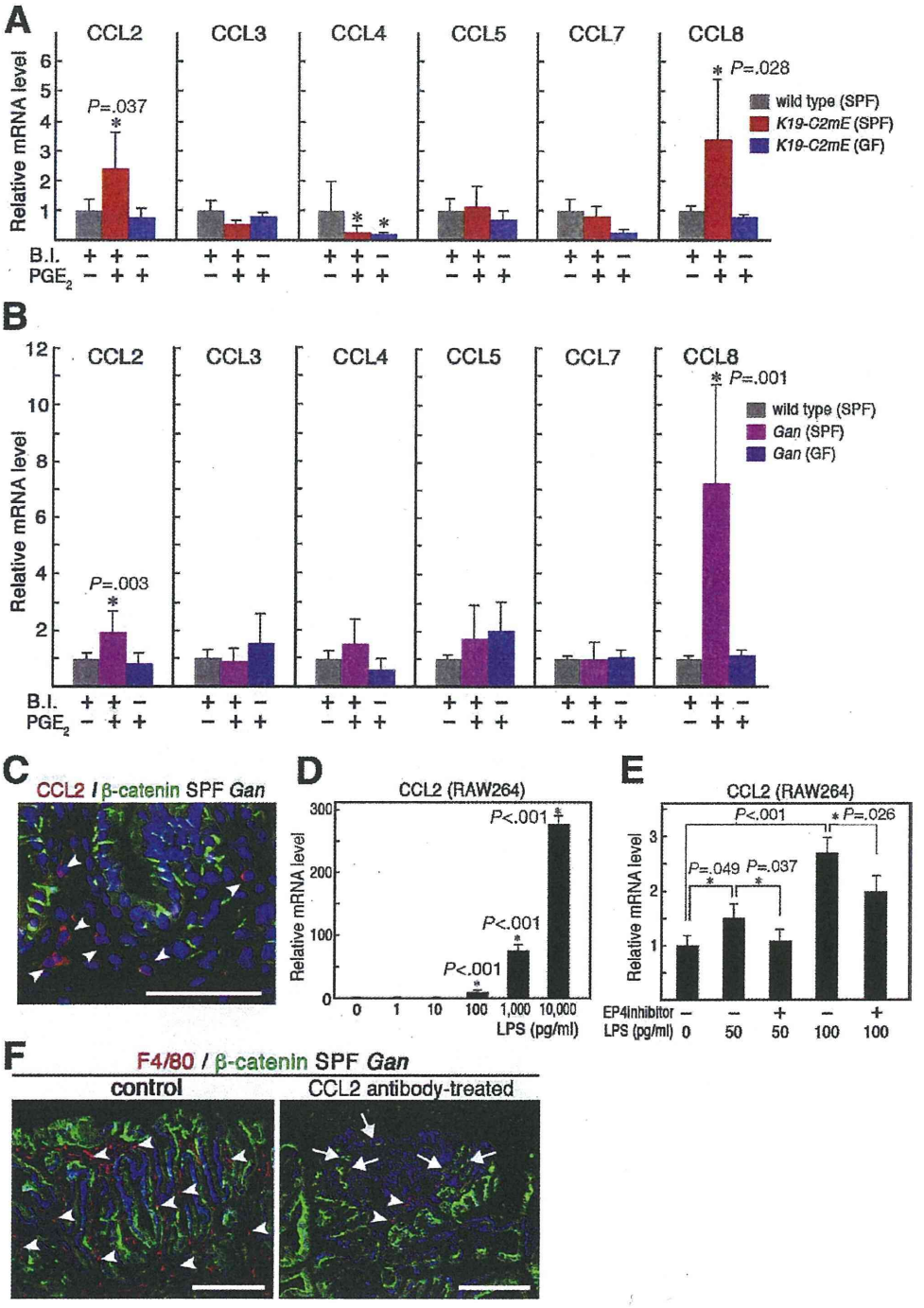

Figure 4. Chemokine induction by bacterial colonization and PGE$_2$ signaling. (*A* and *B*) The messenger RNA (mRNA) levels of the indicated chemokines in SPF-*K19-C2mE* (*red*) and GF-*K19-C2mE* (*blue*) gastric mucosa (*A*) and SPF-*Gan* (*pink*) and GF-*Gan* (*purple*) gastric tumors (*B*) relative to that in the control SPF wild-type mouse stomach (*gray*) (mean ± standard deviation [SD]). The indigenous bacterial colonization (*B.I.*) and PGE$_2$ transgenic status (*PGE$_2$*) are indicated at the bottom. *$P < .05$ vs wild-type level. (*C*) Immunostaining of CCL2 (*red*, *arrowheads*) and β-catenin (*green*) with 4',6-diamidino-2-phenylindole (DAPI) staining (*blue*) in SPF-*Gan* gastric tumor. *Scale bar*, 100 μm. (*D*) The mRNA levels of CCL2 in the LPS-stimulated RAW264 cells relative to that in the unstimulated control cells (mean ± SD). *$P < .001$ vs control level. (*E*) The mRNA levels of CCL2 in RAW264 cells with indicated treatment relative to the level in the unstimulated RAW264 cells (mean ± SD). *$P < .05$. (*F*) Immunostaining for F4/80 (*red*, *arrowheads*) and β-catenin (*green*) with DAPI staining (*blue*) in tumors of control SPF-*Gan* (*left*) and CCL2 antibody-treated SPF-*Gan* mice (*right*). *Scale bars*, 100 μm. *Arrows* in *right panel* indicate regressed tumors in the macrophage-depleted area.

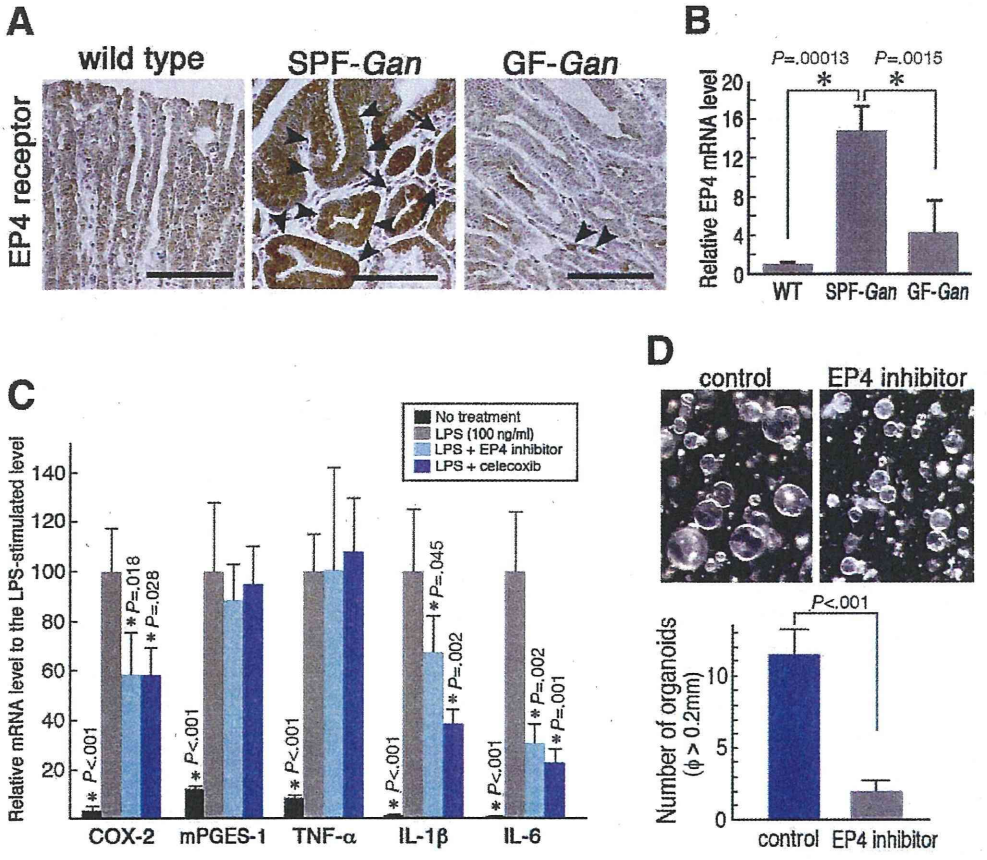

Figure 5. EP4 signaling on macrophages and epithelial cells. (*A*) Immunostaining of EP4 in the normal gastric mucosa of wild-type mouse and gastric tumors of SPF- and GF-*Gan* mice. *Arrowheads* and *arrows* indicate EP4 expression in the tumor epithelia and tumor stromal cells, respectively. *Scale bars*, 100 μm. (*B*) The messenger RNA (mRNA) levels of EP4 in gastric tumors of SPF-*Gan* and GF-*Gan* mice relative to the wild-type mouse level (mean ± standard deviation [SD]). *$P < .01$. (*C*) The mRNA levels of inflammatory cytokines in the control or drug-treated RAW264 cells relative to the level of LPS-stimulated RAW264 cells (mean ± SD). *$P < .05$ to the LPS-stimulated level. (*D*) Representative photographs of organoid structures formed by the primary cultured gastric epithelial cells in matrigel with EP4 inhibitor treatment (*top right*) and no-treatment control (*top left*). The mean number of organoids larger than 0.2 mm in diameter in the microscopic field on day 6 of culture (*bottom*) (mean ± SD). *$P < .001$.

that macrophages in the PGE_2-induced inflammation can be polarized to the M2 type in the absence of T cells.

EP4 Signaling on Macrophages and Epithelial Cells

Expression of the EP4 receptor was detected by immunostaining in both tumor epithelial cells and stromal cells of SPF-*Gan* mice, whereas it was rarely detected in the GF-*Gan* or wild-type mouse stomach (Figure 5*A*). Induction of EP4 in SPF-*Gan* tumors was confirmed by real-time RT-PCR (Figure 5*B*). When RAW264 macrophages were stimulated with LPS, expression of COX-2 and mPGES-1, as well as proinflammatory cytokines, was elevated (Figure 5*C*). Importantly, treatment of LPS-stimulated macrophages with an EP4 inhibitor or celecoxib suppressed the induction of COX-2, IL-1β, and IL-6. Moreover, inhibition of EP4 suppressed proliferation of the primary cultured gastric epithelial cells in matrigel (Figure 5*D*). These results suggest that EP4 signaling is also important for macrophage activation and epithelial cell proliferation.

Wnt Promotion by Bacterial Infection and TNF-α Stimulation

Expression of Wnt-target genes, CD44 and Eph receptor B3 (EphB3), was significantly down-regulated in the GF-*Gan* and EP4i-*Gan* mouse stomachs (Figure 6*A*). In the wild-type mouse stomach, expression of CD44 was found only in the neck of the gastric gland, whereas EphB3 was not detected (Figure 6*B*). Notably, expression of CD44 and EphB3 was significantly induced in tumor epithelial cells of SPF-*Gan* mice, which was suppressed in GF-*Gan* mice. Consistently, the active β-catenin level was decreased in the GF-*K19-Wnt1* stomach and GF-*Gan* mouse gastric tumors compared with SPF mice (Figure 6*C*), indicating that Wnt signaling activity is suppressed under GF conditions. We previously showed that macrophage-derived TNF-α promotes Wnt signaling in gastric

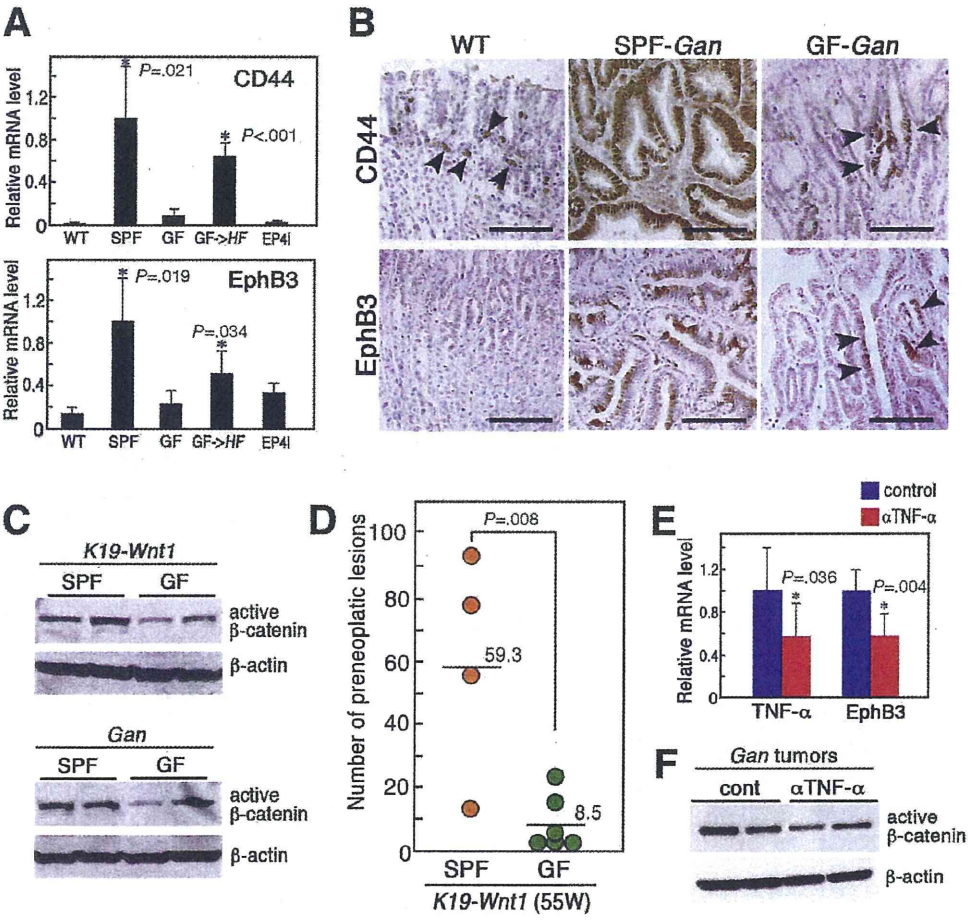

Figure 6. Wnt promotion by bacterial infection and TNF-α. (*A*) Relative messenger RNA (mRNA) levels of Wnt-target genes, CD44 and EphB3, in the wild-type mouse stomach (*WT*) and SPF-*Gan* (*SPF*), GF-*Gan* (*GF*), *H felis*-infected GF-*Gan* (*GF->HF*), and EP4 inhibitor-treated-*Gan* (*EP4i*) mouse gastric tumors (mean ± standard deviation [SD]). *$P < .05$ vs wild-type level. (*B*) Immunostaining of CD44 (*top*) and EphB3 (*bottom*) in the wild-type mouse stomach (*left*) and SPF-*Gan* (*center*) and GF-*Gan* (*right*) mouse tumors. *Arrowheads* indicate immunostained epithelial cells in the wild-type (*WT*) and GF-*Gan* mice. *Scale bars*, 100 μm. (*C*) Immunoblotting of active β-catenin in the SPF-*K19-Wnt1* and GF-*K19-Wnt1* mouse stomach (*top*) and SPF-*Gan* and GF-*Gan* mouse gastric tumors (*bottom*). β-Actin was used as an internal control. (*D*) The number of preneoplastic lesions developed in SPF-*K19-Wnt1* (*SPF*) and GF-*K19-Wnt1* (*GF*) mice. The mean numbers are indicated. (*E*) The mRNA levels of TNF-α and EphB3 in the gastric tumors of the anti-TNF-α neutralizing antibody-treated *Gan* (*αTNF-α*) relative to those of untreated control *Gan* mice (control) (mean ± SD). *$P < .05$ vs control. (*F*) Immunoblotting of active β-catenin in control (*cont*) and anti-TNF-α antibody-treated (*αTNF-α*) *Gan* mouse tumors.

cancer cells.[25] It is therefore possible that the decreased level of TNF-α in the GF-*Gan* and EP4i-*Gan* mouse stomachs resulted in suppression of Wnt signaling.

K19-Wnt1 mice develop preneoplastic lesions caused by promotion of Wnt signaling by macrophage-derived TNF-α[25] (Supplementary Table 1). Importantly, the number of preneoplastic lesions decreased significantly in the GF-*K19-Wnt1* mice compared with the SPF-*K19-Wnt1* mice (Figure 6*D*), suggesting that bacterial colonization is important for macrophage recruitment, which triggers TNF-α-induced Wnt promotion.

We next investigated whether TNF-α promotes Wnt signaling in the gastric tumor tissues. Treatment of the SPF-*Gan* mice with an anti-TNF-α neutralizing antibody resulted in down-regulation of TNF-α in tumors, possibly caused by suppression of macrophage activation (Figure 6*E*). Importantly, expression of EphB3, a Wnt-target gene, was also down-regulated. Consistently, the active β-catenin level decreased in gastric tumors by TNF-α inhibition (Figure 6*F*). These results indicate that macrophage-derived TNF-α enhances Wnt activity in gastric tumors, which may promote gastric tumorigenesis.

Discussion

Accumulating evidence has indicated that infection-associated inflammation plays an important role in cancer development.[2] Bacterial infection stimulates toll-like receptors, which induces activation of the nuclear factor-κB (NF-κB) pathway. The activation of NF-κB causes tumor promotion through induction of growth factors and suppression of apoptosis.[26] NF-κB activation

also induces COX-2 expression, which is followed by induction of the PGE_2 pathway. The COX-2/PGE_2 pathway plays a key role in intestinal tumorigenesis.[8,9] These results indicate that infection plays a key role in the activation of the NF-κB and PGE_2 pathways, which promotes tumorigenesis. In the present study, we infected *H felis* at 30 weeks of age to examine the effect of infection in tumorigenesis because we found a significant suppression of tumorigenesis in GF-*Gan* stomach at 30 weeks of age. Importantly, *H felis* infection in GF-*Gan* mice induced gastric tumor development by 55 weeks. Accordingly, the present study indicates that bacterial infection is still required for tumorigenesis even after the induction of the PGE_2 pathway.

It has been shown that PGE_2 signaling through EP4 receptor is important for intestinal tumorigenesis through the activation of epidermal growth factor receptor.[10] The current results also showed EP4 to play an important role in tumorigenesis. Bacterial colonization and EP4 signaling cooperatively induce expression of macrophage-tropic chemokine CCL2. It has been shown that CCL2 signaling is important for macrophage infiltration in colon cancers in the intestinal tumorigenesis.[22] Accordingly, it is possible that expression of CCL2 induced by bacterial colonization and EP4 signaling is important for macrophage recruitment in gastric tumorigenesis.

Intestinal commensal bacteria stimulate the toll-like receptors in the mucosa, which is important for the proliferation of undifferentiated epithelial cells.[16] Moreover, macrophages are an important niche component for the proliferation of intestinal progenitor cells in the tissue repair process.[27] Accordingly, it is possible that the innate immune response to commensal bacteria is important for the proliferation of tumor epithelial cells through macrophage recruitment. On the other hand, acquired immunity by T cells is essential for *H felis*-associated gastric pathology.[28] It is thus possible that T cells play a role in gastric tumorigenesis in *H felis*-infected GF-*Gan* mice. However, hyperplasia still developed, and the macrophages were polarized to M2, in the SPF-*Rag2*$^{-/-}$ *K19-C2mE* mouse stomach[29] (Supplementary Figure 5*D*), suggesting that increased PGE_2 levels and commensal flora can trigger these gastric phenotypes without T-cell response.

We previously showed that macrophage-derived TNF-α promotes Wnt signaling activity in gastric cancer cells, which contributes to gastric tumorigenesis.[25] Moreover, Wnt activation levels correlate with the incidence of intestinal tumorigenesis in *Apc* knockout mice,[30] and promotion of Wnt signaling activity may play an important role in malignant progression.[31] In the present study, inhibition of TNF-α resulted in a decrease in Wnt signaling activity in gastric tumors, confirming that TNF-α functions as a Wnt promoting factor in vivo. Notably, Wnt activity in the gastric tumors of GF-*Gan* mice was lower than that of SPF-*Gan* mice, which may have been caused by a decreased level of macrophage-derived TNF-α. Accordingly, it is possible that TNF-α-dependent Wnt promotion is one of the important mechanisms by which macrophages induce tumorigenesis, which is triggered by bacterial colonization and EP4 signaling.

The COX-2/PGE_2 pathway has been shown to suppress the T helper 1 immune response in the *H pylori*-infected stomach.[32] Notably, nonsteroidal anti-inflammatory drug treatment suppresses gastric carcinogenesis in the insulin-gastrin transgenic (INS-GAS) gastric tumor model mice. However, nonsteroidal anti-inflammatory drug treatment enhances gastritis in *Helicobacter*-infected INS-GAS mice, which may promote gastric tumorigenesis.[17] These results suggest that the PGE_2 pathway suppresses infection-associated carcinogenesis, which appears to be inconsistent with the present results. However, the present results indicate that commensal flora with low bacterial counts can elicit gastritis when the mucosal PGE_2 level is increased, and such commensal flora and PGE_2-dependent inflammation are important for gastric tumorigenesis. It is therefore possible that the role of PGE_2 for immune responses and tumorigenesis varies according to the level of infection status, such as exogenous aggressive infection by *Helicobacter* or commensal colonization, although this remains to be investigated.

In conclusion, bacterial infection or colonization, in cooperation with PGE_2 signaling through the EP4 receptor, induces expression of CCL2, resulting in macrophage recruitment to gastric mucosa. TNF-α produced by macrophages promotes Wnt signaling in the tumor cells, which may promote gastric tumorigenesis. Accordingly, the eradication and inhibition of the PGE_2 pathway may be an effective strategy for preventing gastric cancer development.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Depletion of Indigenous Bacteria

To deplete indigenous bacteria in the stomach, specific pathogen free (SPF)-*Gan* (*Gan* for Gastric neoplasia) mice (n = 5) were administered ampicillin (1 g/L; Sigma, St. Louis, MO), vancomycin (500 mg/L; Sigma), neomycin sulfate (1 g/L; Sigma), and metronidazole (1g/L; Sigma) in drinking water as previously described.[1] Gastric tumors were examined by x-ray computerized tomography using a LaTheta LCT-100 instrument (Aloka, Tokyo, Japan) after 0, 2, and 4 weeks of antibiotic administration. The mean tumor area was calculated from computerized tomography images using a National Institute of Health (NIH) Image J software program (Bethesda, MD).

Histology and Immunohistochemistry

Stomach tissue specimens were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 4-μm thickness. Frozen sections were used for CD4 immunostaining. The construction of SPF-*Rag2*$^{-/-}$*K19-C2mE* mice was described previously.[2] Antibodies for β-catenin (Sigma), mannose receptor (AbD Serotec, Raleigh, NC), CD3ε (Santa Cruz Biotechnology, Santa Cruz, CA), and CD4 (BD Pharmingen, Columbus, NE) were used as the primary antibodies. Alexa Fluor 594 or Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR) was used as the secondary antibody. Infection of *Helicobacter felis* in the gastric glands was confirmed using hematoxyin-stained paraffin sections of the *H felis*-infected germfree-*Gan* mouse stomach at $\times$1000 magnification.

Expression Analysis of M2 Macrophage Markers

The expression profiles of M2 macrophage markers Ym1, Ym2, arginase 1 (Arg1), and transforming growth factor-β1 in *Gan*, *K19-C2mE*, and wild-type mouse stomachs were downloaded from the National Center for Biotechnology Information Gene Expression Omnibus (GEO; accession number GSE16902). These expression data were transformed to log10 ratios to the average of wild-type mouse samples.

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Supplementary Table 1. Genotypes and Phenotypes of Transgenic Mouse Models

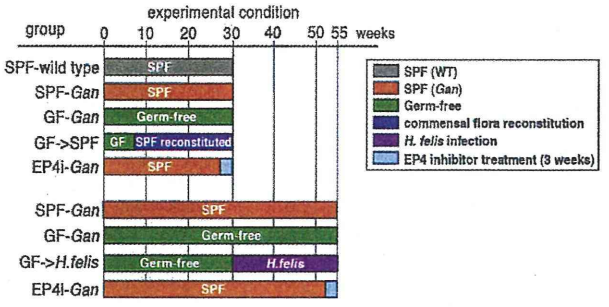
Strain name	Transgene(s)	Phenotypes in the glandular stomach (reference)
K19-Wnt1	*Wnt1*	Activation of canonical Wnt signaling Small preneoplastic lesions consisting of Wnt-promoted epithelial cells with macrophage infiltration and activation.[3]
K19-C2mE	*Ptgs2* and *Ptges*, encoding COX-2 and mPGES-1, respectively	Activation of PGE_2 pathway Hyperplasia consisting of TFF2-positive metaplastic mucous cells associated with inflammatory infiltration.[2,4]
K19-Wnt1/C2mE (*Gan* for Gastric neoplasia)	*Wnt1*, *Ptgs2*, and *Ptges*	Activation of both Wnt and PGE_2 pathways Development of dysplastic tumors with the infiltration of macrophages.[3]

PGE_2, prostaglandin E_2; COX-2, cyclooxygenase-2; *Ptgs2*, gene symbol for mouse COX-2; mPGES-1, microsomal prostaglandin E synthase-1; *Ptges*, gene symbol for mouse mPGES-1; TFF2, trefoil factor 2.

Supplementary Table 2. Results of Germfree Monitoring Tests

Samples	Feces			Bedding			Swab	
Media	TGC[a]		PDB[b]	TGC		PDB	PDB	
Temp[c]	37	RT	RT	37	RT	RT	RT	Fecal smear
Mouse age, wks								
2	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	—	—
31	—	—	—	—	—	—	—	—
45	—	—	—	—	—	—	—	—
50	—	—	—	—	—	—	—	—
55	—	—	—	—	—	—	—	—

RT, room temperature; Temp, temperature.
[a]Thioglycollate medium.
[b]Potato dextrose broth.
[c]Samples were incubated in TGC at 37°C or at room temperature or in PDB at room temperature.



Supplementary Figure 1. A schematic illustration of the experimental schedule. The gastric phenotypes of specific pathogen free (SPF)-*Gan* and germfree (GF)-*Gan* mice were examined at 30 and 55 weeks of age. GF-*Gan* mice were reconstituted with commensal flora at 7 weeks of age, and gastric phenotypes were examined at 30 weeks of age. The EP4 inhibitor was administered for 3 weeks from 27 or 52 weeks of age. GF-*Gan* mice were infected with *Helicobacter felis* at 30 weeks of age, and infected mice were examined at 55 weeks of age.

Supplementary Table 3. List of Excluded Pathogens in Animal Room 4 of SPF Facility, Kanazawa University

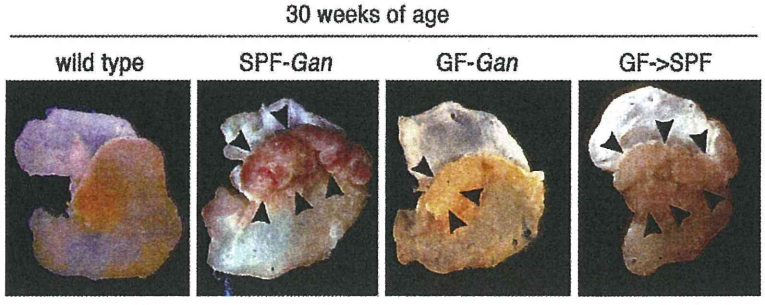
Bordetella bronchiseptica
Citrobacter rodentium
Corynebacterium kutscheri
Mycoplasma pulmonis
Pasteurella pneumotropica
Salmonella spp
Streptococcus pneumoniae
Pseudomonas aeruginosa
Helicobacter hepaticus
Clostridium piliforme
Ectromelia virus
Sialodacryoadenitis virus (SDAV)
Hanta virus
Lymphocytic choriomeningitis virus (LCMV)
Mouse hepatitis virus
Sendai virus
Intestinal protozoas (*Giardia muris*, *Spironucleus muris*, *Tritrichomonas muris*, *Octomitus pulcher*, Coccidiosis)
Helminths (*Aspiculuris tetraptera*, *Syphacia obvelata*)
Parasites (*Myobia* sp, *Polyplaxserrata*)

NOTE. All SPF mice used in the present study were raised in this SPF room.
SPF, specific antigen free.

Supplementary Table 4. Primer Sequences for Real-time RT-PCR

Gene name	Forward primer	Reverse primer
COX-2	GTGTGCGACATACTCAAGCAGGA	TGAAGTGGTAACCGCTCAGGTG
mPGES-1	CTGCAGCACACTGCTGGTCA	CTCCACATCTGGGTCACTCCTGTA
TNF-α	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
IL-1β	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
IL-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC
KC(CXCL1)	GCTTGAAGGTGTTGCCCTCAG	AAGCCTCGCGACCATTCTTG
MIP-2(CXCL2)	GCGCTGTCAATGCCTGAAGA	TTTGACCGCCCTTGAGAGTG
CCL2	GCATCCACGTGTTGGCTCA	CTCCAGCCTACTCATTGGGATCA
CCL3	TGAAACCAGCAGCCTTTGCTC	AGGCATTCAGTTCCAGGTCAGTG
CCL4	CCATGAAGCTCTGCGTGTCTG	GGCTTGGAGCAAAGACTGCTG
CCL5	ACCAGCAGCAAGTGCTCCAA	TGGCTAGGACTAGAGCAAGCAATG
CCL7	GCATCCACATGCTGCTATGTCA	GATGGGCTTCAGCACAGACTTC
CCL8	TGCCTGCTGCTCATAGCTGTC	GACATACCCTGCTTGGTCTGGAA
EP4	GTGGTGCTCATCTGCTCCATTC	CTGCAAATCTGGGTTTCTGCTG
CD44	TTTAACCTATATGCAGCAAGCCACT	CAGAATCATCACCACTATGGCAAG
EphB3	AGCTGTGAATATCACCACCAACCA	TGACTCCATTAGGCCGCTCTG

COX-2, cyclooxygenase-2; IL-1β, interleukin-1β; IL-6, interleukin-6; mPGES-1, microsomal prostaglandin E synthase-1; RT-PCR, reverse-transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α; KC, keratinocyte-derived chemokine; MIP-2, macrophage inflammatory protein-2; CXCL, chemokine (C-X-C motif) ligand; CCL, chemokine (C-C motif) ligand; EP4, PGE$_2$ receptor subtype 4; EphB3, Eph receptor B3.



Supplementary Figure 2. Representative photographs of gastric tumor phenotypes at 30 weeks of age. Wild-type mouse stomach, gastric tumors of SPF-*Gan* mouse, GF-*Gan* mouse, and GF-*Gan* mouse reconstituted with commensal bacteria (GF->SPF) are shown (from left to right). *Arrowheads* indicate gastric tumors in SPF-*Gan*, GF-*Gan*, and GF->SPF *Gan* mice.