

Trefoil Family Factor 2 Is Expressed in Murine Gastric and Immune Cells and Controls both Gastrointestinal Inflammation and Systemic Immune Responses[∇]

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Trefoil family factor 2 (TFF2), also known as spasmodic peptide, is a low-molecular-weight protein that is upregulated in gastric tissues infected with *Helicobacter* or having other inflammatory conditions, but a precise function is yet to be elucidated. The role of TFF2 in the development of gastritis, colitis, and inflammatory cytokine responses was examined both in vivo and in vitro using wild-type and TFF2 knockout mice. TFF2 knockout and wild-type mice were infected with *Helicobacter felis* (*H. felis*) to induce gastritis. Colitis was induced in TFF2 knockout and wild-type mice by administering dextran sodium sulfate (DSS) in drinking water. Histopathology, clinical disease (colitis), and antibody levels (*H. felis*) were examined. TFF2 expression in tissues was determined by reverse transcriptase PCR, and the inflammatory and proliferative responses of TFF2-expressing macrophages and spleen cells were examined by cytokine enzyme-linked immunosorbent assay, thymidine incorporation, and gene array studies. TFF2 knockout mice have increased susceptibility to *H. felis*-induced gastritis, with enhanced gastric inflammation. They were also more susceptible to DSS-induced colitis, with prolonged colonic hemorrhage and persistent weight loss. Remarkably, TFF2 expression was not limited to the gastrointestinal tract, as suggested in previous studies, but was also present in macrophages and lymphocytes. The inflammatory and proliferative responses of these immune cell types were dysregulated in TFF2 knockout mice. TFF2^{-/-} cells were hyperresponsive to interleukin 1 beta stimulation but showed normal responses to lipopolysaccharide, suggesting a specific role for TFF2 in interleukin 1 receptor but not Toll-like receptor 4 signaling via their Toll-interleukin 1 resistance domains. TFF2^{-/-} lymphocytes also produced higher levels of interleukin 2 than wild-type cells. Thus, TFF2 was expressed in the gastrointestinal cells and in immune cells and was a negative regulator of gastrointestinal inflammation and immune cell cytokine responses. Our studies suggest that TFF2 not only controls gastrointestinal repair but also regulates mononuclear cell inflammatory responses.

Trefoil factor family 2 (TFF2), also known as spasmodic polypeptide, is a small peptide upregulated in association with gastric *Helicobacter* infection and other diverse inflammatory conditions of the gastrointestinal tract. There are three known members of the TFF family (TFF1, -2, and -3), which all contain one or more three-looped structural motifs resembling a three-leaf clover or trefoil (36). Under normal conditions, TFF2 is expressed and secreted by gastric mucosal neck cells and antral mucous cells, and it appears to play a role in gastric cytoprotection, at least in part through promotion of epithelial

cell migration. More recently, studies with TFF2-deficient mice showed evidence of decreased gastric cell proliferation and increased susceptibility to gastric mucosal injury (10).

While a number of potential biological roles for TFF2 have been reported, a precise understanding of the function of TFF2 has remained elusive. In addition, the cellular mechanisms by which TFF2 exerts its effects remain uncertain, since cell surface receptors for the TFFs have yet to be identified. Recently, the possibility has been raised that TFF2 may be involved in some aspect of immune function. Cook et al. reported that TFF2 (and TFF3) could be detected at low levels in rat spleen, lymph nodes, and bone marrow (2). To investigate the role of TFF2 in immunity, we undertook studies of immune cells isolated from wild-type and TFF2-deficient mice. These studies reveal a specific role for TFF2 in the negative regulation of interleukin 1 (IL-1) receptor-mediated signaling.

MATERIALS AND METHODS

Animals: TFF2^{-/-} mice (Farrell). Specific-pathogen-free TFF2-deficient (SPKO) and wild-type littermate controls on a mixed C57BL/6 × 129Sv back-

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TABLE 1. Scoring criteria for statistical analysis of histology

Score	Description		
	Colitis	Mucosal erosion/ulcer	Crypt loss/atrophy
0	None	None	None
1	Mild; focal or multifocal	Mild, focal erosion	Mild; focal or multifocal
2	Moderate; focal or multifocal or segmental	Multifocal erosion or focal ulcer \pm submucosal edema (>2 foci)	Moderate; multifocal (>5 foci)
3	Marked; hemorrhagic and diffuse	Diffuse erosion or multifocal/segmental ulcer with submucosal edema	Marked; segmental
4	Severe; hemorrhagic and diffuse	Diffuse ulcer and marked submucosal edema	Severe; diffuse

ground were generated as previously reported (10). The mice were maintained in an Association for Assessment and Accreditation of Laboratory and Animal Care (AAALAC)-approved facility under barrier conditions. Animal protocols were approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School.

DSS colitis. Mice received 4% dextran sodium sulfate (DSS) in their drinking water for 4 days, followed by 18 days of plain water. A second cycle of DSS treatment, for 3 days, was followed by an additional 25 days of plain water. Mice were weighed, and blood in the stool was monitored using Hemocult strips ($n = 11$ mice per group for wild-type and knockout mice). For statistical analysis, the P values were obtained by testing a group \times time interaction in a linear random effects model (where the animal is a random effect) to test whether the time trends were significantly different between the groups. A likelihood ratio test was used to compare nested models (with and without the interaction term) after day 23 (11). The entire length of the colon was examined histologically and scored by a gastrointestinal (GI) pathologist blinded to the treatment group. Statistical analysis of histologic scores were performed using the Mann-Whitney test. Scoring criteria are given in Table 1.

***Helicobacter felis* infection studies.** An *H. felis* (ATCC 49179) strain was used for oral inoculation as previously described (41). The organism was grown for 48 h at 37°C under microaerobic conditions on 5% lysed horse blood agar, harvested, and resuspended in phosphate-buffered saline. Bacteria were assessed by Gram stain and phase microscopy for purity, morphology, and motility. Mice were orally infected with 10^8 CFU of *H. felis* in 0.3 ml phosphate-buffered saline given three times every other day. At 3 and 6 months postchallenge, mice were sacrificed, and gastric tissues were collected from the corpus and antrum and used for histopathologic evaluation ($n = 4$ for the wild type; $n = 8$ for TFF2 knockout mice). Murine tissues were harvested, processed, and blindly scored by a comparative pathologist, using an established scoring rubric which we have previously described in detail (31). The mice were 70% B6 and 30% SV129, but the controls were genetically matched. The inflammation in the wild-type (littermate) controls is slightly less in mice on a completely SV129 background (31).

Evaluation of serum antibody responses to *H. felis*. Serum was collected at intervals up to 12 months postinfection and evaluated by enzyme-linked immunosorbent assay (ELISA) for serum immunoglobulin G2c (IgG2c) (IgG2a-equivalent Th1-promoted isotype in C57BL/6 mice) (25) and IgG1 (Th2-promoted isotype in all strains of mice) using an outer membrane antigen preparation of *H. felis* as previously described (13, 30). Antigen (10 μ g/ml) was coated on Immulon II plates (Thermo Labsystems, Franklin, MA), and sera were diluted 1:100. Biotinylated secondary antibodies were monoclonal antimouse antibodies produced by clones A85-1 and 5.7 (Pharmingen, San Diego, CA) for detecting IgG1 and IgG2c, respectively (25). Assays were developed with extravidin peroxidase (Sigma), followed by the 2,2'-azinobis(3-ethylbenzothiazolinesulfonic acid substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Data were analyzed using the two-sample Student t test, assuming equal variances.

Peritoneal macrophages. Peritoneal exudate cells (PECs) were harvested by peritoneal lavage of mice. In some experiments, 1 ml of 4% thioglycolate was administered intraperitoneally, and cells were harvested 4 days later (elicited macrophages). PECs were cultured at 10^6 per well in 24-well plates in RPMI 1640 plus 10% heat-inactivated fetal calf serum and stimulated with lipopolysaccharide (LPS) or human interleukin 1 beta (IL-1 β). LPS from *Escherichia coli* serotype O111:B4 was purchased from Sigma (St. Louis, Missouri) and phenol reextracted to remove contaminating lipopeptides (21). IL-1 β was purchased from R&D Systems (Minneapolis, MN). Culture supernatants were harvested 18 h after stimulation, and cytokine levels were determined by ELISA (OptEIA; BD-Pharmingen).

For analysis of cytokine gene transcription, resident (nonelicited) peritoneal macrophages were incubated in polypropylene tubes with or without IL-1 β (100 ng/ml) for 2 h. The cells were harvested and lysed, and RNA was prepared using a commercial RNA extraction kit (RNeasy; QIAGEN, Valencia, CA). Total mRNA (5 μ g per blot) was reverse transcribed, labeled with biotin, and hybridized to a Mouse NF- κ B Signaling Pathway SuperArray according to the manufacturer's instructions (SuperArray, Inc., Frederick, MD). (The experiment was repeated on three separate occasions using cells pooled from two to three mice.) Chemiluminescent images on film were transilluminated, scanned, and analyzed using GEArray software.

Spleen cell assays. Spleen cells were isolated using lymphocyte separation medium M and cultured in 96-well plates at 2×10^5 per well with concanavalin A (ConA) (2.5 μ g/ml) and IL-1 β (1 to 100 ng/ml). [3 H]thymidine (1 μ Ci per well) was added for the last 16 h of a 96-h incubation. (The analysis was repeated on at least three separate occasions using spleen cells from pools of two to three mice.) For anti-CD3 stimulation, HighProtein binding plates (Corning) were coated with anti-CD3 antibody (Ab) at 400 ng/ml together with anti-CD28 at 100 ng/ml (BD-Pharmingen). Spleen cells were plated at 1×10^6 per well and incubated for 18 h. Cytokine levels were measured in culture supernatants by ELISA. Statistical analysis was performed using Student's t test (JMP software). For fluorescence-activated cell sorting analysis, spleen cells were harvested, and red blood cells were lysed with Tris-ammonium chloride. Cells were incubated with allophycocyanin- or phycoerythrin-labeled antibody specific for CD3, CD4, CD8, CD11b, or CD19 (BD-Pharmingen) and analyzed using a BD FACScan analyzer.

RT-PCR detection of TFF2 and TFF1 mRNA. RNA was extracted using RNeasy (QIAGEN) reagents and treated with RNase-free DNase (Ambion). Reverse transcriptase PCR (RT-PCR) analysis was performed on tissues from individual mice and on tissues from pools of two to three animals on at least three separate occasions with identical results. Murine embryonic fibroblasts were prepared as previously described (22). RNA was DNase treated, reverse transcribed, and PCR amplified using the QIAGEN One-Step/RT-PCR kit. PCR conditions for all three genes were as follows: 94°C for 2 min, followed by 30 cycles at 94°C for 30 s plus 55°C for 30 s plus 72°C for 30 s and one cycle at 72°C for 10 min. Primers were TFF2 (5'-GCAGTGCTTGGATCTTGGATGC-3' and 5'-TCAGGTTGGAAAAGCAGCAGTT-3'), TFF1 (5'-TGTCCTCGCTGTGGTCTCA-3' and 5'-CGATGGCCATGGGGTGAAGC-3'), and glyceraldehyde-3-phosphate dehydrogenase (5'-GACATCAAGAAGGTGGTGAAGC-3' and 5'-GTCCACCACCTGTTGCTGTAG-3').

RESULTS

TFF2 $^{-/-}$ mice show increased inflammatory responses to *H. felis*. TFF2 is thought to play a role in protection of the gastrointestinal tract from injury. Exogenous administration of TFF2 protects mice and rats from GI injury in several models of gastrointestinal inflammatory disease. We examined the effect of TFF2 on gastrointestinal pathology using knockout mice and studied two models of gastrointestinal inflammation, *Helicobacter felis*-induced gastritis and DSS-induced colitis.

Wild-type and TFF2 $^{-/-}$ mice were inoculated with *H. felis* and assessed at 3 and 6 months postinfection. TFF2 $^{-/-}$ mice showed more severe inflammation in response to *H. felis* than

a. Stomach (6 months post-*H. felis* infection)

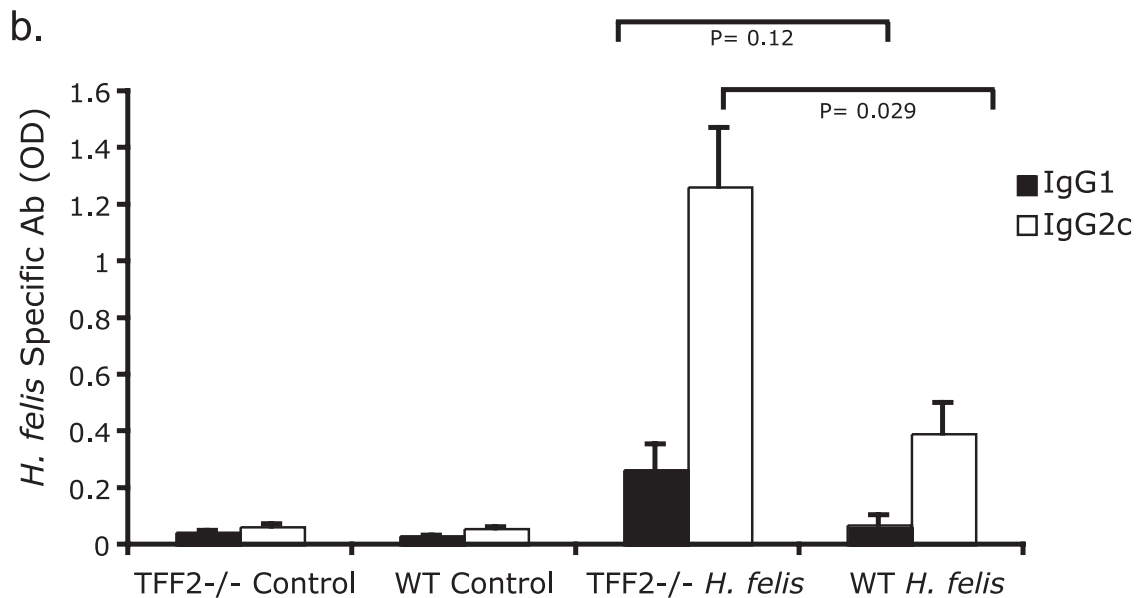
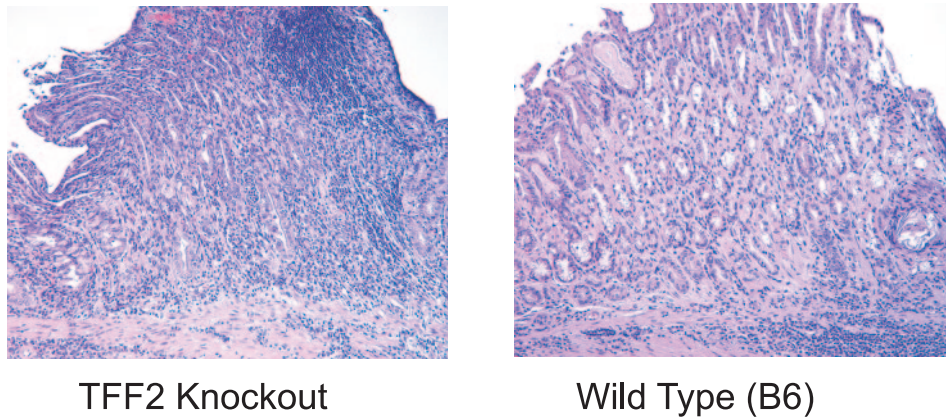


FIG. 1. TFF2^{-/-} mice show more-severe inflammatory responses to *Helicobacter felis*. (a) Histopathology from wild-type and TFF2^{-/-} mice infected for 6 months with *H. felis*, showing marked inflammation in TFF2^{-/-} cells compared to wild-type cells. (b) *H. felis*-specific IgG1 and IgG2c levels in serum (12 months postinfection) determined by ELISA. (IgG2c is the IgG2a-equivalent allele that is uniquely expressed in C57BL/6 mice [25].) IgG2c levels were significantly higher in TFF2^{-/-} mice than in wild-type mice ($P = 0.029$).

wild-type mice (Fig. 1a and b). Histological scoring revealed significant differences between wild-type and TFF2^{-/-} mice in the degree of inflammation, atrophy, mucus metaplasia, and dysplasia at both the 3- and 6-month time points (Table 2). In addition, there was a significantly greater degree of hyperplasia and intestinal metaplasia at 6 months in the TFF2^{-/-} mice ($P < 0.01$) (Table 2).

TFF2^{-/-} mice (like wild-type mice) showed a strong Th1-polarized T-cell response to *H. felis* as revealed by IgG subclass analysis. In fact, the levels of IgG2c were significantly higher in TFF2^{-/-} mice than in wild-type mice ($P = 0.029$) (Fig. 1b). IgG1 levels were not significantly different ($P = 0.12$) between TFF2^{-/-} and wild-type mice.

TFF2^{-/-} mice are susceptible to DSS colitis: delayed recovery compared to wild-type mice. Intermittent DSS treatment is thought to model recurrent inflammatory bowel disease by

TABLE 2. Histological scores of gastric corpus mucosa in TFF2^{-/-} mice and wild-type mice with *Helicobacter felis* infection for 3 or 6 months

Characteristic of <i>H. felis</i> infection	Score ^c			
	3 mo		6 mo	
	Wild type	TFF2 ^{-/-}	Wild type	TFF2 ^{-/-}
Inflammation	0.9 ± 0.5	2.3 ± 0.6 ^b	1.1 ± 0.8	2.4 ± 0.9 ^a
Atrophy	0.1 ± 0.1	1.4 ± 1.0 ^a	1.0 ± 0.6	2.3 ± 1.0 ^a
Mucus metaplasia	0.3 ± 0.3	1.6 ± 1.3 ^a	0.3 ± 0.3	1.6 ± 1.1 ^b
Intestinal metaplasia	0.0 ± 0.0	0.4 ± 0.4	0.0 ± 0.0	0.6 ± 0.4 ^b
Hyperplasia	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	0.7 ± 0.5 ^b
Dysplasia	0.0 ± 0.0	0.5 ± 0.4 ^a	0.0 ± 0.0	0.6 ± 0.4 ^b

^a $P < 0.05$; results for TFF2^{-/-} mice compared with those for wild-type mice.
^b $P < 0.01$; results for TFF2^{-/-} mice compared with those for wild-type mice.
^c $n = 8$ for TFF2^{-/-} mice with *H. felis* infection for 6 months. $n = 4$ for other groups.

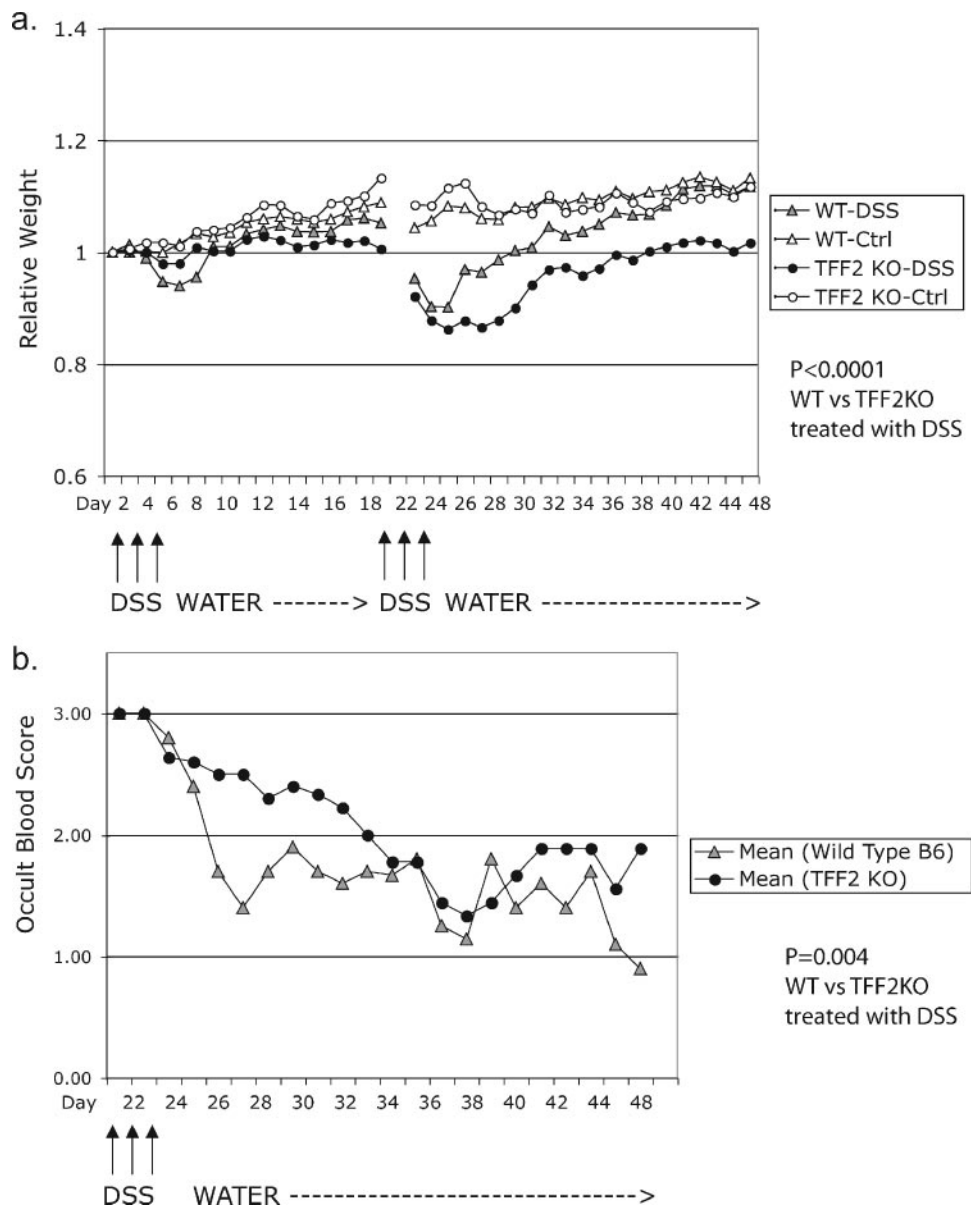
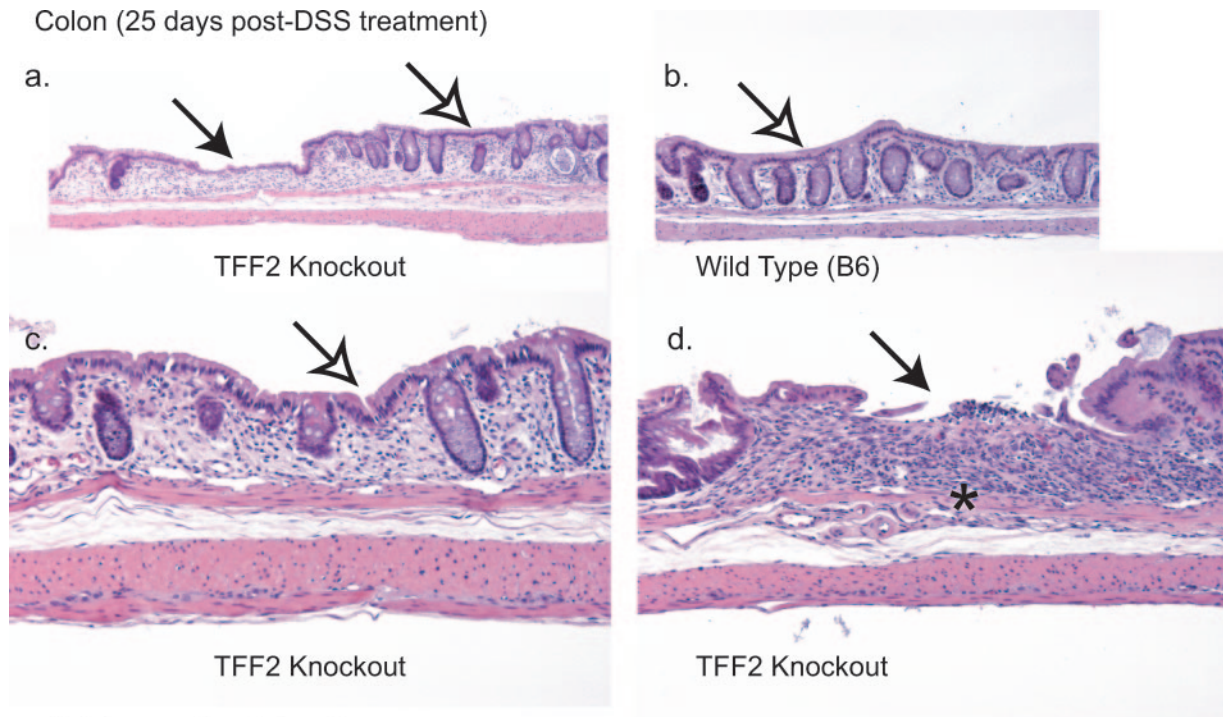


FIG. 2. $TFF2^{-/-}$ mice are susceptible to DSS colitis. $TFF2^{-/-}$ mice showed delayed recovery from DSS colitis (slower weight gain) and prolonged inflammation (occult blood) compared to results for wild-type mice. Data shown are means for 11 animals per group starting at day 22, 1 day prior to the start of the second cycle of DSS. (a) Weights relative to starting weight. (b) Blood in stool was monitored both visually and by Hemocult strip. Scoring for blood in stool was as follows: 0, none; 1, trace using Hemocult strips; 2, strong positive using Hemocult strips; 3, gross hemorrhage.

subjecting the colonic epithelium to acute injury followed by a period of epithelial healing and regeneration (3, 4, 9, 20, 35). Mice were subjected to two cycles of DSS treatment and were weighed daily and scored for colonic hemorrhage. Weight loss was minimal (<10%) during the first cycle of DSS, and no significant differences were noted between wild-type and $TFF2^{-/-}$ mice. However, following the second cycle of DSS, $TFF2^{-/-}$ mice showed greater colonic inflammation and injury than wild-type mice, as measured clinically by sustained weight loss (Fig. 2a) ($P < 0.0001$) and by increases in the frequency and persistence of colonic hemorrhage (Fig. 2b) ($P = 0.004$). Pathological examination of colons from mice recovering from

DSS colitis revealed evidence of persistent inflammation, crypt atrophy, erosion, and ulceration at 3 weeks after the second cycle of DSS (Fig. 3a to d). Histological scores showed a tendency for greater inflammatory changes and ulceration in the colons of $TFF2^{-/-}$ mice than in those of wild-type mice (Fig. 3e), but the differences did not achieve statistical significance ($P > 0.05$).

Expression of TFF2 in murine tissues. We next examined TFF2 expression in gastric and nongastric cells. First, we confirmed that TFF2 was expressed in the stomach. TFF2 mRNA could be detected by RT-PCR in stomach tissue from wild-type and heterozygous ($TFF2^{+/-}$) mice but not in that from ho-



Solid arrow: Focal ulceration
 Open Arrow: Crypt separation, atrophy
 Asterisk: Granulation tissue, inflammation

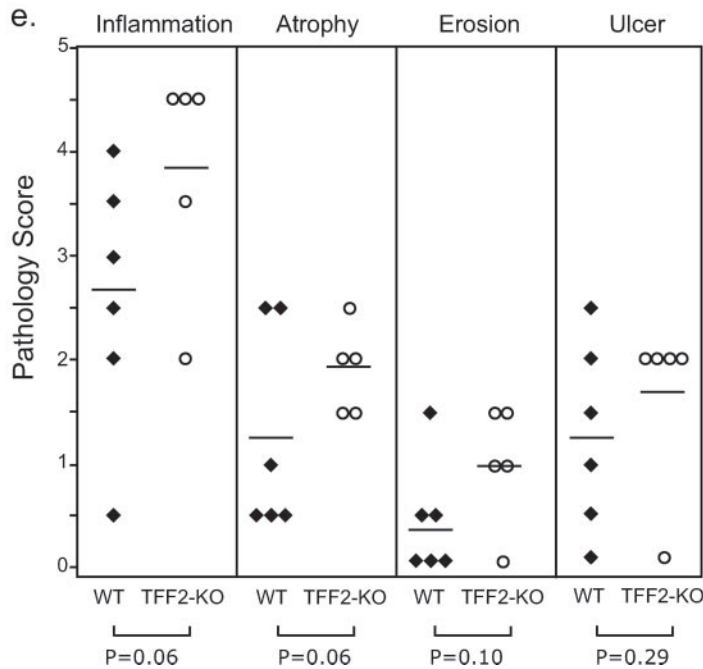


FIG. 3. Inflammatory responses in colons of TFF2^{-/-} and wild-type mice treated with DSS. Histopathology for TFF2^{-/-} (a, c, d) or wild-type (b) mice treated with two cycles of DSS followed by 25 days of water. Colons show inflammatory cell infiltration (asterisk), separation and atrophy of crypts (open arrow), and focal ulceration (solid arrows). (e) Histological scoring of colons from individual wild-type and TFF2^{-/-} mice ($P > 0.05$, Mann-Whitney).

mozygous TFF2^{-/-} knockout animals (Fig. 4a). TFF2 was also expressed in spleen cells (consistent with studies of Cook et al. [2]), suggesting that TFF2 expression was not limited to the GI tract and could be present in cells of the immune system. In

addition to spleen tissue, TFF2 was also expressed in PECs, primarily macrophages, and in murine embryonic fibroblast cell lines (Fig. 4a). Interestingly, TFF2 was not well expressed in the thymus relative to expression in the spleen of wild-type

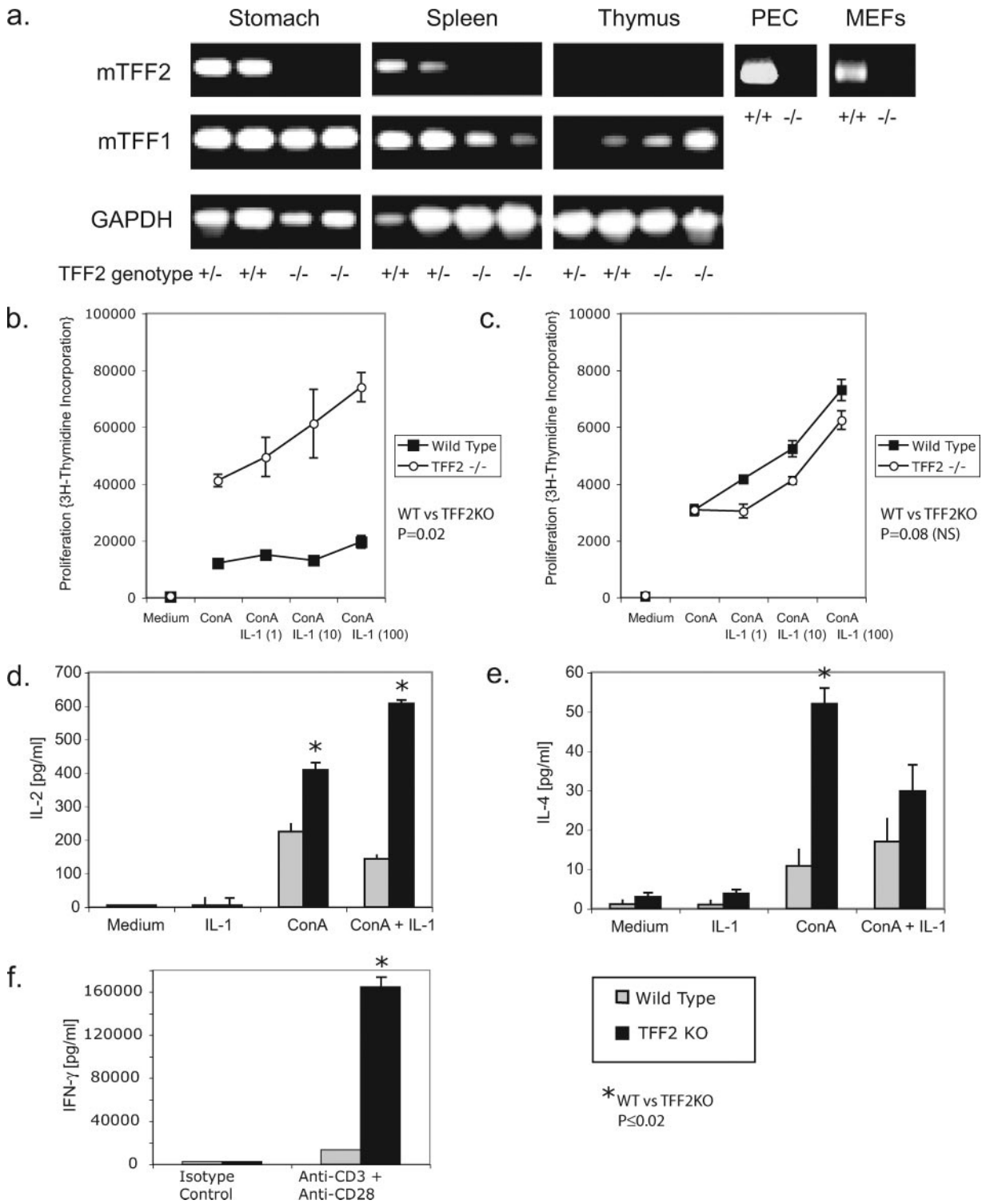


FIG. 4. TFF2 expression in gastric and immune cells. (a) PCR-amplified RNA from wild-type, TFF2^{+/-}, and TFF2^{-/-} mice. TFF2 is expressed in stomach, splenocytes, PEC, and murine embryonic fibroblasts (MEFs) from wild-type mice but not in thymic cells nor in corresponding cells from TFF2-deficient mice. Dose-dependent proliferation of spleen cells (b) and thymocytes (c) cultured with ConA plus various doses of IL-1β (1 to 100 ng/ml). Proliferation of lymphocytes was measured by ³H-thymidine incorporation in cultures incubated for 96 h with ConA plus IL-1β. (d) IL-2 and (e) IL-4 cytokine secretion into culture supernatants 24 h after spleen cell challenge with ConA and IL-1β. (f) Gamma interferon secretion from spleen cells cultured on anti-CD3-coated plates plus anti-CD28.

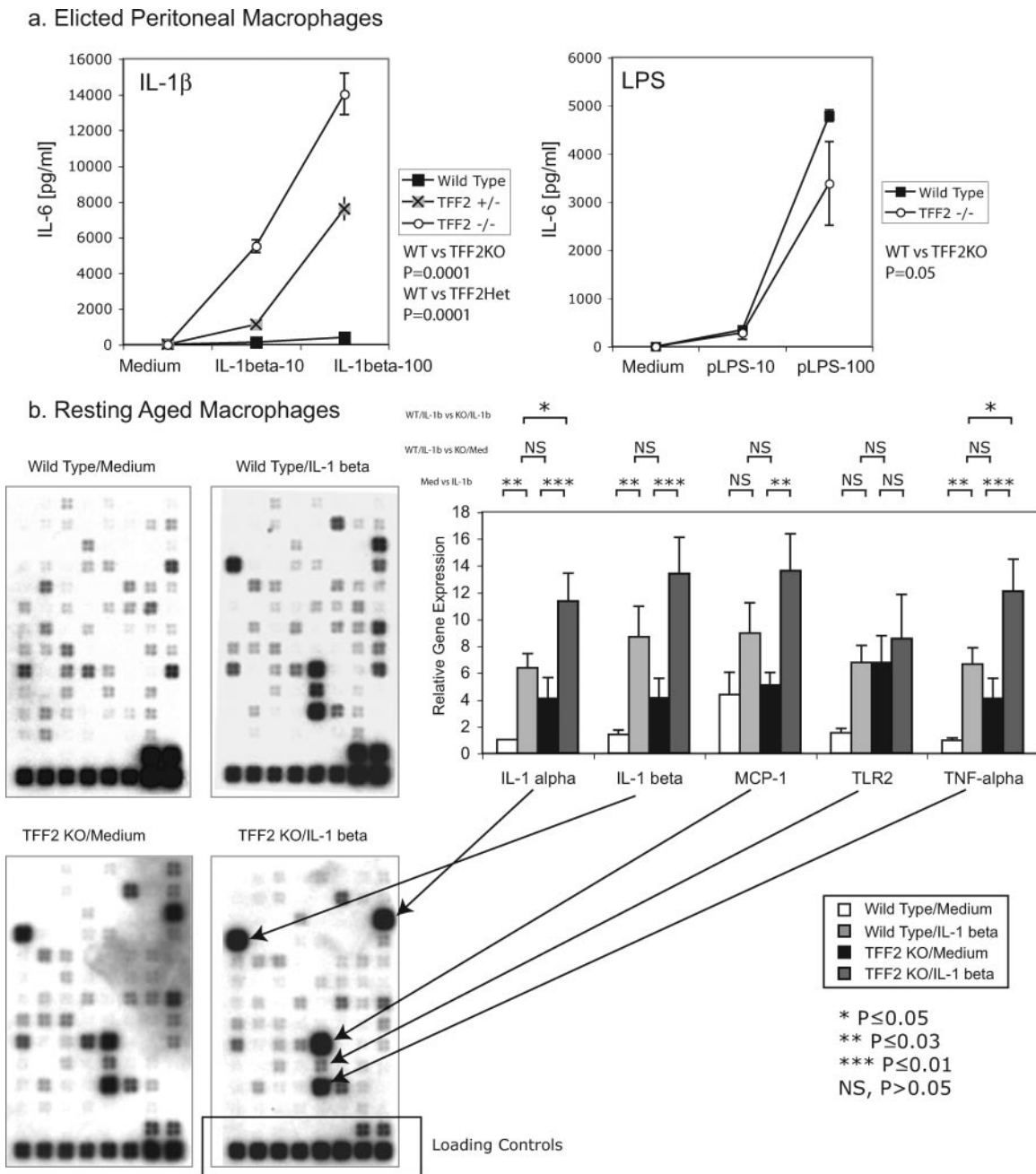


FIG. 5. Macrophages from TFF2^{-/-} mice show increased responses to IL-1β but not TLR4. (a) Elicited macrophages (PEC) from wild-type and TFF2^{-/-} mice were challenged with IL-1β 10 to 100 ng/ml or with LPS, a TLR4 ligand, at 10 to 100 ng/ml. Secretion of IL-6 into culture supernatants was measured by ELISA. (b) Resting macrophages from aged mice with constitutive activation of cytokine genes. Resting (resident) peritoneal macrophages were cultured in medium alone or challenged with IL-1β for 2 h. Inflammatory cytokine mRNA levels were determined using a SuperArray NF-κB Signaling Pathway Array membrane. Membranes probed with wild type or TFF2^{-/-} mRNA with or without IL-1β challenge. Bar graph: Densitometry measurements of selected inflammatory cytokine genes.

animals, suggesting lesser expression in immature T cells. The gene encoding TFF1, which is closely related to that encoding TFF2, lies within the same region of the chromosome as the gene for TFF2 and is an excellent internal control for RNA quality and for PCR and gene targeting specificity, i.e., the gene for TFF2 was specifically targeted in the knockout; thus, TFF2^{-/-} mice should be wild type for TFF1 expression. As predicted, TFF1 was expressed in the thymus as well as in the

spleen and stomach of both wild-type and TFF2^{-/-} mice. Further, thymic TFF1 levels were higher in TFF2^{-/-} mice than in wild-type mice.

Splenic T cells but not thymocytes from TFF2^{-/-} mice are hyperresponsive to ConA-plus-IL-1β-induced proliferative signals. The expression of TFF2 in leukocytes in the spleen and peritoneal cavity raised the possibility that TFF2 could regulate inflammation by directly affecting mononuclear cells

(lymphocytes and macrophages) in inflammatory infiltrates. TFF2^{-/-} splenic T cells exhibited an enhanced proliferative response compared to that of wild-type T cells when stimulated with ConA alone or ConA plus IL-1 β (Fig. 4b). The hyperresponse of spleen cells was dose dependent on the IL-1 β concentration, suggesting that TFF2 may have a direct effect on IL-1R signaling in mature T cells. Interestingly, thymocytes (immature T cells) from TFF2^{-/-} and wild-type mice had very similar proliferative responses to ConA plus IL-1 β (Fig. 4c), consistent with the lack of TFF2 expression in these cells (Fig. 4a). Consistent with the enhanced proliferative response of mature T cells, TFF2^{-/-} splenic T cells secreted higher levels of IL-2 and IL-4 (Fig. 4d and e) than wild-type T cells when cultured with ConA or ConA plus IL-1 β . TFF2^{-/-} cells also exhibited enhanced proliferation and cytokine secretion when activated via their T-cell receptors using anti-CD3 plus CD28 as stimulants (Fig. 4f).

Lymphocyte differentiation in TFF2^{-/-} mice is normal. Our studies suggested that TFF2 was expressed in the spleen but not in the thymus and that splenic responses mediated via IL-1R were dysregulated in TFF2^{-/-} mice. TFF2 deficiency did not affect the differentiation of lymphocytes in the TFF2^{-/-} mice. Fluorescence-activated cell sorting analysis of spleen cells stained for CD3⁺ cells (T and NK cells), CD3⁺ CD4⁺ cells (T helper cells), CD3⁺ CD8⁺ cells (cytolytic T cells), CD19⁺ cells (B cells), and CD11b⁺ cells (dendritic cells and splenic macrophages) indicated that the percentage of each of these subpopulations was indistinguishable when wild-type and TFF2^{-/-} spleen cells were analyzed by flow cytometry (data not shown). Similarly, the percentage of CD4⁺ CD8⁺ double- and single-positive thymic lymphocytes was unchanged for TFF2^{-/-} mice compared to that for wild type mice (data not shown). Thus, TFF2 deficiency did not affect the differentiation of lymphocytes in the TFF2^{-/-} mice, although the proliferative response of these cells to IL-1 β challenge (i.e., IL-1R signaling) was enhanced.

Wild-type and TFF2^{-/-} macrophages responded similarly to TLR4 LPS but were hyperresponsive to IL-1 β stimulation in vitro. Peritoneal macrophages, like spleen cells, expressed TFF2 mRNA. We therefore wanted to determine if the hyperresponsiveness to IL-1 β we found in TFF2^{-/-} spleen cells was limited to lymphocytes or occurred in other IL-1-responsive cell types. Peritoneal macrophages isolated from wild-type and TFF2^{-/-} mice were challenged with IL-1 β to engage IL-1R, and the secretion of IL-6 into culture supernatants was measured. TFF2^{-/-} macrophages secreted much higher levels of IL-6 than wild-type macrophages in response to IL-1R stimulation (Fig. 5a, left panel). In contrast, wild-type macrophages secreted low levels of IL-6 even at the highest IL-1 β concentration tested (100 ng/ml), suggesting that IL-1R activation was limited in wild-type mice. Interestingly, macrophages from TFF2^{+/-} heterozygous mice secreted IL-6 at levels intermediate between those of homozygous knockout cells and wild-type cells, suggesting a gene dosage effect of TFF2 on IL-1R signaling (Fig. 5a).

Unlike the case with the IL-1 β response, wild-type and TFF2^{-/-} mice had very similar responses to LPS (Fig. 5a, right panel), suggesting that the hyperresponsiveness of TFF2^{-/-} mice was specific for IL-1 β /IL-1R signaling pathways despite

the fact that the IL-1R pathway shares many common adaptors with the LPS/TLR4 pathway (1).

Resident (resting) macrophages from TFF2^{-/-} mice are highly activated compared to wild-type macrophages. Our in vivo and in vitro studies suggested that TFF2 deficiency produced a dysregulated, proinflammatory response. We examined the inflammatory gene expression profile of resident peritoneal macrophages from older adult mice (>8 weeks of age). RNA was prepared from freshly isolated, resident macrophages, labeled with biotin, and hybridized to a commercial NF- κ B signaling pathway gene array (SuperArray). Wild-type resident macrophages expressed few inflammatory cytokine genes, but the expression of these genes could be induced by a 2-h incubation with IL-1 β (Fig. 5b). In contrast, TFF2^{-/-} resident macrophages expressed a large number of inflammatory genes even in the absence of exogenous IL-1 β (Fig. 5b). In fact, the pattern of gene expression in unstimulated TFF2^{-/-} macrophages is remarkably similar to the gene expression pattern in wild-type macrophages incubated with IL-1 β . This included changes in genes such as those encoding IL-1 α , IL-1 β , monocyte chemoattractant protein 1, etc. (Fig. 5B). Thus, TFF2^{-/-} macrophages appeared to have been activated via their IL-1R in vivo, consistent with the hyperinflammatory phenotype of these mice.

DISCUSSION

Since 1989, it has been well documented that TFFs are expressed along the length of the normal gastrointestinal tract, with TFF2 expression being primarily localized to the stomach (27, 28). In previously published experiments using either rats or mice, our group has shown that TFF2 is constitutively expressed in the GI mucosa within specialized epithelial cells and is markedly upregulated in response to inflammation, injury, and repair, as demonstrated using immunohistochemistry of tissue sections (18, 34). Here we demonstrate that immune cells are an important additional source of TFF2, a novel and potentially important finding, and that immune cell TFF2 is an important regulator of the innate immune response in these cells.

In the gut, TFFs are typically expressed in mucus-secreting cells, and consequently, most of the focus has been on their role in cytoprotection and reparation of the mucosal lining of the gut (29, 37, 40). However, given that administration of exogenous TFFs reduces inflammation in the gastrointestinal tract (40), Cook et al. (2) first explored the possibility of expression of TFFs in nongastric cells. They reported the expression of TFF2 in rat spleen and lymphoid tissues (2). Thus, the current study extends these findings but also provides the first demonstration that TFF2 functions as an anti-inflammatory peptide with specificity for IL-1 β signaling and immune cell cytokine secretion.

In this study, we demonstrate a novel role for TFF2 in the regulation of IL-1 signaling. We confirmed that TFF2 is expressed not only in gastric epithelial cells (18, 34) but also in spleen cells and that TFF2 deficiency is associated with increased cytokine secretion by macrophages and T-cell proliferation in response to IL-1 β . These in vitro responses correlated with increased inflammatory responses to LPS injection, *H. felis* infection, and DSS models of inflammatory bowel dis-

ease. The effect of TFF2 deficiency on macrophages was highly specific for the IL-1 pathway, since no alteration was seen in the responses of PECs to the TLR4 ligand LPS, which shares many/most of the same signaling pathways.

Several previous observations suggested that TFF2 might play a role in controlling or dampening inflammation. Several groups have reported that application of recombinant trefoils can reduce inflammatory indices in animal models of colitis (16, 33, 40). For example, luminal application of recombinant TFF2/spasmocytic peptide in a rat model of colitis revealed markedly accelerated colonic mucosal rebuilding and reduced inflammatory indices (40) through mechanisms that could possibly include inhibition of inducible nitric oxide synthase and NO in monocytes (17). In addition, TFF2 is clearly upregulated in epithelial cells in chronic inflammatory conditions of the gastrointestinal tract, such as peptic ulcer disease and Crohn's disease, and in other organ systems, such as the lung; in fact, TFF2 may be regulated directly by a variety of cytokines (26). TFF2 may also function in promoting restitution of the epithelium, and it has always been assumed that such increased epithelial expression functioned mainly in the context of epithelial regeneration and repair. Recently, TFF2 has been shown to affect the development of asthma in mice (26). However, given our results, it would seem reasonable to suggest that TFF2 may also represent a signal by the epithelium to the immune system to down-regulate or otherwise dampen an acute inflammatory response. Thus, TFF2 would serve as a key player in the epithelial-immune signaling system.

IgG subclass responses have been used as estimates of Th cell type, with IgG1 being produced in Th2 processes and IgG2c (the C57BL/6 allelic equivalent of IgG2a) in Th1 responses of C57BL/6 mice (25, 32, 38). Thus, the IgG1/IgG2c ratio represents a quantifiable measure of balance between mucosal Th2/Th1 activity, and in previous studies by our group, a low ratio (indicating a predominant Th1 response) has correlated well with the tendency toward neoplastic progression (12). TFF2^{-/-} mice, like wild type mice, had a Th1-dominated response to *H. felis* and in fact had significantly higher levels of IgG2c than wild-type mice following *H. felis* infection. Thus, the Ab response to infection was consistent with an enhanced proinflammatory response in the TFF2^{-/-} mice.

The expression of TFF2 in spleen cells raised the possibility that TFF2 might affect lymphocyte responses. We examined the effect of TFF2 deficiency on spleen cell responses to B- and T-cell mitogens. Splenic B cells proliferate in response to Pokeweed mitogen, while splenic T cells proliferate and secrete cytokines in response to ConA. IL-1 β (an inflammatory cytokine which has been linked to human gastrointestinal disease and gastric cancer progression) synergizes with ConA to stimulate T-cell proliferation. T cells are also activated by cross-linking T-cell receptors with plate-bound anti-CD3 Ab.

T cells from TFF2^{-/-} mice exhibited an enhanced proliferative response to ConA and ConA plus IL-1 and to anti-CD3-coated plates. Thymocytes, on the other hand, were unaffected by TFF2 deficiency. Thus, the hyperproliferative response of the TFF2^{-/-} T cells was limited to mature cells and/or peripheral lymphoid tissues, i.e., mature T lymphocytes in the spleen but not immature T cells in the thymus. The sensitivity to TFF2 follows the pattern of TFF2 gene expression in these lymphoid organs. In addition to excess proliferation, TFF2^{-/-} lympho-

cytes secreted increased levels of IL-2 and IL-4, suggesting a generalized enhancement of T-lymphocyte activation. Although TFF2 deficiency markedly increased T-cell proliferative responses in the spleen, TFF2 deficiency did not affect B-cell proliferation induced by Pokeweed mitogen (data not shown). Thus, mature T cells may be the major target of TFF2 regulation in the spleen.

Our current study suggests that in addition to excess cytokine production and lymphocyte proliferation, a major phenotype associated with a deficiency of TFF2 is unrestrained IL-1R signaling. In fact, the TFF2^{-/-} mouse resembled in some ways a mouse with constitutive IL-1R activation. IL-1 β signals via the type I IL-1R. The cytoplasmic domain of IL-1RI contains a Toll-interleukin 1 resistance (TIR) domain which mediates receptor signaling via MyD88-dependent pathways leading to cytokine secretion. Like IL-1R, TLR4 utilizes TIR domain-mediated and MyD88-dependent signaling cascades. Thus, LPS-induced signaling via TLR4 engages many of the same downstream adapters that are triggered by IL-1 β /IL-1R engagement. To determine if TFF2 deficiency globally enhanced TIR signaling, we examined the response of wild-type and TFF2 knockout macrophages to LPS. Interestingly, TFF2^{-/-} mice were hyperresponsive to IL-1 receptor signaling but not to LPS signaling, suggesting a selective enhancement of IL-1R-induced pathways.

Interleukin 1 receptor signaling has been shown to be particularly important in chronic inflammatory diseases of the gastrointestinal tract, including both inflammatory bowel disease and *Helicobacter pylori* gastritis. IL-1R signaling induces the expression and secretion of multiple inflammatory cytokines and chemokines, including IL-6, monocyte chemoattractant protein 1, and tumor necrosis factor alpha, as well as autocrine secretion of IL-1 β . These inflammatory cytokines were up-regulated in resident peritoneal macrophages from older TFF2 knockout mice, suggesting an ongoing inflammatory response in vivo. Mononuclear cells isolated from the intestinal mucosa of patients with Crohn's disease or ulcerative colitis show enhanced production of IL-1 β (24), and patients with ulcerative colitis show an increased carrier rate for the intron 2 IL-1 receptor antagonist polymorphism (39). Infection with *H. pylori* also results in a strong inflammatory response led by the early release of IL-1 β along with numerous other cytokines and chemokines (5). Further, we have noted that *H. felis* infection in C57BL/6 mice induces a significant IL-1 β response (12). However, the progression of gastric inflammation to gastric atrophy and cancer is closely related to the overall balance between pro- and anti-inflammatory cytokines and specifically to the expression of IL-1 β . Thus, proinflammatory polymorphisms in the IL-1B (511 T/T) and IL-1RN (*2) genes are associated with an increased risk for gastric carcinoma in *H. pylori*-infected individuals (6–8, 14, 23). *H. pylori*-infected carriers of these polymorphisms have been shown to exhibit higher mucosal levels of IL-1 β and more severe inflammation than *H. pylori*-infected noncarriers (19). Recently, mice lacking SIGIRR/Tir8, an important regulator of IL-1 signaling, were shown to exhibit increased susceptibility to inflammatory bowel disease (15). Given the central role of IL-1 in the inflammatory process and the potential harm associated with excessive IL-1-dependent signaling, it would be reasonable to speculate that vertebrates have evolved diverse mechanisms, including TFF2,

to counterregulate the response to IL-1 β . The recognition of trefoil peptides as a novel class of cytokines may provide new insight into the modulation of the immune system.

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REFERENCES

- Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**:143–150.
- Cook, G. A., M. Familiari, L. Thim, and A. S. Giraud. 1999. The trefoil peptides TFF2 and TFF3 are expressed in rat lymphoid tissues and participate in the immune response. *FEBS Lett.* **456**:155–159.
- Cooper, H. S., S. Murthy, K. Kido, H. Yoshitake, and A. Flanigan. 2000. Dysplasia and cancer in the dextran sulfate sodium mouse colitis model. Relevance to colitis-associated neoplasia in the human: a study of histopathology, B-catenin and p53 expression and the role of inflammation. *Carcinogenesis* **21**:757–768.
- Cooper, H. S., S. N. Murthy, R. S. Shah, and D. J. Sedergran. 1993. Clinico-pathologic study of dextran sulfate sodium experimental murine colitis. *Lab. Investig.* **69**:238–249.
- El-Omar, E. M. 2001. The importance of interleukin 1beta in Helicobacter pylori associated disease. *Gut* **48**:743–747.
- El-Omar, E. M., M. Carrington, W. H. Chow, K. E. McColl, J. H. Bream, H. A. Young, J. Herrera, J. Lissowska, C. C. Yuan, N. Rothman, G. Lanyon, M. Martin, J. F. Fraumeni, Jr., and C. S. Rabkin. 2000. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* **404**:398–402.
- El-Omar, E. M., M. Carrington, W. H. Chow, K. E. McColl, J. H. Bream, H. A. Young, J. Herrera, J. Lissowska, C. C. Yuan, N. Rothman, G. Lanyon, M. Martin, J. F. Fraumeni, Jr., and C. S. Rabkin. 2001. The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer. *Nature* **412**:99.
- El-Omar, E. M., C. S. Rabkin, M. D. Gammon, T. L. Vaughan, H. A. Risch, J. B. Schoenberg, J. L. Stanford, S. T. Mayne, J. Goedert, W. J. Blot, J. F. Fraumeni, Jr., and W. H. Chow. 2003. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* **124**:1193–1201.
- Elson, C. O., R. B. Sartor, G. S. Tennyson, and R. H. Riddell. 1995. Experimental models of inflammatory bowel disease. *Gastroenterology* **109**:1344–1367.
- Farrell, J. J., D. Taupin, T. J. Koh, D. Chen, C. M. Zhao, D. K. Podolsky, and T. C. Wang. 2002. TFF2/SP-deficient mice show decreased gastric proliferation, increased acid secretion, and increased susceptibility to NSAID injury. *J. Clin. Investig.* **109**:193–204.
- Fitzmaurice, G. M., N. M. Laird, and J. H. Ware. 2004. Applied longitudinal analysis. John Wiley & Sons, Hoboken, N.J.
- Fox, J. G., P. Beck, C. A. Dangler, M. T. Whary, T. C. Wang, H. N. Shi, and C. Nagler-Anderson. 2000. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. *Nat. Med.* **6**:536–542.
- Fox, J. G., A. B. Rogers, M. Ihrig, N. S. Taylor, M. T. Whary, G. Dockray, A. Varro, and T. C. Wang. 2003. Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific. *Cancer Res.* **63**:942–950.
- Furuta, T., E. M. El-Omar, F. Xiao, N. Shirai, M. Takashima, H. Sugimura, and H. Sugimura. 2002. Interleukin 1beta polymorphisms increase risk of hypochlorhydria and atrophic gastritis and reduce risk of duodenal ulcer recurrence in Japan. *Gastroenterology* **123**:92–105.
- Garlanda, C., F. Riva, N. Polentarutti, C. Buracchi, M. Sironi, M. De Bortoli, M. Muzio, R. Bergottini, E. Scanziani, A. Vecchi, E. Hirsch, and A. Mantovani. 2004. Intestinal inflammation in mice deficient in Tlr8, an inhibitory member of the IL-1 receptor family. *Proc. Natl. Acad. Sci. USA* **101**:3522–3526.
- Giraud, A. S. 2000. X. Trefoil peptide and EGF receptor/ligand transgenic mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278**:G501–G506.
- Giraud, A. S., P. M. Pereira, L. Thim, L. M. Parker, and L. M. Judd. 2004. TFF-2 inhibits iNOS/NO in monocytes, and nitrated protein in healing colon after colitis. *Peptides* **25**:803–809.
- Houghton, J., C. Stoicov, S. Nomura, A. B. Rogers, J. Carlson, H. Li, X. Cai, J. G. Fox, J. R. Goldenring, and T. C. Wang. 2004. Gastric cancer originating from bone marrow-derived cells. *Science* **306**:1568–1571.
- Hwang, I. R., T. Kodama, S. Kikuchi, K. Sakai, L. E. Peterson, D. Y. Graham, and Y. Yamaoka. 2002. Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1beta production in Helicobacter pylori infection. *Gastroenterology* **123**:1793–1803.
- Kitajima, S., S. Takuma, and M. Morimoto. 2000. Histological analysis of murine colitis induced by dextran sulfate sodium of different molecular weights. *Exp. Anim.* **49**:9–15.
- Kurt-Jones, E. A., L. Mandell, C. Whitney, A. Padgett, K. Gosselin, P. E. Newburger, and R. W. Finberg. 2002. Role of toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. *Blood* **100**:1860–1868.
- Kurt-Jones, E. A., F. Sandor, Y. Ortiz, G. N. Bowen, S. L. Counter, T. C. Wang, and R. W. Finberg. 2004. Use of murine embryonic fibroblasts to define Toll-like receptor activation and specificity. *J. Endotoxin Res.* **10**:419–424.
- Machado, J. C., C. Figueiredo, P. Canedo, P. Pharoah, R. Carvalho, S. Nabais, C. Castro Alves, M. L. Campos, L. J. Van Doorn, C. Caldas, R. Seruca, F. Carneiro, and M. Sobrinho-Simoes. 2003. A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. *Gastroenterology* **125**:364–371.
- Mahida, Y. R., K. Wu, and D. P. Jewell. 1989. Enhanced production of interleukin 1-beta by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease. *Gut* **30**:835–838.
- Martin, R. M., J. L. Brady, and A. M. Lew. 1998. The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. *J. Immunol. Methods* **212**:187–192.
- Nikolaides, N. M., N. Zimmermann, N. E. King, A. Mishra, S. M. Pope, F. D. Finkelman, and M. E. Rothenberg. 2003. Trefoil factor-2 is an allergen-induced gene regulated by Th2 cytokines and STAT6 in the lung. *Am. J. Respir. Cell Mol. Biol.* **29**:458–464.
- Nomura, S., T. Baxter, H. Yamaguchi, C. Leys, A. B. Vartapetian, J. G. Fox, J. R. Lee, T. C. Wang, and J. R. Goldenring. 2004. Spasmolytic polypeptide expressing metaplasia to preneoplasia in H. felis-infected mice. *Gastroenterology* **127**:582–594.
- Otto, W. R., and K. Patel. 1999. Trefoil factor family (TFF)-domain peptides in the mouse: embryonic gastrointestinal expression and wounding response. *Anat. Embryol. (Berlin)* **199**:499–508.
- Poulsen, S. S., J. Thulesen, L. Christensen, E. Nexø, and L. Thim. 1999. Metabolism of oral trefoil factor 2 (TFF2) and the effect of oral and parenteral TFF2 on gastric and duodenal ulcer healing in the rat. *Gut* **45**:516–522.
- Pronovost, A. D., S. L. Rose, J. W. Pawlak, H. Robin, and R. Schneider. 1994. Evaluation of a new immunodiagnostic assay for *Helicobacter pylori* antibody detection: correlation with histopathological and microbiological results. *J. Clin. Microbiol.* **32**:46–50.
- Rogers, A. B., N. S. Taylor, M. T. Whary, E. D. Stefanich, T. C. Wang, and J. G. Fox. 2005. Helicobacter pylori but not high salt induces gastric intraepithelial neoplasia in B6129 mice. *Cancer Res.* **65**:10709–10715.
- Romagnani, S. 1997. The Th1/Th2 paradigm. *Immunol. Today* **18**:263–266.
- Soriano-Izquierdo, A., M. Gironella, A. Massagué, F. E. May, A. Salas, M. Sans, R. Poulosom, L. Thim, J. M. Pique, and J. Panes. 2004. Trefoil peptide TFF2 treatment reduces VCAM-1 expression and leukocyte recruitment in experimental intestinal inflammation. *J. Leukoc. Biol.* **75**:214–223.
- Stoicov, C., X. Cai, H. Li, K. Klucsevsek, J. Carlson, R. Saffari, and J. Houghton. 2005. Major histocompatibility complex class II inhibits Fas antigen-mediated gastric mucosal cell apoptosis through actin-dependent inhibition of receptor aggregation. *Infect. Immun.* **73**:6311–6321.
- Strober, W., I. J. Fuss, and R. S. Blumberg. 2002. The immunology of mucosal models of inflammation. *Annu. Rev. Immunol.* **20**:495–549.
- Taupin, D., and D. K. Podolsky. 2003. Trefoil factors: initiators of mucosal healing. *Nat. Rev. Mol. Cell. Biol.* **4**:721–732.
- Thim, L. 1997. Trefoil peptides: from structure to function. *Cell Mol. Life Sci.* **53**:888–903.
- Toellner, K. M., S. A. Luther, D. M. Sze, R. K. Choy, D. R. Taylor, I. C. MacLennan, and H. Acha-Orbea. 1998. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *J. Exp. Med.* **187**:1193–1204.
- Tountas, N. A., V. Casini-Raggi, H. Yang, F. S. Di Giovine, M. Vecchi, L. Kam, L. Melani, T. T. Pizarro, J. I. Rotter, and F. Cominelli. 1999. Functional and ethnic association of allele 2 of the interleukin-1 receptor antagonist gene in ulcerative colitis. *Gastroenterology* **117**:806–813.
- Tran, C. P., G. A. Cook, N. D. Yeomans, L. Thim, and A. S. Giraud. 1999. Trefoil peptide TFF2 (spasmolytic polypeptide) potentially accelerates healing and reduces inflammation in a rat model of colitis. *Gut* **44**:636–642.
- Wang, T. C., C. A. Dangler, D. Chen, J. R. Goldenring, T. Koh, R. Raychowdhury, R. J. Coffey, S. Ito, A. Varro, G. J. Dockray, and J. G. Fox. 2000. Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. *Gastroenterology* **118**:36–47.