

STAT3-Driven Upregulation of TLR2 Promotes Gastric Tumorigenesis Independent of Tumor Inflammation

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<http://dx.doi.org/10.1016/j.ccr.2012.08.010>

SUMMARY

Gastric cancer (GC) is associated with chronic inflammation; however, the molecular mechanisms promoting tumorigenesis remain ill defined. Using a GC mouse model driven by hyperactivation of the signal transducer and activator of transcription (STAT)3 oncogene, we show that STAT3 directly upregulates the epithelial expression of the inflammatory mediator Toll-like receptor (TLR)2 in gastric tumors. Genetic and therapeutic targeting of TLR2 inhibited gastric tumorigenesis, but not inflammation, characterized by reduced proliferation and increased apoptosis of the gastric epithelium. Increased STAT3 pathway activation and TLR2 expression were also associated with poor GC patient survival. Collectively, our data reveal an unexpected role for TLR2 in the oncogenic function of STAT3 that may represent a therapeutic target in GC.

INTRODUCTION

Gastric cancer (GC) is the second most lethal cancer worldwide (Parkin et al., 2005) and represents a growing number of cancers, such as colon, lung, liver, and prostate, that are associated with inflammation (Gao et al., 2005; Kawada et al., 2006; Ogata et al., 2006; Walser et al., 2008). Although the molecular mechanisms underlying the pathogenesis of these cancers remain unclear, a causal correlation has been established between inflammation triggered by microbes and gastrointestinal cancers, as evidenced by chronic gastritis caused by infection with the Gram-negative pathogen *Helicobacter pylori* being a major risk factor for human GC (Uemura et al., 2001).

The involvement of pathogenic microbes in gastrointestinal inflammation and carcinogenesis has implicated Toll-like receptors (TLRs), a key family of microbial sensors of the host innate and adaptive immune systems (Kawai and Akira, 2010), in mediating chronic inflammatory responses that promote tumorigenesis. For instance, genetic ablation of the common TLR and interleukin (IL)-1 receptor family signaling adaptor MyD88 in mice alleviates intestinal tumorigenesis induced in *Apc*^{Min/+} mice (Rakoff-Nahoum and Medzhitov, 2007). With respect to human gastric disease, *TLR2* and *TLR4* gene expression is elevated in *H. pylori*-positive gastritis patients (Uno et al., 2007), and *TLR2* and *TLR4* gene polymorphisms are associated with an increased GC risk (Hold et al., 2007; Tahara et al., 2007). However, the

Significance

Hyperactivation of the STAT3 oncogene is a hallmark of many epithelial tumors, including gastric, however, the full spectrum of actions by which STAT3 elicits its oncogenicity remains ill defined. We have discovered that in gastric epithelial cells, STAT3 directly upregulates the expression of the innate immune pathogen recognition receptor TLR2. While gastric tumorigenesis is associated with inflammation, we unexpectedly revealed that TLR2 promoted gastric epithelial cell survival and proliferation rather than tumor inflammation. Given the emergence of TLR signaling cascades as key oncogenic drivers in various tumors also characterized by STAT3 hyperactivation (e.g., liver, colon), our studies suggest that selective blockade of TLR2 may provide a therapeutic approach to suppress tumorigenesis.

expression status of these TLRs in human GC is unclear. In addition to the link between TLRs and the inflammatory response, more recent studies have revealed that epithelial-expressed TLRs can promote noninflammatory-related epithelial responses including cell survival, proliferation and migration (Shaykhiev et al., 2008), and angiogenesis (West et al., 2010). While these findings constitute compelling circumstantial evidence for the involvement of TLRs in GC and other inflammatory-related cancers, a causal role for specific TLRs in these cancers remains to be defined.

We have previously established the *gp130^{F/F}* mouse, which by 6 weeks of age spontaneously develops gastric inflammation and intestinal-type gastric tumors (Tebbutt et al., 2002). These mice are homozygous for a knockin phenylalanine substitution at tyrosine 757 in the cytoplasmic domain of the IL-6 cytokine family receptor signaling subunit gp130, which disrupts the negative feedback mechanism on gp130 signaling mediated by suppressor of cytokine signaling (SOCS)3 (Tebbutt et al., 2002). The gastric phenotype of *gp130^{F/F}* mice is driven by hyperactivation of the oncogenic latent transcription factor STAT3 via the IL-6 cytokine family member, IL-11 (Ernst et al., 2008; Jenkins et al., 2005), the clinical relevance of which is underscored by upregulated IL-11 production and STAT3 hyperactivation being common traits of human GC (Ellmark et al., 2006; Gong et al., 2005; Yakata et al., 2007).

Although the full spectrum of molecular mechanisms underlying the potent oncogenic properties of STAT3 in cancers remains undetermined, the oncogenicity of STAT3 has been attributed to, at least in part, its direct transcriptional upregulation of genes, which promote angiogenesis, cell cycle progression, and cell survival (Bromberg et al., 1999). Moreover, the complex role of STAT3 in cancer is evidenced by the fact STAT3 can promote both a protumorigenic chronic inflammatory microenvironment (Bollrath and Greten, 2009) and conversely suppress antitumor innate and adaptive immune responses (Kortylewski et al., 2005). Regarding the former, emerging evidence invokes functional overlap between STAT3 and nuclear factor- κ B (NF- κ B) oncogenic and inflammatory signaling pathways in driving inflammation-associated cancers (Bollrath and Greten, 2009; Yang et al., 2007). Here, we investigate whether crosstalk between STAT3 and TLR signaling contributes to the molecular pathogenesis of GC.

RESULTS

Augmented TLR2 Expression in Gastric Tumors Correlates with STAT3 Hyperactivation

We initially identified changes in the expression profile of genes associated with the TLR network during gastric tumorigenesis in *gp130^{F/F}* mice by using RT² Profiler PCR Arrays specific for 84 key TLR-associated genes. The majority of genes upregulated in gastric antrum tumor tissue of 24-week-old *gp130^{F/F}* mice compared to tumor-free antrum tissue from *gp130^{+/+}* mice were NF- κ B-regulated, including *Tnfa* and *Il1b* proinflammatory cytokine genes, as well as *Tlr2*, previously implicated in human GC (El-Omar et al., 2003; Tahara et al., 2007) (Table S1 available online). Independent quantitative real-time PCR (qPCR) assays confirmed that among TLRs and other pattern recognition receptors (PRRs) implicated in gastric disease,

including Nod1 and Nod2 (Rosenstiel et al., 2006), only *Tlr2* messenger RNA levels were significantly upregulated in *gp130^{F/F}* gastric tumor tissue compared to gastric tumor and tumor-free tissue from *gp130^{F/F}:Stat3^{-/-}* mice and gastric tumor-free tissue from *gp130^{F/F}:Il11r^{-/-}* mice displaying reduced STAT3 activation (Ernst et al., 2008) and expression of STAT3-target genes, *Stat3* and *Il11* (Figures 1A and S1A). In support of the notion that TLR-induced responses are associated with gastric tumorigenesis, the gastric expression of TLR-regulated proinflammatory genes *Il1b*, *Cxcl2*, and *Tnfa*, as well as the *Cd14* gene encoding the coreceptor for TLR2 and TLR4, was significantly increased in *gp130^{F/F}* mice compared to compound mutant mice (Figure S1A). The coexistent hyperactivation of STAT3, augmented IL-11 expression, and specific upregulation of TLR2 in gastric tumorigenesis was also independently observed in the transgenic K19-Wnt/C2mE (Gan) mouse model of GC that simultaneously overexpresses the cyclooxygenase-2/prostaglandin E2 and Wnt signaling pathways in the gastric mucosa (Itadani et al., 2009; Oshima et al., 2006) (Figures S1B–S1E).

The augmented gastric expression profile of specific STAT3-target genes and inflammatory mediators in *gp130^{F/F}* mice translated to human disease, since *TLR2*, *IL1B*, *CD14*, *IL8* (human homolog of *Cxcl2*), *STAT3*, and *IL11* were significantly upregulated in tumor tissue from GC patients compared to adjacent nontumor tissue and antrum tissue from healthy individuals (Figure 1B). Furthermore, analysis of 78 primary human gastric tumors (Ooi et al., 2009) revealed an overall positive correlation between increased activation of the STAT3 pathway and elevated *TLR2* expression (Figure 1C). Among these 78 gastric tumors, we also compared 25 tumors in which STAT3 activation-*TLR2* gene expression was high against 21 tumors in which STAT3 activation-*TLR2* expression was low. These 46 GC patients segregated into two distinct groups whereby those displaying a combined high STAT3-*TLR2* status had a significantly impaired overall survival compared to patients with a combined low STAT3-*TLR2* status, thus suggesting that STAT3 hyperactivation and *TLR2* upregulation are poor prognostic factors in GC (Figure 1D). In further support of a STAT3-*TLR2* axis in human GC, analysis of 18 human GC cell lines previously characterized by gene expression signatures representing the activity status of various oncogenic pathways, including STAT3 (Ooi et al., 2009), revealed a significant positive correlation between the levels of STAT3 pathway activation and *TLR2* gene expression (Figure 1E).

STAT3-Mediated Upregulation of TLR2 in Gastric Epithelial Cells

Since the gastric epithelium plays a key role in modulating the immune response to microbes, we next evaluated whether TLR2 was transcriptionally regulated by STAT3 in gastric epithelial cells. Overexpression of the hyperactive STAT3-C mutant (Bromberg et al., 1999) in mouse gastric epithelial IMGE-5 cells induced gene expression for *Sox3*, a known STAT3-target gene (Table S2), and *Tlr2*, but not other TLR family members (Figures 2A and 2B), and this result was also confirmed in human GC epithelial cell lines MKN-28 and AGS (Figures 2C and 2D). The transcriptional induction of TLR2 by STAT3-C was further confirmed by the STAT3-C-driven transactivation

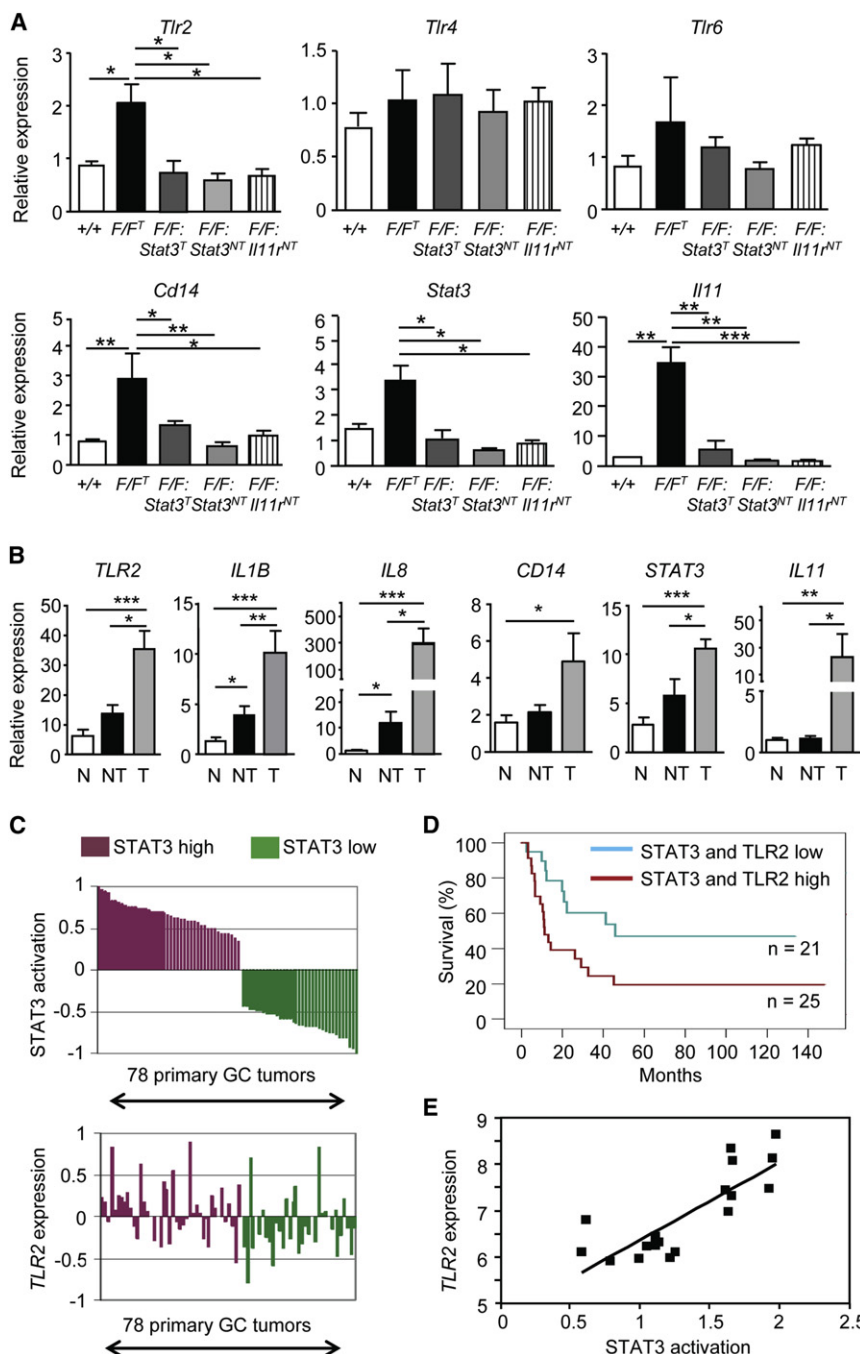


Figure 1. Augmented STAT3 Activation Correlates with Increased Expression of TLR2 in Gastric Tumors of *gp130^{F/F}* Mice and Human GC

(A) qPCR expression analyses on antral gastric tumor (T) or nontumor (NT) tissue of 24-week-old mice of the indicated genotypes. Expression data from at least five samples per genotype are shown following normalization for 18S expression. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

(B) qPCR of the indicated genes in human gastric antral biopsies corresponding to normal (N) tissue from healthy individuals (*n* = 6), as well as tumor (T) and matched adjacent nontumor (NT) tissue from GC patients (*n* = 12). Expression data are shown following normalization for 18S. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

(C) Top panel, STAT3 activation levels in 78 human gastric tumors and tumors with positive STAT3 activation scores (STAT3 high), and those with negative STAT3 activation scores (STAT3 low) are indicated by different color schemes. Bottom panel, TLR2 gene expression levels in 78 tumors in the same order as the top panel. The y axis values are presented on a log scale, with positive and negative values representing high and low TLR2 gene expression, respectively. Spearman rank correlation coefficient (*r*) = 0.3395. *p* = 0.0025.

(D) Kaplan-Meier survival analysis of a subset of 46 human GC patients with nonzero STAT3 activation scores stratified into two groups based on either low (*n* = 21) or high (*n* = 25) combined STAT3-TLR2 status.

(E) Correlation between STAT3 activation and TLR2 expression in human GC cell lines. STAT3 activation levels in 18 human GC cell lines (■) were determined based on the average STAT3 pathway activation scores (x axis). The y axis represents the log2-transformed TLR2 gene expression value. *r* = 0.6633, *p* = 3.86 × 10⁻⁵.

In (A and B), data are presented from replicate analysis as the mean ± SEM. See also Figure S1 and Table S1.

of the full-length TLR2 promoter-luciferase reporter TLR2-2391 (Figure 2E). Moreover, bioinformatic analyses identified five putative STAT3-binding sites in the 5' promoter region of TLR2 (Figure 2F), and chromatin immunoprecipitation on either IL-11-stimulated gastric epithelial cells or *gp130^{F/F}* tumor tissue revealed that tyrosine-phosphorylated (pY) STAT3 was recruited to the TLR2 5' region spanning the putative STAT3 site at bases -1241/-1249 (Figures 2F-2H), thus suggesting that TLR2 is a direct STAT3 target gene. In support of these data, STAT3-C did not transactivate either the truncated (TLR2-1248) or site-directed mutant (TLR2-TT > AA) TLR2-specific

promoter reporters that disrupted the -1241/-1249 STAT3 site (Figure 2E). The functional relevance of these findings was validated by the augmented production of IL-6 and IL-1β in primary gastric epithelial cells from *gp130^{F/F}* compared

TLR2 Promotes Gastric Tumorigenesis in *gp130^{F/F}* Mice

To genetically define a causal role for TLR2 in STAT3-driven gastric tumorigenesis, we generated *gp130^{F/F}* mice lacking TLR2 (*gp130^{F/F};Tlr2^{-/-}*). The stomachs of 24-week-old *gp130^{F/F};Tlr2^{-/-}* mice were noticeably smaller in size compared to those of age-matched *gp130^{F/F}* littermates (Figure 3A), analogous to the reduced stomach size observed previously in *gp130^{F/F};Stat3^{-/-}* mice due to suppression of STAT3-driven hyperplasia

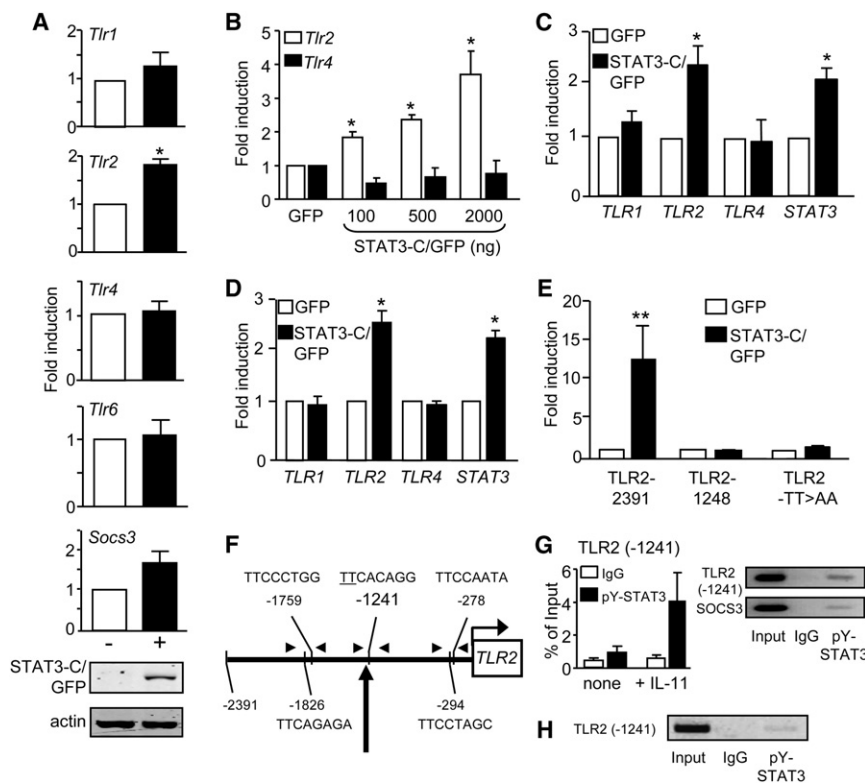


Figure 2. STAT3 Directly Regulates TLR2 Gene Expression in Gastric Epithelial Cells

(A) qPCR of *Socs3* and *Tlr* genes in gastric epithelial IMGE-5 cells stably-transfected with STAT3-C/green fluorescent protein (GFP) (+) or GFP alone (-). Data from three independent experiments are shown following normalization for 18S. *p < 0.05. Cell lysates were immunoblotted with STAT3 and actin antibodies with exogenous STAT3-C/GFP fusion protein (~120kDa) detected in transfected cells only.

(B) qPCR of endogenous *Tlr2* and *Tlr4* gene expression in IMGE-5 cells transiently-transfected with increasing amounts of STAT3-C/GFP. Data from three independent experiments are shown following normalization for 18S. *p < 0.05 versus cells transfected with GFP alone.

(C and D) qPCR of endogenous gene expression for TLR family members and STAT3 in human MKN-28 (C) and AGS (D) cells transiently-transfected with 500ng STAT3-C/GFP or GFP alone. Data from four independent experiments are shown for each cell line following normalization for *ACTIN*. *p < 0.05.

(E) Human epithelial 293 cells were cotransfected with luciferase reporter plasmids for the full-length TLR2 promoter (TLR2-2391), the truncated TLR2 promoter (TLR2-1248), and the site-directed mutant TLR2 promoter (TLR2-TT > AA) together with the STAT3-C/GFP construct or an empty GFP vector (GFP). Data from four independent experiments are expressed as the mean ± SEM-fold change relative to TK-renilla normalized reporter activity in GFP-transfected cells. **p < 0.01.

(F) Schematic diagram of the *TLR2* gene promoter. The vertical arrow indicates where the TLR2 promoter fragment was truncated in the TLR2-1248 construct and the underlined "TT" bases at the -1241/-1248 STAT3 site (bold type) were mutated to "AA" in the TLR2-TT > AA construct. Arrowheads depict primers (used in ChIP assays) spanning five putative STAT3 binding sites in the TLR2 promoter.

(G) Cells were incubated without or with IL-11 for 1 hr and subjected to ChIP assays using pY-STAT3 or IgG isotype control antibodies. The graph represents quantification by qPCR of the immunoprecipitated TLR2 promoter fragment containing the -1241/-1248 STAT3 site bound to each antibody. Data from four independent experiments are expressed as the mean ± SEM % of input DNA. Gels represent semiquantitative PCR of specific promoter fragments that were amplified from coprecipitated DNA from IL-11-treated cells using primers spanning the -1241 STAT3 site in the TLR2 promoter (F) and, as a control, the STAT3 binding site at position -70 in the *SOCS3* promoter.

(H) Gel representing semiquantitative PCR of ChIP assay for pY-STAT3 interaction with the TLR2 gene promoter as per (G), except on gastric tumor tissue from *gp130^{F/F}* mice.

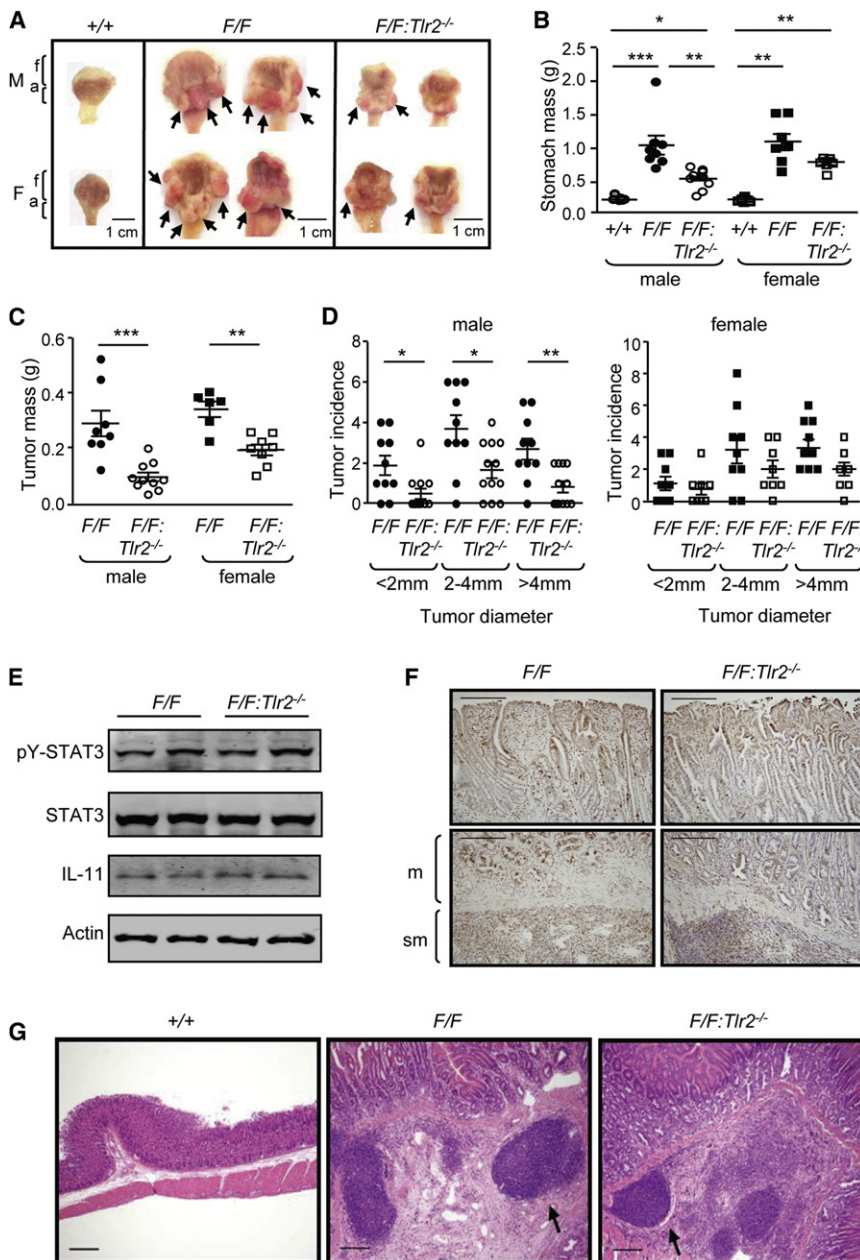
In (A)–(D), all data are presented from replicate analysis as the mean ± SEM-fold induction relative to cells transfected with GFP alone. See also Figure S2 and Table S2.

throughout the gastric epithelium (Jenkins et al., 2005). The reduction in stomach size and mass of *gp130^{F/F};Tlr2^{-/-}* mice was more pronounced in males (50% reduction in mass) than females (30%) when compared to their *gp130^{F/F}* counterparts (Figures 3A and 3B). The gastric tumor mass of *gp130^{F/F};Tlr2^{-/-}* mice was also significantly reduced (70% in male and 40% in female) compared to *gp130^{F/F}* mice of the corresponding sex (Figure 3C). The total incidence of gastric lesions in *gp130^{F/F};Tlr2^{-/-}* male (2.76 ± 0.28) and *gp130^{F/F};Tlr2^{-/-}* female (4.22 ± 0.76) mice was also lower than in *gp130^{F/F}* males (8.30 ± 0.49) and *gp130^{F/F}* females (7.50 ± 1.22), with a noticeable reduction in the numbers of large (>4 mm diameter) tumors (Figure 3D). We also note that STAT3 tyrosine phosphorylation and IL-11 expression levels in *gp130^{F/F};Tlr2^{-/-}* tumors remained elevated and were comparable to those of *gp130^{F/F}* gastric tumors (Ernst et al., 2008) (Figures 3E and 3F), consistent with the IL-11/STAT3 signaling axis being an upstream regulator of TLR2. The pathological requirement for TLR2 in gastric tumorigenesis was specific, since the gross appearance of stomachs and extent

of gastric tumorigenesis in *gp130^{F/F};Tlr4^{-/-}* male and female mice were comparable to those of *gp130^{F/F}* littermates (Figures S2B–2F).

TLR2 Promotes Gastric Epithelial Cell Survival and Proliferation, but Not Inflammation

We have previously reported that gastric tumor formation in *gp130^{F/F}* mice is characterized by chronic inflammatory cell (lymphoplasmacytoid) aggregates in the submucosa (Ernst et al., 2008). However, despite reduced gastric tumorigenesis in *gp130^{F/F};Tlr2^{-/-}* mice, histological examination of hematoxylin and eosin (H&E)-stained gastric sections from 24-week-old mice indicated that the extent of these submucosal inflammatory aggregates was comparable to that of *gp130^{F/F}* mice (Figure 3G). Further histological and immunohistochemical analyses revealed a similar composition of both innate and adaptive inflammatory cell infiltrates between the two genotypes (Figures S3A–S3C). These observations were further verified by flow cytometry, which indicated comparable



frequencies of Gr-1⁺CD11b⁺ (myeloid-derived suppressor cells [MDSC]), CD11b⁺F4/80⁺ (monocytes/macrophages), B220⁺ (B), and CD3⁺ (T) cells in the stomachs and perigastric lymph nodes of $gp130^{F/F}$ and $gp130^{F/F};Tlr2^{-/-}$ mice (Figures S3D and S3E). In addition, the activation status of B (B220⁺CD86⁺) and T (CD4⁺CD69⁺ and CD8⁺CD69⁺) cells, which are the predominant gastric tumor-infiltrating immune cells in $gp130^{F/F}$ and $gp130^{F/F};Tlr2^{-/-}$ mice, in the stomach and perigastric lymph node tissue of the two genotypes was comparable (Figure S3F), as were the percentages of TNF α - or IFN γ -producing gastric or perigastric lymph node lymphocytes (Figure S3F). Despite these observations supporting the notion that TLR2 does not promote the activation of these inflammatory cells, the expression of a subset of TLR2-regulated cytokines and chemokines

These data therefore suggest that gastric epithelial stimulation by TLR2 does not promote the infiltration or activation of inflammatory cells.

We next investigated whether the reduced gastric tumorigenesis in $gp130^{F/F};Tlr2^{-/-}$ mice correlated with any proliferative, apoptotic, and/or angiogenic changes within the gastric mucosa. By contrast to the expansion of proliferating gastric cells observed throughout the mucosal epithelium of $gp130^{F/F}$ mice, the proliferation zone in the gastric epithelium of $gp130^{F/F};Tlr2^{-/-}$ mice was more defined and resembled that of $gp130^{+/+}$ mice with no apparent proliferating-cell nuclear antigen (PCNA)-positive staining in the surface epithelium (Figures 4A and 4B). Furthermore, apoptotic TUNEL-positive cells were found predominantly within the surface gastric

epithelial region of $gp130^{F/F};Tlr2^{-/-}$ mice, whereas little to no TUNEL-positive gastric cells were detected within the mucosal epithelium of $gp130^{F/F}$ mice (Figure 4C). The extent of cellular proliferation and apoptosis within the gastric submucosal inflammatory cell infiltrates, however, was similar between $gp130^{F/F}$ and $gp130^{F/F};Tlr2^{-/-}$ mice (Figures S4A and S4B). With respect to angiogenesis, the expression of the CD31 angiogenic marker in the gastric mucosa and submucosa of $gp130^{F/F}$ and $gp130^{F/F};Tlr2^{-/-}$ tumors was comparable (Figures S4C and S4D). Collectively, these results unexpectedly suggest that TLR2 promotes gastric epithelial cell growth rather than gastric tumor inflammation or angiogenesis.

The notion that TLR2 signaling can promote cell proliferation was also confirmed in two human GC epithelial cell lines, whereby TLR2 ligand stimulation of MKN-28 and NUGC4 cells resulted in a dose-dependent significant increase in cell proliferation (Figure 4D). TLR2 ligand stimulation of these human GC cell lines also significantly upregulated the expression of numerous cell cycle progression and cell survival/antiapoptosis genes (Figures 4E and 4F), among which *CCND2*, *BCL2A1*, *IER3*, and *BIRC3* are highly expressed in human GC (Li et al., 2011; Park et al., 1997; Sasada et al., 2004; Takano et al., 2000). These observations were also supported in vivo in the $gp130^{F/F}$ tumor model, where the expression of these cell survival and cell cycle progression genes was significantly reduced in $gp130^{F/F};Tlr2^{-/-}$ tumors compared to $gp130^{F/F}$ tumors (Figure 4G).

To identify signaling pathways that facilitated the TLR2 ligand-induced proliferation of human GC cells, cells were pretreated with a series of well-documented signaling pathway inhibitors at doses shown to suppress the TLR2-driven activation of NF- κ B; phosphatidylinositol 3-kinase (PI3K)/Akt; stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK); p38; and extracellular signal-related kinase (ERK)1/2 mitogen-activated protein kinases (MAPKs) (Figure 5A). Suppression of the PI3K/Akt, ERK1/2, and JNK MAPK, or NF- κ B pathways resulted in a significant inhibition of TLR2-stimulated cell growth, whereas blocking the activity of p38 MAPK did not impair TLR2-induced cell proliferation (Figure 5B). In support of these findings, blockade of at least one of these known TLR2 signaling pathways in human GC cells significantly suppressed the TLR2-induced expression of 6/8 cell cycle regulation and survival genes (Figure 6A). These in vitro findings were also validated in vivo, where treatment of $gp130^{F/F}$ mice with the U0126 mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor suppressed the TLR2-induced expression of *Bcl2a1*, *Ccnd1*, and *c-Myc* (Figures 6B and 6C). Taken together, these results suggest that TLR2 utilizes multiple signaling cascades to promote the growth of GC cells.

TLR2-Expressing Bone Marrow-Derived Inflammatory Cells Do Not Promote Gastric Tumors

We have previously employed bone marrow chimeras to demonstrate that bone marrow-derived inflammatory cell infiltrates displaying STAT3 hyperactivation do not contribute to gastric inflammation-associated tumorigenesis (Ernst et al., 2008). However, since TLR2 was expressed on both epithelial cells (surface mucosa) and inflammatory cells (submucosa) in the stomachs of $gp130^{F/F}$ mice (Figure 7A), we investigated whether TLR2-expressing inflammatory cells played a role in gastric

tumorigenesis. Reconstitution of the hematopoietic compartment of irradiated $gp130^{F/F}$ recipient mice with bone marrow from donor $gp130^{F/F};Tlr2^{-/-}$ mice (Figure S5A) did not alleviate the size and number of gastric tumors, nor the overall size of hyperplastic stomachs of the recipient mice (Figures 7B–7E). Furthermore, the reciprocal reconstitution of $gp130^{F/F};Tlr2^{-/-}$ recipient mice with $gp130^{F/F}$ donor bone marrow did not exacerbate gastric tumorigenesis (Figures S5B and S5C). As predicted, gastric tumorigenesis and the overall appearance of stomachs of control $gp130^{F/F}$ and $gp130^{F/F};Tlr2^{-/-}$ recipient mice reconstituted with autologous $gp130^{F/F}$ and $gp130^{F/F};Tlr2^{-/-}$ donor bone marrow, respectively, were indistinguishable from their naive $gp130^{F/F}$ and $gp130^{F/F};Tlr2^{-/-}$ counterparts (Figures 7B–7E). Collectively, these data further support a role for gastric epithelial TLR2-mediated responses in promoting tumorigenesis.

Therapeutic Targeting of TLR2 in $gp130^{F/F}$ Mice Alleviates Gastric Tumorigenesis

To determine whether TLR2 may serve as a bona fide therapeutic target for GC, we next explored whether therapeutic suppression of TLR2 activity would alleviate the growth of established gastric tumors in $gp130^{F/F}$ mice. We initially examined the efficacy of the OPN-301 TLR2 blocking antibody (Meng et al., 2004) in suppressing acute TLR2-driven gastric responses by pretreating $gp130^{F/F}$ mice by intraperitoneal (i.p.) injection with OPN-301 antibody or isotype control for 30 min prior to administration with Pam₃Cys over 6 hr. qPCR confirmed that OPN-301 significantly suppressed the TLR2-dependent induction of *Cxcl2* and *Tnfa* genes in gastric antrum tissue (Figure 8A). Since the onset of gastric hyperplasia and tumors in $gp130^{F/F}$ mice occurs from 4–6 weeks of age and progresses in severity up to 24 weeks of age (Jenkins et al., 2005; Tebbutt et al., 2002), we undertook a therapeutic approach by injecting 12-week-old $gp130^{F/F}$ mice displaying established gastric disease with OPN-301 or isotype control twice weekly over 10 weeks. Notably, OPN-301 treatment substantially reduced the stomach size and overall tumor burden, including the number of gastric tumors, of $gp130^{F/F}$ mice (Figures 8B–8D). Collectively, these data demonstrate that therapeutic targeting of TLR2 suppresses both the initiation and growth of gastric tumors in $gp130^{F/F}$ mice.

DISCUSSION

Here, we reveal that uncontrolled activation of the oncogenic transcription factor STAT3 promotes gastric tumorigenesis through the specific upregulation of TLR2 in the gastric epithelium. Surprisingly, the composition and activation status of gastric inflammatory cell infiltrates in tumors of $gp130^{F/F}$ mice was comparable in the presence or absence of TLR2, suggesting that TLR2 signaling does not contribute to tumor-associated gastric inflammation. Rather, we observed that TLR2 promoted cell survival and proliferation in the mouse gastric tumor epithelium. Furthermore, our bone marrow reconstitution studies imply a protumorigenic role for TLR2 in a tumor-cell intrinsic rather than extrinsic (i.e., inflammatory cell) manner, consistent with our previous data for STAT3 (Ernst et al., 2008). A role for the STAT3-TLR2 axis in gastric tumorigenesis is also supported by the comparable reductions observed in both stomach size and tumor mass upon either ablation of TLR2 or genetically reducing

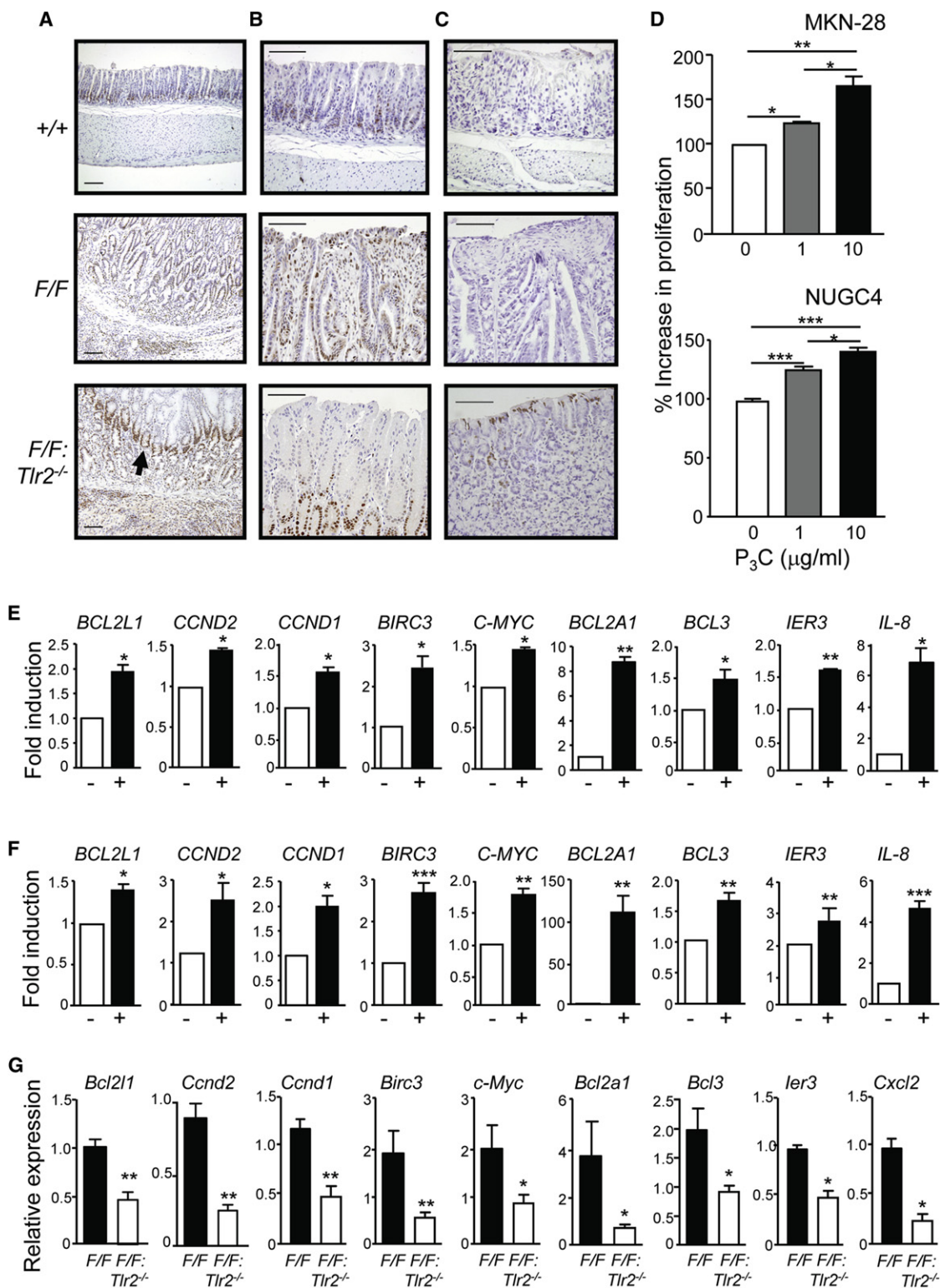


Figure 4. TLR2 Promotes Gastric Epithelial Tumor Cell Survival and Proliferation

(A and B) Representative photomicrographs showing PCNA-stained cross-sections under low power (A) and high power (B) magnification through the antral region of stomachs from 24-week-old mice of the indicated genotypes. In (A), arrows point to the defined zone of proliferating mucous neck cells of the gastric epithelium. Scale bars = 100 μm.

(C) Representative photomicrographs of TUNEL-stained cross-sections through the antral stomach region from mice of the indicated genotypes. Scale bars = 100 μm.

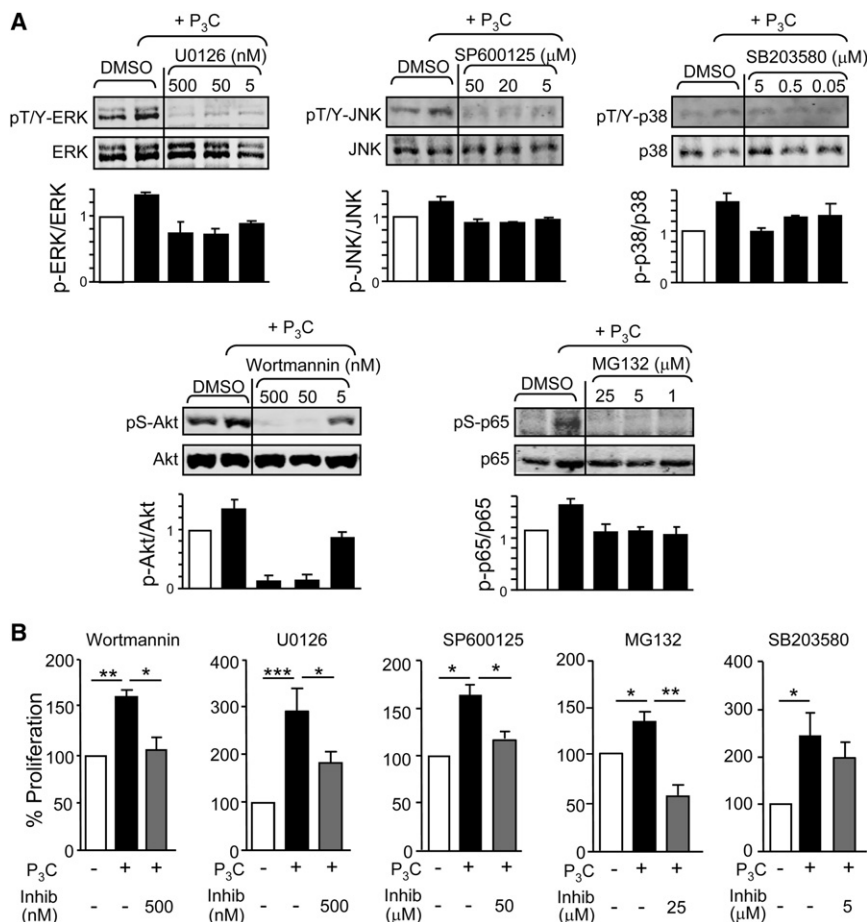


Figure 5. TLR2-Driven Cell Proliferation in Human GC Cells Occurs via Activation of Multiple Signaling Pathways

(A) Representative western blots with the indicated antibodies of lysates from MKN-28 cells pretreated with DMSO vehicle or specific pathway inhibitors at the indicated concentrations for 30 min, and then stimulated without or with 10 μg/ml Pam₃Cys for 30 min. Densitometry quantification from two independent experiments for the indicated phosphorylated signaling molecules and relative expression was determined against the corresponding amount of total protein and is presented as the mean ± SD.

(B) Proliferative response of MKN-28 cells incubated over 24 hr without (–) or with (+) Pam₃Cys (10 μg/ml) in the presence of pathway inhibitors for PI3K (Wortmannin), ERK MAPK (U0126), JNK MAPK (SP600125), NF-κB (MG132), and p38 MAPK (SB203580). Data are from four independent experiments in which cells were pretreated for 30 min with either DMSO vehicle (–) or the indicated concentrations of specific inhibitors (Inhib) and are expressed as the mean ± SEM % increase in proliferation relative to DMSO pretreated cells in the absence of Pam₃Cys. *p < 0.05, **p < 0.01, and ***p < 0.001 versus Pam₃Cys-incubated cells pretreated with DMSO.

the level of STAT3 activation in *gp130^{F/F}* mice, the latter of which diminishes the STAT3-driven hyperplastic response throughout the entire gastric epithelium (Jenkins et al., 2005). Collectively, our findings not only support emerging evidence for a broader role of TLRs in regulating noninflammatory processes, including epithelial cell proliferation and survival (Shaykhiev et al., 2008), but also demonstrate in a STAT3-driven cancer model that TLR2 is required for tumorigenesis in a cell autonomous manner independently of inflammation.

Another key finding of our current study was that TLR2 stimulation promoted the proliferation of human gastric epithelial (cancer) cells, as well as the transcriptional induction of numerous genes associated with cell cycle regulation and antiapoptosis, via multiple downstream signaling pathways, namely PI3K/Akt, ERK1/2 and JNK MAPKs, and NF-κB. The notion that these signaling pathways represent an important mecha-

nism promoting gastric epithelial cell growth is supported by previous observations that blockade of either the PI3K/Akt, ERK1/2 MAPK, or NF-κB pathways, which are frequently overexpressed and/or overactivated in human GC (Cinti et al., 2008; Liang et al., 2005; Sasaki et al., 2001), in human GC cell lines suppresses cell proliferation (Asciutti et al., 2011; Lim et al., 2001; Yokota et al., 2010). We also note that a role for the PI3K/Akt pathway in the TLR2-induced survival of epithelial cells has been previously reported (Caro et al., 2007). Furthermore, several of these antiapoptotic and cell proliferation genes (e.g., *BCL2A1*, *BIRC3*) have previously been reported to be highly expressed in human GC (Li et al., 2011; Park et al., 1997; Sasada et al., 2004; Takano et al., 2000). We note that despite a subset of these genes, such as *CCND1* and *C-MYC*, being STAT3-regulated genes (Bromberg et al., 1999; Ernst et al., 2008), their reduced expression in tumors of *gp130^{F/F};Tlr2^{-/-}* versus *gp130^{F/F}* mice occurs despite comparable STAT3 hyperactivation in tumors from both genotypes. Such a discrepancy can be reconciled by the complex transcriptional regulation of these genes by both

(D) Proliferative response of human MKN-28 and NUGC4 cells to the TLR2 ligand Pam₃Cys over 24 hr. Data are from at least three independent experiments in which cells were either untreated or treated with the indicated concentrations of Pam₃Cys, and are expressed as the mean ± SEM % increase in proliferation relative to untreated cells. *p < 0.05, **p < 0.01, ***p < 0.001.

(E and F) qPCR gene expression analyses in MKN-28 (E) and NUGC4 (F) cells stimulated (+) with TLR2 ligand (100 ng/ml) for 6 hr. Expression data from at least three independent experiments are shown following normalization for *ACTIN*. *p < 0.05, **p < 0.01, ***p < 0.001 versus untreated (–) cells.

(G) qPCR gene expression analyses in antral gastric tumor tissue of 24-week-old *gp130^{F/F}* (n = 5) and *gp130^{F/F};Tlr2^{-/-}* (n = 5) mice. Expression data are shown following normalization for *18S*. *p < 0.05, **p < 0.01.

In (E)–(G), data are presented from replicate analysis as the mean ± SEM. See also Figure S4.

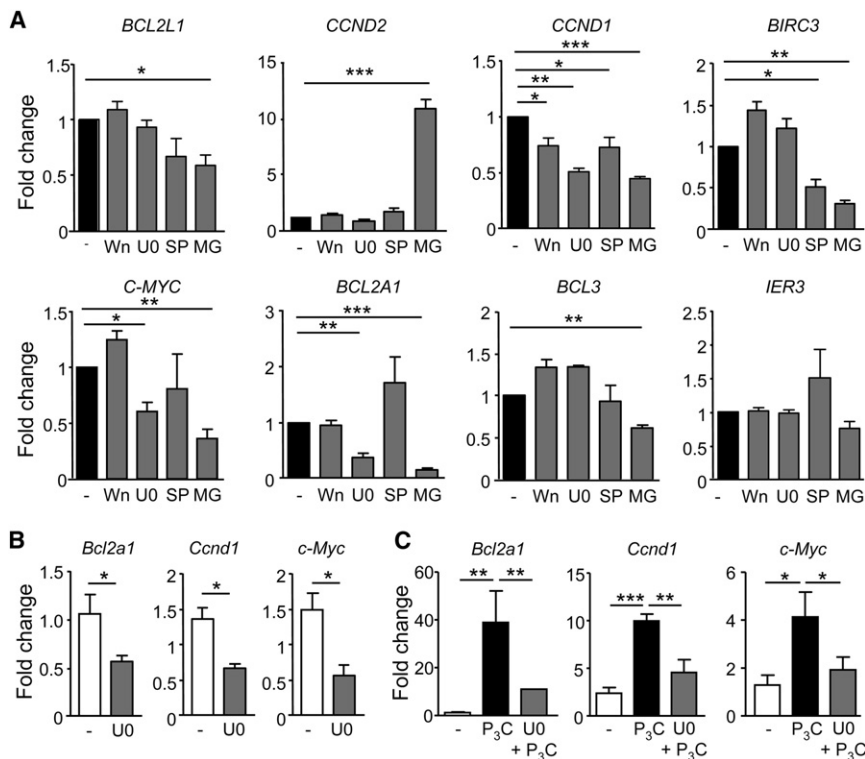


Figure 6. TLR2 Upregulates the Expression of Cell Cycle Regulation and Survival Genes in Human GC Cells via Multiple Signaling Pathways

(A) qPCR gene expression analyses in MKN-28 cells pretreated for 30 min with either DMSO vehicle (black bars; -) or specific inhibitors (gray bars) and stimulated with TLR2 ligand (100 ng/ml) for 6 hr. Expression data from three independent experiments are shown following normalization for *ACTIN* and are presented as the mean \pm SEM % relative to DMSO pretreated cells stimulated with TLR2 ligands. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus TLR2 ligand-stimulated cells pretreated with DMSO. Wn, Wortmannin; U0, U0126; SP, SP600125; MG, MG132.

(B and C) qPCR gene expression analyses in gastric tumor tissue from *gp130^{F/F}* mice that were either untreated (-, white bars) or treated with MEK inhibitor U0126 for 18 hr (U0, gray bars) (B) and either untreated (-, white bars), administered with Pam₃Cys over 6 hr (P₃C, black bars), or pretreated with U0126 for 18 hr prior to administration with Pam₃Cys for 6 hr (U0 + P₃C, gray bars) (C). Expression data from at least three samples per treatment group are shown following normalization for *18S* and are presented from replicate analysis as the mean \pm SEM fold induction. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

STAT3-dependent and STAT3-independent mechanisms, the latter of which have been widely reported for *CCND1* and *C-MYC* to involve transcriptional regulatory factors such as NF- κ B, activating transcription factor 2, activator protein 1, and cAMP response element-binding (Beier et al., 1999; Boulon et al., 2002; Joyce et al., 1999; Kim et al., 2000; Vartanian et al., 2011). Since these transcription factors are also activated by TLR2 (Kawai and Akira, 2006; Mellett et al., 2011; Wang et al., 2011), the reduced gene expression in *gp130^{F/F}; Tlr2^{-/-}* gastric tumors may be a consequence of, at least in part, the loss of gastric TLR2-mediated activation of such transcription factors.

Several recent studies support a role for TLR2 in promoting tumorigenesis, albeit by divergent mechanisms, which most likely reflect the complex role of TLR2 in innate and adaptive immunity, as well as nonimmune (e.g., epithelial) cellular responses. For instance, TLR2-dependent NF- κ B signaling has been associated with WEHI-3B tumor cell survival and disease progression in an in vivo mouse model of myelomonocytic leukemia (Shcheblyakov et al., 2011). In addition, TLR2 can promote the proliferation of hepatocarcinoma tumor cells in a bacterial (*Listeria monocytogenes*) infection-associated ectopic tumor model independent of influencing immunosuppressive immune cells (e.g., MDSC) (Huang et al., 2007). By contrast, in another ectopic tumor model involving metastatic melanoma cells, it was proposed that TLR2 promoted tumor metastasis via STAT3 activation leading to suppression of antitumor immunity (Yang et al., 2009), an oncogenic mechanism previously assigned to STAT3 (Kortylewski et al., 2005). However, the role of TLR2 in STAT3-mediated gastric tumorigenesis is unlikely to involve such a mechanism; since we have previously

demonstrated that gastric tumorigenesis in *gp130^{F/F}* mice is independent of antitumor immunity (Ernst et al., 2008). Furthermore, we demonstrate here that gastric STAT3 hyperactivation in *gp130^{F/F}* mice is unaffected by targeting TLR2, and bone marrow reconstitution studies confirm that gastric tumor growth is independent of TLR2 expression on hematopoietic-derived tumor-infiltrating immune cells.

While our current study demonstrates a causal role for TLR2 in gastric tumorigenesis, the role of TLRs in other inflammation-associated cancers, in particular colon, is controversial. For instance, our work presented here builds upon recent reports that the MyD88 adaptor protein, which is essential for TLR signaling, promotes intestinal tumorigenesis (Rakoff-Nahoum and Medzhitov, 2007; Schiechl et al., 2011). Similar to our current findings for gastric tumorigenesis, reduced intestinal tumor formation in MyD88-deficient *Apc^{Min/+}* mice was associated with higher numbers of apoptotic tumor cells without any change in the frequency of infiltrating leukocytes (Rakoff-Nahoum and Medzhitov, 2007). In addition, MyD88-deficiency protected mice from colonic tumor development but not inflammation in a colitis-associated colorectal cancer (CAC) model induced by oxazolone and azoxymethane (AOM) (Schiechl et al., 2011). On the other hand, others have reported that *Myd88^{-/-}* mice are more susceptible to AOM/dextran sodium sulfate (DSS) CAC (Salcedo et al., 2010). The contrasting roles for TLR2 in CAC induced by AOM/DSS have also been reported, with one study suggesting that TLR2 is dispensable for colonic tumorigenesis (Salcedo et al., 2010), whereas another that tumorigenesis was exacerbated in *Tlr2^{-/-}* mice (Lowe et al., 2010). Collectively, these discrepancies most likely reflect inherent differences in the experimental approaches

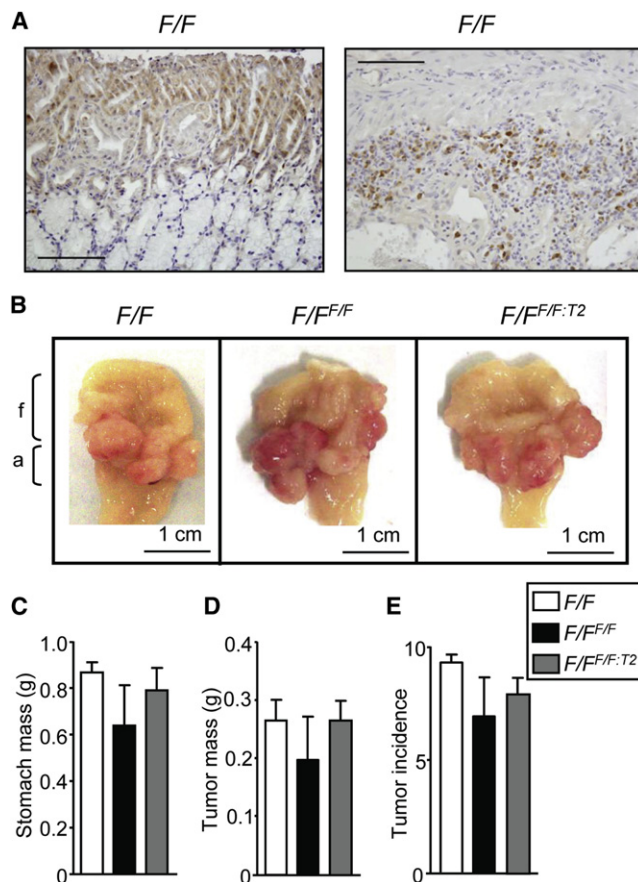


Figure 7. TLR2-Mediated Gastric Tumorigenesis Is Independent of Bone Marrow-Derived Inflammatory Cells

(A) Representative TLR2-stained cross-sections through the antral stomach tumor region of a 24-week-old *gp130^{F/F}* mouse showing staining around the surface area of the glandular gastric epithelium (left panel), as well as in submucosal inflammatory cell infiltrates (right panel). Scale bars = 100 μ m.

(B) Representative appearance of stomachs from 22-week-old *gp130^{F/F}* naive mice, or recipient *gp130^{F/F}* mice reconstituted with autologous *gp130^{F/F}* (*F/F/F*), or heterologous *gp130^{F/F}:Tlr2^{-/-}* (*F/F/F:TLR2*) donor bone marrow. a, antrum; f, fundus.

(C–E) Bar graphs depict the total mass (grams) of stomachs (C) and gastric tumors (D) as well as the incidence of tumors (E) from the indicated mice. Data for each group are expressed as the mean \pm SEM. *gp130^{F/F}*, n = 3; *F/F/F*, n = 3; and *F/F/F:TLR2*, n = 10.

See also Figure S5.

used by investigators to study chemical-induced intestinal carcinogenesis.

In summary, our current study defines that an endogenously expressed PRR promotes gastric tumorigenesis by regulating the expression of genes involved in cell survival and proliferation, and importantly highlights the need to further investigate the broader role of TLR2 in other inflammatory-related cancers, for instance colon, prostate, and liver. In this regard, it is noteworthy that deregulated STAT3 activation, including by *gp130*-acting cytokines, has been linked to the pathogenesis of tumor formation in these organs (Bollrath et al., 2009; Gao et al., 2005; Kawada et al., 2006; Ogata et al., 2006; Rebouissou et al., 2009). Collectively, our findings that increased STAT3 activation

and TLR2 expression in human GC correlates with a poor prognosis, together with the suppression of gastric tumorigenesis in a STAT3-driven preclinical GC mouse model following genetic and therapeutic targeting of TLR2, suggest that TLR2 represents a compelling biomarker and therapeutic target for GC.

EXPERIMENTAL PROCEDURES

Human Gastric Biopsy Tissue Collection

Antral gastric biopsies were collected at Monash Medical Centre (Melbourne, Australia) from 18 patients undergoing upper gastrointestinal endoscopy or surgical resection for clinical indications. Patients with a history of taking nonsteroidal anti-inflammatory drugs, proton pump inhibitors, or antibiotics were excluded from the study. Biopsies were either snap-frozen in liquid nitrogen or stored in 10% formalin, the latter for histopathological assessment on H&E-stained tissue sections using the revised version of the Sydney System (Dixon et al., 1996). Biopsies free of any lesions were considered normal. Full and informed consent was obtained from all patients, and biopsy collection was approved by the Southern Health Human Research Ethics Committee.

Mice and Treatments

The generation of *gp130^{F/F}*, *gp130^{F/F}:Stat3^{+/-}*, and *gp130^{F/F}:IL-11^{-/-}* mice has been previously described (Ernst et al., 2008; Jenkins et al., 2005; Tebbutt et al., 2002). Mice homozygous null for either *Tlr2* (Takeuchi et al., 1999) or *Tlr4* (Hoshino et al., 1999) were used to generate *gp130^{F/F}:Tlr2^{-/-}* and *gp130^{F/F}:Tlr4^{-/-}* mice. The specificity and pharmacokinetics of the OPN-301 monoclonal TLR2 blocking antibody (Opsona Therapeutics), analogous to clone T2.5 (Meng et al., 2004), have been previously described (Arslan et al., 2010). For acute studies, 12-week-old male *gp130^{F/F}* mice were i.p. injected with 10mg/kg of OPN-301 antibody or IgG1 isotype control for 30 min prior to i.p. administration with 2mg/kg of Pam₃Cys (EMC Microcollections) for 3 or 6 hr. For chronic studies, 12-week-old male *gp130^{F/F}* mice were i.p. injected with 10mg/kg of OPN-301 or IgG1 isotype control twice weekly over 10 weeks. For treatment with the MEK 1/2 inhibitor U0126 (Cell Signaling Technology), *gp130^{F/F}* mice were i.p. injected with U0126 (10mg/kg) or vehicle (Dimethyl sulfoxide; DMSO) for 18 hr prior to i.p. injection of Pam₃Cys (2 mg/kg) for 6 hr.

All experiments were endorsed by the Monash University Animal Ethics Committee and, where appropriate, included genetically-matched (mixed 129Sv \times C57BL/6 background) *gp130^{+/+}* (wild-type) littermate controls. Mice were housed under specific pathogen-free conditions and were age-matched for each experiment.

Gastric Epithelial Cells

The generation of primary mouse gastric epithelial cells (Viala et al., 2004) and maintenance of gastric IMGE-5, AGS, and MKN-28 cells (Jenkins et al., 2005) have been described previously. Transfections of mouse IMGE-5 and human MKN-28 and AGS cells were performed with the Nucleofector II System (Amaxa) and FuGene-6 (Roche), respectively. Chromatin immunoprecipitations were performed using the Imprint ChIP kit (Sigma-Aldrich) with pTyr705-STAT3 and IgG antibodies (Santa Cruz Biotechnology). For experiments involving the use of specific pathway inhibitors, cells were pretreated for 30 min with either DMSO vehicle, U0126, the PI3K inhibitor Wortmannin (Sigma), the NF- κ B inhibitor MG132 (Calbiochem), the p38 MAPK inhibitor SB203580 (Calbiochem), or the JNK MAPK inhibitor SP600125 (Calbiochem) at the indicated concentrations prior to stimulation with Pam₃Cys.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism V5.0 software. The normality of data was assessed using the D'Agostino and Pearson omnibus K2 normality test. Statistical significance ($p < 0.05$) between the means of two groups was determined using Student's t tests (normal distribution) or Mann-Whitney U tests (abnormal distribution). Statistical comparisons of the means of multiple (≥ 3) groups were determined using repeated-measures one-way ANOVA (normal distribution) or Kruskal Wallis nonparametric analyses (abnormal distribution).

Additional procedures are described in Supplemental Experimental Procedures.

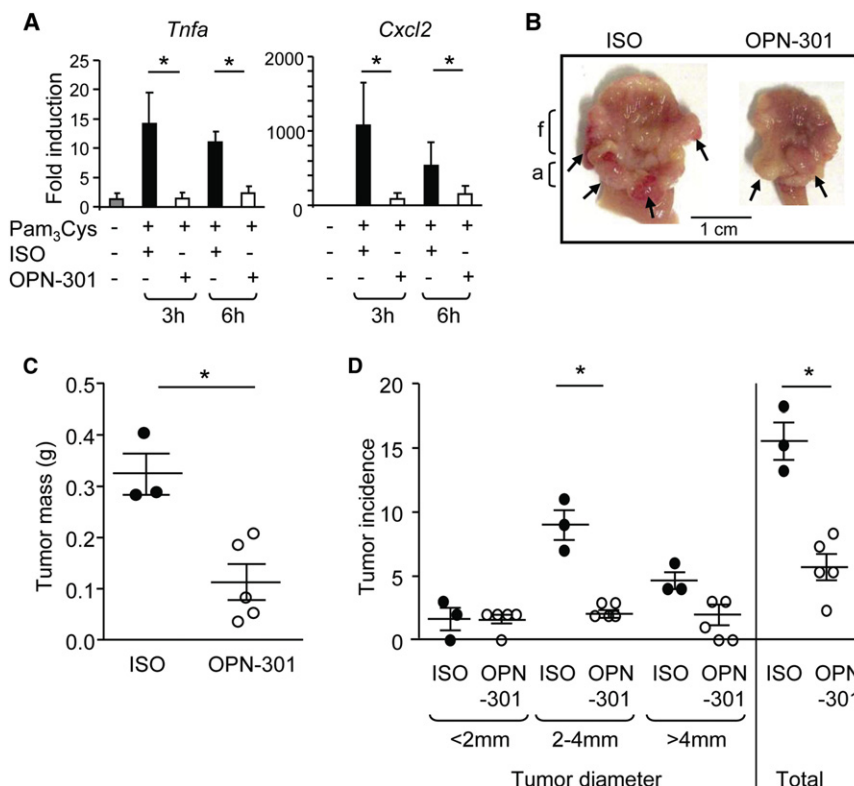


Figure 8. Therapeutic Suppression of Gastric Tumorigenesis in *gp130^{F/F}* Mice Treated with a TLR2 Blocking Antibody

(A) qPCR of *Tnfa* and *Cxcl2* gene expression was performed on antral gastric tissue of 12-week-old *gp130^{F/F}* mice that were untreated (gray bars) or pretreated with either OPN-301 (white bars) or isotype (black bars) control antibodies for 30 min prior to administration with Pam₃Cys over 6 hr. Expression data from at least two samples per time point and treatment group are shown following normalization for 18S and are presented from replicate analysis as the mean \pm SEM fold induction. *p < 0.05.

(B) Representative appearance of stomachs from 22-week-old *gp130^{F/F}* mice treated twice weekly over 10 weeks with either OPN-301 or isotype control antibodies. Arrows indicate macroscopically visible tumors >4 mm in diameter. Fundic (f) and antral (a) stomach regions are labeled.

(C and D) Scatter plots depicting the total mass (grams) of gastric tumors (C), as well as the incidence of tumors (D), from 22-week-old *gp130^{F/F}* mice treated twice weekly over 10 weeks with either OPN-301 or isotype control antibodies. Data for each group are expressed as the mean \pm SEM. *p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables, five figures, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2012.08.010>.

ACKNOWLEDGMENTS

We thank E. Vidacs and M. Meilak for technical assistance and N. Watkins and P. Hertzog for critical reading of the manuscript. We are grateful to S. Akira and S. Uematsu (University of Osaka, Japan) for kindly providing the *Tlr2^{-/-}* and *Tlr4^{-/-}* mice. This work was supported by grants from the Association for International Cancer Research (U.K.), Cancer Council of Victoria (Australia), and the National Health and Medical Research Council (Australia), as well as the Operational Infrastructure Support Program by the Victorian Government of Australia. B.J.J. is supported by both a Senior Medical Research Fellowship awarded by the Sylvia and Charles Viertel Foundation and a Monash University Fellowship. We also acknowledge the generosity of the family and friends of the late L. Hender for supporting cancer research. W.M. is a current employee and A.E.P. is a former employee and shareholder of Opsona Therapeutics, a drug discovery and development company focused on the role of TLR signaling in human immunology.

Received: July 28, 2011

Revised: April 12, 2012

Accepted: August 14, 2012

Published: October 15, 2012

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