Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Accelerated Progression of Gastritis to Dysplasia in the Pyloric Antrum of TFF2^{-/-} C57BL/6 × Sv129 *Helicobacter pylori*-Infected Mice

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Trefoil factor family 2 (TFF2) is up-regulated in *Helicobacter* spp.-infected gastric tissues of both humans and mice. To ascertain the biological effects of TFF2 *in vivo*, TFF2^{-/-} C57BL/6 × Sv129 and wild-type (WT) C57BL/6 × Sv129 mice were orally infected with *Helicobacter pylori* SS1. Mice were evaluated for gastric *H. pylori* colonization, pathology, and cytokine profiles at 6 and 19 months post inoculation (pi). At 6 months pi, there was a significant difference ($P < 0.05$) for epithelial criteria (mucosal defects, atrophy, hyperplasia, pseudopyloric metaplasia, and dysplasia) in the corpus of TFF2^{-/-} versus WT mice. At 19 months pi, a similar statistical difference in epithelial parameters was noted in the antrum of TFF2^{-/-} versus WT mice ($P < 0.01$). All of the TFF2^{-/-} *H. pylori*-infected mice had high-grade antral dysplasia, including gastric intraepithelial neoplasia, which was statistically significant ($P < 0.05$) compared with the infected WT mice. Levels of interferon- γ were markedly elevated in the gastric mucosa of infected TFF2^{-/-} mice at both 6 and 19 months pi. TFF2 provided a cytoprotective and/or anti-inflammatory effect against the progression of premalignant lesions of the gastric corpus at 6 months pi and in the pyloric antrum in *H. pylori*-infected mice at 19 months pi. These data support a protective role for TFF2 in part by modulating levels of gastric interferon- γ in the development of *H. pylori*-associated premalignancy of the distal stomach. (Am J Pathol 2007, 171:1520–1528; DOI: 10.2353/ajpath.2007.070249)

found in the epithelium of the gastric surface pits¹ and is a known tumor suppressor. TFF1^{-/-} mice develop spontaneous antral dysplasia and adenomas,² and somatic mutations in TFF1 have been reported in human gastric cancers.³ TFF2, also known as spasmolytic polypeptide, is mainly secreted by gastric mucous neck cells and is up-regulated after a variety of injuries to the gut, as is TFF3, intestinal trefoil factor.¹ TFF3 is expressed primarily in intestinal goblet cells where it plays a role in intestinal repair and anti-apoptosis.¹

The increase in TFF2-expressing cells that develops in the setting of chronic gastric *helicobacter* infection was first noticed by our group in C57BL/6 mice infected with *Helicobacter felis*.^{4,5} These *helicobacter*-infected mice developed hyperplasia of the surface-type epithelial cells (fundic atrophy) with a loss of parietal and chief cells, which were replaced by TFF2-immunoreactive mucous cells similar in some respects to antral glands or Brunner's glands. This TFF2-expressing lineage appears to be associated with a strong interferon (IFN)- γ - or Th1-polarized immune response^{6–9} and appears to be associated with gastric cancer in this and other gastric *helicobacter* mouse models.^{10,11} A similar spasmolytic polypeptide-expressing metaplasia lineage, also known as pseudopyloric metaplasia, has been identified in humans consisting of mucous cells with Brunner's or antral gland morphology in fundic areas devoid of chief, parietal, and gastrin cells. TFF2-expressing spasmolytic polypeptide-expressing metaplasia is also noted in a high percentage of dysplastic glands of gastric adenocarcinoma cases in populations surveyed in several countries, and expression of TFF2 was also recognized in one study of metastatic gastric cancer.^{12–15} However, although both murine and human studies suggest that lineages expressing TFF2 often precede and can give

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There are three known peptides of mammalian origin that belong to the trefoil factor family (TFF). TFF1 is normally

Table 1. Number of WT and TFF2^{-/-} Mice Infected with *H. Pylori* or Vehicle and Analyzed at 6 and 19 Months After Infection

Time	WT control	TFF2 ^{-/-} control	WT <i>H. pylori</i>	TFF2 ^{-/-} <i>H. pylori</i>
6 months	4 (2/2)	4 (2/2)	8 (4/4)	10 (4/6)
19 months	5 (2/3)	4 (2/1) (1 female, 14 months)	9 (3/6)	9 (2/7)

Data are *n* (male/female).

rise to cancerous lesions of the stomach, the possibility that TFF2 plays a direct role in gastric cancer progression has not been investigated.

In an effort to address further the role of TFF2 in carcinogenesis and to study the physiological role of TFF2, Wang and colleagues¹⁶ developed a TFF2/spasmolytic polypeptide-deficient mouse model. Characterization of these TFF2^{-/-} B6129 mice at 8 weeks of age indicated that the TFF2^{-/-} mice had a twofold increase in parietal cells with a similar increase in gastric acid output and an undetectable serum gastrin level.¹⁶ Histologically, there was an overall decrease in gastric mucosal thickness and gastric proliferation rates, suggesting that TFF2^{-/-} mice would be less susceptible to gastric cancer. The mice were also more vulnerable to indomethacin-induced gastric injury compared with their wild-type counterparts.¹⁶ These data suggested a gastric cytoprotective role for TFF2 via an epithelial cell proliferation response coupled with a down-regulation of acid secretion.¹⁶ However, more recent data have suggested that TFF2 may function in part as an anti-inflammatory cytokine, showing some similarity in this respect to interleukin (IL) 10.¹⁷ TFF2 expression is not limited to the gastrointestinal tract but is also present in both macrophages and lymphocytes.¹⁸ Both TFF2^{-/-} and IL-10^{-/-} C57BL/6 mice show immune dysregulation and, when infected with *H. felis*, develop more severe gastric inflammation early in the course of the disease than their WT counterparts.¹⁷ In addition, TFF2^{-/-} mice are more susceptible to DSS-induced colitis and have a more prolonged response to LPS.¹⁷ These data raised the possibility that long-term helicobacter infection in TFF2^{-/-} may induce accelerated and more severe gastric lesions.

The availability of TFF2^{-/-} mice on a B6129 background afforded us the opportunity to study the long-term effects of *Helicobacter pylori* infection in gastric tissue in the absence of TFF2. We were specifically interested in ascertaining whether the lack of TFF2 affects the progression of gastric cancer in this model.¹⁹

Materials and Methods

Animals

Specific pathogen-free TFF2-deficient and wild-type littermate controls on a mixed B6129Sv background were generated as previously reported.¹⁶ Weanling, specific pathogen-free (free of enteric *Helicobacter* spp., *Citrobacter rodentium*, *Salmonella* spp. endoparasites, ectoparasites, and serum antibodies to murine viral pathogens) mice obtained from an in-house breeding colony were used in the study.

Mice were housed in microisolator, solid-bottomed polycarbonate cages, fed a commercially prepared pelleted diet, and given water ad libitum. The mice were all maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility under barrier conditions for the duration of the 19-month experiment. The protocol was approved by the Committee on Animal Care of the Massachusetts Institute of Technology.

Bacteria

H. pylori Sydney strain was used for oral inoculation as previously described.²⁰ The bacteria were grown for 48 hours at 37°C under microaerobic conditions on 5% lysed horse blood agar. The bacteria were harvested after 48 hours of growth; resuspended in PBS; assessed by Gram stain and phase microscopy for purity, morphology, and motility; and tested for urease, catalase, and oxidase activity.

Experimental Infection

Twenty TFF2^{-/-} B6129 and 20 B6129 WT mice were orally infected with 10⁸ cfu *H. pylori*, Sydney strain in 0.3 ml of PBS given three times every other day. Ten TFF2^{-/-} and 10 control mice were dosed with PBS only. At 6 and 19 months after challenge, infected and uninfected mice were euthanized with CO₂ (Table 1).

Histopathology

At necropsy, stomach and duodenum were collected for histopathology and scored by a comparative pathologist blinded to sample identity according to previously defined criteria.²¹ Briefly, inflammation reflected the extent of leukocyte infiltration in the mucosa and submucosa. Epithelial defects were scored based on degeneration of the surface epithelium and underlying gastric units, ranging in severity from mild irregularities of the outermost layer to full-thickness ulceration. Hyalinosis is the accumulation of brightly eosinophilic droplets and/or crystals in surface epithelium. It is composed of Ym2 protein encoded by the chitinase 3-like 4 gene and is expressed in multiple epithelial compartments in response to non-specific injury, particularly in mice on a 129Sv strain background.^{22,23} Mucous metaplasia is the expansion of gastric mucous neck cells secreting a mixture of neutral and acidic mucins in the oxyntic mucosa. This is associated with loss of parietal and chief cells, and it has been suggested that metaplastic chief cells contribute to the mucus-secreting population.^{5,24} Oxyntic atrophy is the

loss of chief and parietal cells in the corpus. (The fundus of the mouse stomach is lined by stratified squamous epithelium.) Chief cell disappearance usually precedes parietal cell loss. Hyperplasia refers to elongation of gastric units lined by foveolar-type epithelial cells. Pseudopyloric metaplasia is the replacement of normal corpus with a mucosa resembling the pyloric antrum with regard to glandular phenotype and mucin expression. It is a pre-neoplastic change commonly found in mouse models of gastric carcinogenesis and also has been described in *H. pylori*-infected humans.^{24,25} Dysplasia is defined by cellular and glandular atypia during cancer development and progression. For all criteria, we graded corpus and antrum separately. Statistical comparisons between all groups were made by Kruskal-Wallis one-way analysis of variance with Dunn's posttest and between group pairs, by Mann-Whitney U using Prism 4.0 for Macintosh (GraphPad, San Diego, CA). *P* values <0.05 were considered significant.

Special Stains and Immunohistochemistry

Assessment of neutral (gastric-type) and acidic (intestinal-type) mucins in selected cases was made with pH 2.5 Alcian blue/periodic acid-Schiff stain. TFF2 immunohistochemistry using an in-house polyclonal rabbit antibody (T.C.W.) was performed on formalin-fixed tissue sections from WT and TFF2^{-/-} mice after heat-induced epitope retrieval as described previously.²⁶ Parietal cells were identified with a mouse anti-H⁺/K⁺ ATPase β subunit antibody (Sigma, St. Louis, MO). Assessment of intestinal metaplasia was performed with mouse anti-Cdx2 (Biogenex, San Ramon, CA). Mucous neck cells were identified using biotinylated Griffonia (Bandeiraea) simplicifolia lectin II (GSL II) in fluorescence histochemistry with streptavidin-fluorescein isothiocyanate, and slides were mounted with Vectashield plus DAPI (4,6-diamidino-2-phenylindole) (all from Vector Laboratories, Burlingame, CA). The ARK kit (DakoCytomation, Carpinteria, CA) was used for mouse-on-mouse immunohistochemistry applications.

Enzyme-Linked Immunosorbent Assay for Serum IgG2c and IgG1 Responses to *H. pylori* SS1

Serum Th1-associated IgG2c and Th2-associated IgG1 responses to outer membrane antigens of *H. pylori* SS1 were measured by enzyme-linked immunosorbent assay as previously described.²¹ Antigen was coated on Immulon II plates at a concentration of 10 μ g/ml (IgG2c and IgG1) with sera diluted 1:100. Biotinylated secondary antibodies included monoclonal anti-mouse antibodies produced by clones A85-1 and 5.7 (Pharmingen-BD Biosciences, San Jose, CA) for detecting IgG1 and IgG2c, respectively. Incubation with extravidin peroxidase (Sigma, St Louis, MO) was followed by 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate (Kirkegaard and Perry Laboratories,

Gaithersburg, MD) for color development. Absorbance (or optical density) development at 405/562 nm was recorded by an enzyme-linked immunosorbent assay plate reader (Dynatech MR7000; Dynatech Laboratories, Inc., Chantilly, VA). An analysis of variance was performed to examine the effect of helicobacter infection, gender, host phenotype, and duration of infection (time) on helicobacter colonization and to examine serological responses of TFF2^{-/-} and wild-type mice.

Quantitative PCR for *H. pylori* SS1

To quantify colonization levels of *H. pylori* strain SS1 within the gastric mucosa, a real-time quantitative PCR assay was developed based on the nucleotide sequence of the *H. pylori* ureB gene in the ABI Prism TaqMan 7700 sequence detection system (A/B Applied Biosystems, Foster City, CA). Two primers (forward, 5'-CAAAA TCGCTGGCATTGGT-3', and reverse, 5'-CTTCACCG-GCTAAGGCTTCA-3'), and an internal probe (5'-AA-CAAAGACATGCAAGATGGCGTTAAAAACA-3') were designed to hybridize within the 100-bp region (nucleotides 273 to 373) of the single-copy ureB gene (GenBank accession no. AF508016) of *H. pylori* SS1 using Primer Express software (Applied Biosystems).²⁷ Quantitative PCR conditions used were similar to those described previously.²⁸ The specificity of these oligonucleotides for *H. pylori* was tested using DNA isolated from *H. felis* (ATC49179), *Helicobacter mustelae* (ATCC43772), and *Helicobacter heilmannii*. To generate a standard curve, serial 10-fold dilutions (from 5 $\times$ 10⁵ to 5) of *H. pylori* SS1 genome copies, estimated from an average mass value (1.66 Mb) obtained from the two published *H. pylori* genomes, were used.^{29,30} Copy numbers of gastric mucosal *H. pylori* SS1 DNA in mice were then calculated and normalized to micrograms of murine chromosomal DNA determined by quantitative PCR using a mammalian 18S rRNA gene-based primers and probe mixture (Applied Biosystems) as described previously.³¹

Quantitation of Gene Expression

For RNA preparation, gastric tissues from all four groups were collected and snap-frozen in liquid nitrogen. Total RNA was prepared using TRIzol reagent according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA). Five micrograms of total RNA was used to generate cDNA using the High Capacity Achieve kit from Applied Biosystems according to the recommendations of the manufacturer. Levels of IL-1 β , IL-4, IL-10, IFN- γ , tumor necrosis factor- α (TNF- α), and inducible nitric-oxide synthase (iNOS) transcripts were quantified with Applied Biosystems predesigned primers and probes (TaqMan Gene Expression Assays) in an ABI Prism Sequence Detection System 7700 (Applied Biosystems). Transcript levels were normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase and expressed as fold change compared with the sham-dosed C57BL $\times$ 129 controls using the comparative C_T method (Applied Biosystems User Bulletin no. 2).

Figure 1. Histopathology summary of *H. pylori* infection in wild-type and TFF2^{-/-} mice at 6 and 19 months postinoculation (MPI). **Top:** *H. pylori*-infected TFF2^{-/-} proximal glandular stomach has more severe epithelial defects and oxyntic atrophy despite similar inflammation when compared with WT. There were no significant differences between groups in the antrum at this time point (not shown). **Middle:** Disease severity of the corpus in TFF2^{-/-} stomach at 19 months is roughly similar to that of WT. **Bottom:** Gastric intraepithelial neoplasia in *H. pylori*-infected TFF2^{-/-} pyloric antrum at 19 months (**inset**, disorganized gland architecture shown at higher magnification) versus mild-moderate inflammation and hyperplasia in WT mouse. H&E, bar = 100 μ m.

Results

Histopathology

When compared with lesion-free, sham-inoculated controls at 6 months, wild-type C57BL/6x129 mice infected with *H. pylori* displayed moderately severe gastritis comprising chiefly mononuclear cells, with multifocal foci of intramucosal granulocytes (Figure 1). This level of inflammation was consistent with previous observations in B6129 mice by us and others.^{32,33} In addition, epithelial defects, mucous metaplasia, oxyntic atrophy, and foveolar hyperplasia were significantly elevated in *H. pylori*-infected WT mice when compared with uninfected mice, whereas there was no significant increase in hyalinosis or dysplasia (data not shown). Spontaneous lesions such as mild hyalinosis (red droplets or crystals associated with surface epithelium containing the chitinase-like molecule Ym2)²² and/or mucous metaplasia (defined below) in uninfected TFF2^{-/-} mice were slightly more common than in uninfected WT mice, but these differences were not significant (not shown). In contrast, TFF2^{-/-} mice

infected with *H. pylori* developed significantly greater epithelial defects, oxyntic atrophy, epithelial hyperplasia, and pseudopyloric metaplasia in the corpus than infected WT mice (Figure 1). Interestingly, these pronounced epithelial changes in TFF2^{-/-} mice were not associated with greater inflammation. Dysplasia also was increased in TFF2^{-/-} mice but fell just short of statistical significance ($P = 0.08$). Antral lesions, when present, also tended to be more severe in TFF2^{-/-} mice at 6 months, although not to a statistically significant degree (data not shown).

At 19 months after inoculation, there was no difference in corpus lesion severity between *H. pylori*-infected WT and TFF2^{-/-} mice, with both groups displaying severe gastritis with attendant mucosal changes. However, in the pyloric antrum, TFF2^{-/-} mice displayed significantly greater epithelial defects, hyalinosis, hyperplasia and dysplasia (Figure 1). All *H. pylori*-infected TFF2^{-/-} mice developed marked antral dysplasia, and two developed pyloric lesions consistent with gastric intraepithelial neoplasia.²¹ In contrast, all WT mice displayed no or minimal

Figure 2. Immunohistochemistry and special stains to characterize *H. pylori* infection in WT and *Tff2*^{-/-} B6129 mice. **A:** In normal uninfected mice (**top row**), WT mice (**left**) express TFF2 within mucous neck cells of the corpus, in the pyloric region at the base of antral glands and within duodenal Brunner's glands. Aberrant Tff2 expression is manifest multifocally in the corpus of *H. pylori*-infected mice with mucous and pseudopyloric metaplasia and is expressed more intensely within antral glands (**bottom row**). In contrast, there is no TFF2 expression in knockout mice (**right**) regardless of infection or lesion status. **B:** In the normal corpus of *Tff2*^{-/-} mice (**top left**), the subisthmus gastric column comprises chiefly parietal cells (brown). Fewer interspersed mucous neck cells are identified by their pale pink periodic acid-Schiff staining, and the surface epithelium is covered by a neutral (red) gastric-type mucous lining. Mucous neck cells also were identified in relatively small numbers by GSL II fluorescence lectin histochemistry (**bottom left**). In contrast, mice with mucous metaplasia (**top right**) exhibited a marked loss of parietal cells and expansion of mucus-secreting cells expressing both gastric (red) and intestinal (blue) type mucins. All of these cells labeled with the mucous neck cell marker GSL II (**bottom right**). An identical staining pattern was observed in normal and *H. pylori*-infected WT mice (not shown). **C:** Compared with normal uninfected mice (**top left**), there is a marked increase in the Ki-67 labeling index in *H. pylori*-infected mice with hyperplasia and dysplasia (**top right**). In contrast to normal duodenal epithelium (**bottom left**), there was no nuclear staining for the intestinal epithelial marker Cdx2 in dysplastic or cancerous lesions of the stomach (**bottom right**), suggesting that the corporal metaplasia in this group of animals was pseudopyloric and not intestinal in nature. Bar: 160 μ m corpus and 200 μ m antrum (A), 80 μ m (B and C), and 40 μ m (**bottom left** C).

antral dysplasia, with the exception of a single animal that developed pyloric gastric intraepithelial neoplasia.

Special Stains and Immunohistochemistry

Both WT and *Tff2*^{-/-} mice infected with *H. pylori* developed mucous and pseudopyloric metaplasia of the corpus as determined by lesion scores for H&E-stained sections. Mucous metaplasia was defined as the expression of a mucus subtype inappropriate for a given anatomical compartment (ie, intestinal-type mucin expression within the gastric corpus). Pseudopyloric metaplasia was defined by columnar elongation of epithelial cells, often with

intestinal or mixed mucin expression, and by loss of normal cell layers comprising gastric columns in the corpus. Because the spasmodic polypeptide-expressing metaplasia phenotype has been associated with up-regulated TFF2 expression,³⁴ we performed immunohistochemistry to confirm the absence of TFF2 expression in knockout mice with mucous and pseudopyloric metaplasia. In uninfected WT mice, TFF2 was expressed at the base of antral glands, within duodenal Brunner's glands, and in mucous neck cells of the corpus (Figure 2A). This distribution pattern agrees with results reported elsewhere.⁶ In infected WT mice with gastric lesions, TFF2 stained with greater intensity in the antrum and was

Figure 3. Wild-type and TFF2^{-/-} mice were bled at 6 and 19 months pi with *H. pylori* SS1 ($n = 8$ –11 per group). Uninfected control sera did not contain specific antibody (data not shown), whereas the TFF2^{-/-} mice made a more robust Th1-associated IgG2c responses earlier in the course of infection compared with wild-type mice.

aberrantly expressed multifocally in the corpus in conjunction with mucous and/or pseudopyloric metaplasia (Figure 2A). In contrast, TFF2^{-/-} mice showed no immunohistochemical staining for TFF2 regardless of infection or gastric lesion severity (Figure 2A). Thus, TFF2 overexpression was associated with, but not required for, mucous and pseudopyloric metaplasia in mice infected with *H. pylori*. To characterize further the mucous metaplasia and its association with loss of oxyntic cells, we performed parietal cell immunohistochemistry followed by Alcian blue/periodic acid-Schiff staining. In the normal mouse stomach, parietal cells comprised the major cell population in the oxyntic mucosa, whereas interspersed mucous neck cells stained very weakly for neutral gastric mucins (Figure 2B). In contrast, mice with mucous metaplasia demonstrated a significant loss of parietal cells, whereas the expanded mucous-secreting cell population expressed large quantities of either intestinal or gastric-type mucins. Regardless of the type of mucin produced, cells involved in mucous metaplasia labeled with the lectin GSL II (recognizing α - or β -linked *N*-acetylglucosamine residues (Figure 2B). GSL II-positive cells in WT mice also express TFF2 and Muc6 and include mucous neck, deep antral, and duodenal Brunner's gland cells.⁶ Finally, pseudopyloric metaplasia but not intestinal metaplasia was documented in dysplastic and cancerous gastric lesions based on the absence of nuclear staining for the intestinal epithelium-specific marker Cdx2 (Figure 2C).

H. pylori Immune Response

Uninfected control wild-type and TFF2^{-/-} mice did not have detectable serum antibody to *H. pylori* SS1 in sera obtained at 6 and 19 months pi (data not shown). As previously shown for *H. pylori*-infected mice on a C57BL/6 background,³⁵ wild-type and TFF2^{-/-} mice developed robust Th1-associated IgG2c³⁶ responses to *H. pylori* that predominated over the Th2-associated IgG1 responses (Figure 3). By 6 months pi, infected TFF2^{-/-} mice developed higher Th1-associated IgG2c and Th2-associated IgG1 responses to *H. pylori* than infected wild-type mice

($P < 0.0001$, <0.03 , respectively). By 19 months pi, the levels of IgG2c had marginally increased in infected TFF2^{-/-} mice, but the increase of IgG2c in infected wild-type mice had produced an equivalent response by this later time point. Notably, the IgG2c response of the TFF2^{-/-} mice to *H. pylori* had essentially peaked by the earlier 6-month time point, whereas the wild-type response was more delayed and had become equivalent to that of the TFF2^{-/-} mice over the ensuing 13 months. The IgG1 responses to *H. pylori* trended lower in both TFF2^{-/-} and WT of mice between 6 and 19 months pi, suggesting that the predominant Th1-associated host responses inhibited the Th2 responses over the course of the chronic infection.

Gastric Cytokine Analysis

Infection with *H. pylori* SS1 significantly enhanced mRNA levels of IFN- γ at both 6 and 19 pi in TFF2^{-/-} infected mice when compared with infected WT mice (Figure 4; $P < 0.01$). mRNA levels of IL-10 were also statistically increased in TFF2^{-/-} versus WT infected mice at 6 months pi but not at 19 months pi. There was significant increase of mRNA levels of TNF- α and IL-1 β at both time points in *H. pylori*-infected TFF2^{-/-} and WT mice when compared with those in the sham-dosed TFF2^{-/-} mice; however, there was no significant difference in the levels of these two proinflammatory cytokines between *H. pylori*-infected TFF2^{-/-} and infected WT mice (Figure 4, $P > 0.05$). iNOS levels were statistically increased ($P < 0.001$) in infected TFF2^{-/-} mice (but not WT infected mice) versus TFF2^{-/-} controls at 6 months, but at 19 months pi, iNOS levels were statistically higher in both TFF2^{-/-} and WT infected mice when compared with sham-dosed TFF2^{-/-} mice. There was no significant difference of IL-4 mRNA levels among the groups at both time points (data not shown).

H. pylori SS1 Colonization Levels Did Not Differ between Wild-Type and TFF2^{-/-} Mice

All of the infected B6129 mice were positive for *H. pylori* SS1 at both time points; *H. pylori* SS1 was detected in all of the infected TFF2^{-/-} mice at 6 months pi and in five of seven mice sampled at 19 months pi. There was no significant difference for the average numbers of *H. pylori* SS1 among the groups at either of the two time points (Figure 5).

Discussion

TFF2^{-/-} mice chronically infected with *H. pylori* over a period of 19 months developed significantly more severe disease, including features of *in situ* carcinoma, when compared with their WT counterparts. Although corpus lesions in the TFF2^{-/-} mice were more severe at 6 months pi but not at 19 months pi, this pattern appears to be consistent with the natural history of the murine *helicobacter* gastritis model, in which initial oxyntic atrophy

Figure 4. Relative mRNA levels of selected cytokines and iNOS. In each sample, mRNA levels of the individual cytokines in the samples were normalized to that of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* and expressed as mean fold change of their mRNA levels in reference to sham-dosed C57BL/6 group. Bars = SEM. * $P < 0.05$.

or dysplasia is followed later on by progressive antral dysplasia. Our group has previously reported that wild-type C57BL/6 mice infected with *H. felis* for 18 to 22 months show initial oxyntic atrophy progressing to severe antral dysplasia and antral predominant polypoid adenocarcinoma.³⁷ Similar results have been observed in a bacterial overgrowth model in the achlorhydric, gastrin-deficient mouse.³⁸ Given that *H. felis* in general produces a more robust and progressive gastric disease than *H. pylori* in C57BL/6 mice, the accelerated antral dysplasia noted in TFF2^{-/-} infected mice is comparable with the more aggressive gastric disease noted in *H. felis*-infected mice. We would predict that long-term infection of TFF2^{-/-} with *H. felis* would most probably result in even more rapid onset of antral adenocarcinoma.

The finding that TFF2^{-/-} mice have a more accelerated progression to gastric neoplasia would be consistent with a role for TFF2 in gastric cytoprotection and as

Colonization levels of *H. pylori* SS1

Figure 5. Colonization levels of *H. pylori* SS1 determined using QPCR with the primers and probe derived from the *H. pylori ureB* gene as described previously. The numbers of bacterial cells are expressed as copies of the *H. pylori* genome per microgram of mouse DNA. There is no significant difference in *H. pylori* colonization among the groups.

an inhibitor of chronic inflammation. Although we cannot currently exclude an important role of TFF2 in gastric cytoprotection in the current study, recent studies by our group¹⁷ have strengthened a role for TFF2 in the modulation of inflammation and pointed to regulation of both macrophage and T-cell function. Our finding at 6 months pi of a statistically higher *H. pylori*-specific IgG2c in infected TFF2^{-/-} mice is consistent with a more robust Th1 immune response in these animals. In addition, the study has demonstrated significant elevations in IFN- γ and IL-10 levels and a trend toward increased levels of iNOS, IL-1 β , and TNF- α in infected TFF2^{-/-} mice compared with WT mice at the 6 months pi time point.

The elevation of iNOS, which produces reactive nitrogen species, in both WT and TFF2^{-/-} mice is probably produced by macrophages or dendritic cells in the inflamed mucosa, and previous studies have reported up-regulation of iNOS in gastric tissues from both humans and mice infected with *H. pylori* or *H. felis*.⁷ The biological relevance of increased iNOS mRNA in this model and in humans may be less important, given that *H. pylori* produces arginase, which competes for the substrate arginine, necessary for the production of NO by macrophages.³⁹ Giraud et al⁴⁰ recently reported that TFF2 inhibits iNOS/NO in monocytes in the rat colon during the healing process after exposure to a single intracolonic dose of dinitrobenzene sulfonic acid. Rats treated with TFF2 had lower nitrated protein levels and reduced levels of inflammatory infiltrates in their colon compared with rats treated with vehicle alone. The pro-inflammatory cytokines TNF- α and IL-1 β were also elevated in both TFF2^{-/-} and WT infected mice when compared with control mice and slightly higher in TFF2^{-/-} infected mice at 6 months pi, although these latter differences were not statistically significant. Interestingly, mRNA levels of the anti-inflammatory cytokine, IL-10, were elevated in in-

infected TFF2^{-/-} versus WT infected mice at 6 months pi, even though corpus inflammation was more severe in infected TFF2^{-/-} mice. However, IL-10 is a known downstream target of IL-1 β and other pro-inflammatory cytokines, and the IL-10 elevation may in part reflect an early host response in its attempt to dampen the inflammatory response due to the infection.⁴¹

Of the cytokines measured, IFN- γ was markedly elevated at both time points (6 and 19 months pi) in the infected TFF2^{-/-} mice compared with those recorded in infected WT mice. It has been previously reported that IFN- γ , a major mediator of the proinflammatory Th1 response, is elevated in gastric tissue of both mice and humans infected with helicobacter.^{7,8,42,43} LPS present in *H. pylori* is known to stimulate IFN- γ production in T cells in an antigen-dependent manner. *H. pylori*-induced gastritis in splenocyte-replenished SCID mice resulted in statistically more severe gastritis and elevated IFN- γ mRNA expression when compared with similarly treated SCID mice infected with a mutant *H. pylori* with truncated LPS.⁴⁴ As expected, IFN- γ ^{-/-} mice infected with *H. pylori* have markedly reduced gastritis and lower iNOS and MIP-2 levels when compared with their wild-type counterpart.^{8,9} A pivotal role for IFN- γ in inducing gastric atrophy was also recently demonstrated by direct infusion of IFN- γ into C57BL/6 mice, which resulted in the subsequent development of atrophy, metaplasia, and dysplasia in the absence of gastric helicobacter infection.⁴⁵ Interestingly, infusion of IFN- γ had no significant effect in BALB/c mice, which were previously shown to be resistant to *H. felis*-dependent gastric atrophy.^{5,45} Kang et al⁶ and others have also reported that infusion into C57BL mice of IFN- γ recapitulated the mucous metaplasia observed in *H. felis*-infected mice.⁵ Using mouse-specific recombinant cytokines in an *in vitro* model of pharmacologically characterized gastric glands isolated from BALB/c and C57BL/6 mice, investigators convincingly demonstrated that only two (among IFN- γ , IL-2, IL-12, IL-4, IL-6, and IL-10) mouse cytokines, IL-2 and IFN- γ , had a direct, dose-dependent inhibitory effect on acid secretion.⁴⁶ These data are consistent with these two cytokines being elevated in a Th1 proinflammatory response due to gastric helicobacter infection, which over time develop gastric atrophy, dysplasia, and cancer.^{19,47} In an *H. felis* C57BL mouse model, coinfection with a helminth significantly reduced IFN- γ , IL-1 β , and TNF- α mRNA and attenuated premalignant lesions.⁴⁷

Most likely, the markedly elevated IFN- γ mRNA in the TFF2^{-/-} mouse accelerated acid hyposecretion, rapid *H. pylori* colonization in the corpus, and initiation of a progressive inflammation, atrophy, and epithelial damage resulting, over time, in the development of severe antral inflammation, dysplasia, and *in situ* carcinoma. The importance of these experimental data is augmented by the recent reports demonstrating that polymorphisms in proinflammatory cytokines, including IFN- γ , influence the susceptibility of *H. pylori*-infected individuals to development of gastric cancer.^{48,49}

In summary, these data strongly support a model in which progression to antral gastric cancer in TFF2^{-/-} mice is primarily a cytokine-driven and immune-mediated

disease. Likewise our results indicate that TFF2 present in WT mice significantly down-regulates IFN- γ in the gastric mucosa of *H. pylori* in infected mice compared with TFF2^{-/-} mice and may in part explain why infected TFF2^{-/-} mice had an accelerated progression of antral gastric lesions. Further studies will be needed to clarify whether the mechanism involves gastric cytoprotection or direct immune regulation.

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