Role of Tumor Necrosis Factor Alpha in *Helicobacter pylori* Gastritis in Tumor Necrosis Factor Receptor 1-Deficient Mice

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Increased gastric production of interleukin 8 and tumor necrosis factor alpha (TNF-α) has been implicated in the pathogenesis of *Helicobacter pylori*-associated gastroduodenal disease. In the present study we used a mouse model to demonstrate whether loss of the tumor necrosis factor receptor 1 (TNF-R1) function leads to differences in gastric inflammation or the systemic immune response in *H. pylori* infection. Six different clinical isolates of *H. pylori* (three cytotoxin-positive and three cytotoxin-negative strains) were adapted to C57BL/6 mice. TNF-R1-deficient (TNF-R1−/−) mice (*n* = 19) and isogenic controls (*n* = 24) were infected and sacrificed after 4 weeks of infection. Inflammation of the stomach and the humoral immune response to *H. pylori* were evaluated by histological, immunohistochemical, and serological methods. There was no detectable difference in the grade or activity of gastritis in TNF-R1−/− mice when they were compared with wild-type mice, but the number of lymphoid aggregates was slightly reduced in the gastric mucosa of TNF-R1−/− mice. Interestingly, total immunoglobulin G (IgG), as well as IgG1, IgG2b, and IgG3, *H. pylori*-specific antibody titers were significantly higher in wild-type mice. As revealed by immunoblot analysis, the difference in reactivity against *H. pylori* antigens was not based on a failure to recognize single *H. pylori* antigens in TNF-R1−/− mice. We therefore suggest that TNF-R1-mediated TNF-α signals might support a systemic humoral immune response against *H. pylori* and that the gastric inflammatory response to *H. pylori* infection seems to be independent of TNF-R1-mediated signals.

*Helicobacter pylori* colonization of the human gastric mucosa has been shown to be the main causative agent of chronic active type B gastritis and is closely associated with peptic and duodenal ulcer disease (6, 23, 42, 58), as well as gastric carcinoma (19, 45) and low-grade gastric B-cell lymphoma of the MALT type (19, 30, 59). There are two proposed explanations for how chronic *H. pylori* infection may lead to such diverse clinical outcomes. First, genetic diversity of virulence factors and antigenic profiles of various *H. pylori* strains may account for different disease entities. Second, genetically based differences in the individual immune responses to the pathogen may result in failure to eradicate the infection and lead to chronic mucosal damage (25); for example, interleukin-1 (IL-1) gene cluster polymorphisms followed by enhanced production of IL-1β seem to be associated with an increased risk of *H. pylori*-induced hypochlorhydria and gastric cancer (20).

Gastric mucosal damage in *H. pylori* infection depends on special virulence factors of *H. pylori*, including enhanced motility (28); production of adaptive enzymes, such as urease (41), catalase (27), and phospholipase C (12); specific adherence to gastric epithelial cells (17); production of a vacuolating cytotoxin (VacA) by some *H. pylori* strains (37); and the presence of a bacterial gene cluster (a pathogenicity island) (22) with cagA (cytotoxin-associated gene A) as a marker for the presence of the cag pathogenicity island (7). CagA- and VacA-expressing *H. pylori* strains enhance gastric inflammation in *H. pylori* infections and are strongly associated with gastroduodenal ulceration (13, 24), in which allelic variants of the vacA gene appear to regulate cytotoxic activity; vacA s1m1 strains produce higher levels of cytotoxin than s1m2 strains, which are essentially nontoxic in the HeLa cell assay but may be able to induce vacuolization in primary gastric cells or other cell lines. vacA s2m2 strains do not show detectable cytotoxin activity (2, 3, 47). Also important is the inflammatory reaction of the host, which is modulated by secretion of various cytokines, like IL-8 (11), gamma interferon (IFN-γ) (33), and tumor necrosis factor alpha (TNF-α) (12).

TNF-α is a key mediator in a host’s response against gram-negative bacteria and in the septic shock syndrome induced by either lipopolysaccharide (LPS) or bacterial superantigens (5). Secretion of TNF-α from LPS-activated mononuclear phagocytes or antigen-stimulated T cells can be enhanced by IFN-γ. In *H. pylori* gastritis (13) the cytokine response is of the Th1 type since IFN-γ but not IL-4 is predominant (38). In mice lacking interferon regulatory factor 1 the defective Th1 response was associated with the total lack of gastritis and atrophy despite severe colonization with *H. pylori* (55). The multiple biological activities of TNF-α, like stimulation of expression of adhesion molecules such as intercellular adhesion molecule 1 on endothelial cells, which facilitates the extravasation of neutrophils into the lamina propria of mucosal tissue, activation of leukocytes and T-lymphocytes, stimulation of the production of cytokines by macrophages and monocytes (26, 56), and induction of apoptosis (34), are mediated by two distinct cell surface receptors. Tumor necrosis factor receptor
1 (TNF-R1), binding TNF-α and lymphotxin alpha (LT-α) (= TNF-β), is generally known to mediate most of the TNF-α effects, especially apoptosis (57), whereas TNF-R2 is mainly implicated in lymphocyte proliferation (21).

Mice deficient for TNF-R1 are resistant to lethal doses of either LPS or *Staphylococcus aureus* endotoxin B but are severely impaired with respect to clearing *Listeria monocytogenes* and readily succumb to infection (51). Moreover, mice lacking TNF-R1 show a complete lack of Peyer’s patches (46), and LT-α-deficient mice have defects in forming germinal centers (43), whereas the development of lymph nodes is not inhibited.

Marchetti et al. (40) developed in 1995 a mouse model of *H. pylori* infection that mimics human disease. The pathogenesis of *H. pylori* infection in vivo was studied by adapting fresh clinical isolates of bacteria to colonize the stomachs of mice, and a gastric pathology resembling human disease was observed, especially in infections with cytotoxin-producing strains.

In this study we used TNF-R1-deficient mice and isogenic controls that were infected orally with different *H. pylori* strains and sacrificed after 4 weeks to show whether the loss of TNF-R1 function leads to differences in the systemic immune response or gastric inflammation. Our findings demonstrate that the systemic humoral immune response to *H. pylori* antigens might be enhanced by TNF-α mediated by the TNF-R1 pathway, whereas gastric inflammation in *H. pylori* infections seems to be independent of this pathway.

**MATERIALS AND METHODS**

**Animals.** Twenty-four female TNF-R1-deficient C57BL/6 mice (GSF, Munich, Germany) (51) and 19 female isogenic controls (Charles River, Sulzfeld, Germany), all of which were 10 weeks old, were housed in Mikrolon type stainless steel isolators; five mice of each group were used as uninfected controls. All materials were sterilized with pressurized steam, and the animals were fed a sterile, totally resorbable liquid nutrition diet (10 ml per mouse per day; Biosorbin MCT; Pfrimmer Nutricia, Erlangen, Germany), as well as water ad libitum.

**Bacteria and growth conditions.** We used six different fresh clinical isolates of *H. pylori* to colonize the stomachs of mice, and a gastric pathology resembling human disease was observed, especially in infections with cytotoxin-producing strains.

**Evaluation of cytotoxicity.** Detection of vacuolating cytotoxic activity was evaluated with HeLa and Vero cell assay (American Type Culture Collection, Rockville, Md.), as published previously (29, 37). *H. pylori* cells were grown for 48 h in BBEFC–8% Dent (Oxoid). Culture supernatants were centrifuged, sterilized filtered with a 0.22-μm-pore-size Millex-GV filter (Millipore, Eschborn, Germany), and tested for vacuolating cytotoxic activity with Vero cells (ATCC CCL 81) and HeLa cells (ATCC CCL 2) under standard conditions. After inoculation of 96-well microtiter plates with 2 × 10^6 cells per well, serial dilutions (1/2 to 1/8) of *H. pylori* culture supernatants were inoculated onto the coated plates and incubated in a humid atmosphere containing 5% CO2 at 37°C. After 24 h, the level of vacuolization was determined by inverse microscopy (magnification, ×100 to ×200). *H. pylori* cell lines were considered cytotoxic positive if vacuolization was observed in more than 50% of Vero and HeLa cells. *H. pylori* ATCC 49503 and ATCC 51932 were used as positive and negative controls. The oligonucleotide primer and PCR amplification conditions for vacA alleles (s1, s2, m1, m2) used have been described previously (2).

For *cagA* we used our own primers, cagAF (5′-AAAGGATGTCGCCCAAGA-GAA-3′) and cagAR (5′-TCCGTTACCTCTTGATGTAGTA-3′).

**Determination of IL-8 protein.** AGS cells (– ATCC CRL 1739) were cultured in RPMI 1640 medium (PAN Biotech, Aidenbach, Germany) supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin G, and 1% streptomycin in a humid atmosphere containing 5% CO2 at 37°C. After 24 h, the supernatants were carefully collected and stored at −80°C until they were used for analysis.

**Characterization of *H. pylori* strains.** The characteristics of the six different fresh clinical isolates evaluated by cell culture assay for vacuolating activity, by PCR for vacA and *cagA*, and by an IL-8 assay are summarized in Table 1.

**Antibiotics and dosing scheme.** As the growth of *H. pylori* may be suppressed by lactobacilli previously inhabiting the stomachs of mice (32), every mouse was treated with ciprofloxacin (0.5 mg per day orally), amikacin (375 μg per day orally and intraperitoneally [i.p.], imipenem (1.25 mg per day orally and i.p.), vancomycin (1 mg per day orally and i.p.), and fluconazole (150 μg per day orally) to decontaminate the gastrointestinal tract on three consecutive days. The doses were in accordance with the highest permissible human doses per day expressed in milligrams per kilogram of body weight. *H. pylori* infection. Beginning 2 days after the treatment with antibiotics, the mice were inoculated orally three times at 2-day intervals with the six different *H. pylori* strains described above. The mice were each given 0.25 ml of a solution of 0.2 M NaHCO3 orally to neutralize acidity, and 10^9 CFU of each strain in 100 to 200 μl of an *H. pylori* suspension was administered immediately after the bicarbonate treatment by using a blunt stainless steel tube.

**Collection of gastric tissue and serum samples.** Mice infected with adapted *H. pylori* strains were sacrificed 4 weeks after infection. At the time of killing, a blood sample was drawn from each mouse from the vena cava to assess the postinfection immune status. The stomach was removed, washed in phosphate-buffered saline (PBS), and dissected longitudinally into four tissue fragments (so that each fragment contained parts of cardia, body, and antrum). Uninfected controls were treated in the same manner.

**Assessment of *H. pylori* infection in mice.** For each stomach, one fragment was immediately placed in urea-containing medium (Helicobacter urease test; Astra, Wedel, Germany), and another was prepared for frozen tissue sectioning for use in immunohistochemistry. A third was placed on a special agar plate containing Wilkins-Chalgren agar (Oxoid), 10% horse blood, 125 mg of vancomycin per 0.5 liter, 5 mg of polymyxin B per 0.5 liter, 62.5 mg of trimethoprim per 0.5 liter, and 12.5 mg of amphotericin B (Sigma, Deisenhofen, Germany) per 0.5 liter and then incubated under microaerophilic conditions at 37°C for 48 to 72 h for bacterial isolation. The remaining fragment was placed in neutral buffered formalin for histological analysis. The presence of urease activity in tissue fragments was determined by monitoring biopsy urease agar for ≤24 h at room temperature.

**Detection of antibodies by ELISA.** *H. pylori* antigen-specific immunoglobulin G (IgG) and IgG subclass antibodies in serum were detected by an ELISA

**Table 1.** Characterization of the six fresh clinical isolates of *H. pylori* used for infection of mice

<table>
<thead>
<tr>
<th>Fresh clinical isolate of <em>H. pylori</em></th>
<th>Cell assay for vacuolating activity</th>
<th>PCR for vacA</th>
<th>PCR for <em>cagA</em></th>
<th>IL-8 assay (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Ly27a)</td>
<td>Negative s1m1 Positive</td>
<td></td>
<td></td>
<td>827.0</td>
</tr>
<tr>
<td>II (SE888)</td>
<td>Negative s1m2 Positive</td>
<td></td>
<td></td>
<td>895.4</td>
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<tr>
<td>III (OM1605)</td>
<td>Negative ND* ND</td>
<td></td>
<td></td>
<td>13.0</td>
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<tr>
<td>IV (OM1626)</td>
<td>Positive s1m1 Negative</td>
<td></td>
<td></td>
<td>33.7</td>
</tr>
<tr>
<td>V (Ca177)</td>
<td>Positive s1m4 Positive</td>
<td></td>
<td></td>
<td>1,174.1</td>
</tr>
<tr>
<td>VI (Ca117)</td>
<td>Positive m1</td>
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*ND,* not determined. *H. pylori* strain OM1605 could not be recultivated for molecular analysis.

**TABLE 1.** Characterization of the six fresh clinical isolates used for infection of mice

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technique by using the Enzygnost anti-Helicobacter ELISA (Behring, Marburg, Germany) with 96-well plates coated with extracts from cytotoxin-positive H. pylori strain NCTC 11637. Diluted (1:231) serum samples were added in 220-µl aliquots to antigen-coated microtiter wells. Bound H. pylori-specific immunoglobulins were detected with peroxidase-conjugated sheep anti-mouse antibodies (Dia mond, Hamburg, Germany). Immune complexes were detected by reaction with a solution containing tetramethylbenzidine and hydrogen peroxide. Optical densities at 450 nm were determined with an Easy Reader EAR 400 AT (SLT- Lab instruments, Crailsheim, Germany). As the Behring Enzygnost ELISA is made for human serum samples, we determined the quantities (in mouse units) of IgG antibodies in sera by interpolation of a standard curve derived from a mouse serum sample with a high optical density value and an H. pylori infection confirmed by a biopsy urease reaction and H. pylori isolation from a gastric tissue sample. An optical density of 1.500 was determined to be 100 U (mouse units), and the cutoff was evaluated by using mean optical density values plus 2 standard deviations for native uninfected mice (optical density of 0.300 or 20 U). An IgG antibody concentration of ≥35 U (plus 3 standard deviations) was determined to confirm H. pylori infection serologically. For the IgG subclass antibodies detected as described above by using specific peroxidase-conjugated sheep anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antibodies (Dianova), the optical densities were compared directly with a cutoff value (optical density, 0.100) evaluated as described above.

Immunoblotting. Fresh clinical H. pylori isolate Ca17 was used as a standard antigen preparation for a native immunoblot. Bacteria were grown under microaerobic conditions on Wilkins-Chalgren agar (Oxoid) with 10% horse blood and H. pylori selective supplement Dent (Oxoid) at 37°C for 48 h. Bacteria were harvested from agar plates, washed in 1 ml of PBS, and pelleted by centrifugation at 10,500 × g for 1 min. The pellet was resuspended in H2O (200 µl), 250 µl of sodium dodecyl sulfate (N-lauryl-sarcosine, sodium salt) sample buffer (Mikrogen, Munich, Germany) was added, and the suspension was then incubated at 100°C for 5 to 10 min. After a second centrifugation at 10,500 × g for 1 min, the pellet was used as an H. pylori protein sample for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples were separated by using a 5% polyacrylamide stacking gel and a 15% polyacrylamide separation gel. Separated proteins were transferred to nitrocellulose paper (Schleicher & Schuell, Dassel, Germany) by semidy blotting for 2 h at 25 V, 102 mA, and 100 W in a Trans-Blot SD semidy transfer cell blotter (Bio-Rad, Munich, Germany). After blocking of nonspecific binding by incubation in Tris-buffered saline containing 0.2% Tween 20 (Serva, Heidelberg, Germany) for 2 h, the nitrocellulose sheets were incubated overnight with serum samples (1 in 200 dilution) in incubation buffer (Mikrogen). Bound IgG was detected by sequential incubation with peroxidase-conjugated sheep anti-mouse antibody (1 in 200 dilution) and 3,3’-diaminobenzidine–30% hydrogen peroxide. For interpretation of the results we used a scoring system that differentiated between specific and cross-reacting bands. The scoring system was evaluated for a commercial IgG blot in a study performed by one of us (N. Lehn, Institute of Medical Microbiology and Hygiene, Technical University of Munich, Munich, Germany) and Mikrogen in 1995 with 99 human sera from 59 H. pylori-positive and 40 H. pylori-negative individuals were well characterized by histopathology and microbiology. Reactions to individual antigens were classified by points depending on immunodominant potency, and then all points were added to obtain a score. The following antigens in accordance with the commercial Mikrogen immunoblot were assessed and identified by size and monoclonal antibodies (Mikrogen): CagAVacA (120 and 87 kDa), OMP (67 kDa), UreB (62 kDa), HspA/B (58 kDa), FlaA (54 kDa), 47 kDa, 33 kDa, UreA (29 kDa), 28 kDa, 25 kDa, and 19 kDa.

Immunohistochemistry. Longitudinal sections of gastric tissue including cardia, body, and antrum were mounted in Jung tissue-freezing medium (Leica, Nussloch, Germany) and frozen in liquid-nitrogen-cooled 2-methylbutane (Al drich, Steinheim, Germany). Tissue sections (thickness, 8 µm) were fixed with acetone and incubated with biotinylated rat anti-mouse monoclonal antibodies (MAb) and then with mouse anti-rat antibody conjugated to biotinylated horseradish peroxidase (Dianova). The controls included incubation with an MAb having unrelated specificity. Reagents were applied to tissue sections for 30 min, and the sections were washed three times with PBS. Cell-bound peroxidase was visualized with 5% 3-amin-9-ethylcarbazole (Sigma), 30% H2O2, and 50 mM acetate buffer (pH 5.2), and sections were counterstained with hematoxylin. The degree of gastric infiltration defined by the MAB was scored by determining the number of cells per field of vision (magnification, ×400).

MAb. The following MAb were used in this study; anti-CD4 (clone H129.19, dilution, 1:100), anti-CD8 (clone 53.6-6.7, dilution, 1:100), anti-CD11b (clone M1/70, dilution, 1:2), anti-CD45 (B220, clone RA3-6B2, dilution, 1:100), and anti-Gr1 (clone RB6-8C5; dilution, 1:50). All MAb were obtained from Pharmingen, San Diego, Calif., and all dilutions were with PBS.

**FIG. 1.** Box plots with medians, minimum and maximum values, upper and lower quartiles, and extremes (asterisks) of IgG antibody titers (ELISA units) to H. pylori (Hp) in infected TNF-R1-deficient mice (TNF-R1−/−, n = 19) and controls (TNF-R1+/+, n = 24). H. pylori-specific IgG antibody titers were significantly higher in infected isogenic control mice than in TNF-R1-deficient mice (P = 0.0049).

**RESULTS**

Detection of H. pylori infection in mice. H. pylori was isolated by culturing mouse gastric tissue for 4 weeks postinoculation from 100% of TNF-R1-deficient mice and isogenic controls inoculated orally with different strains of H. pylori. Likewise, we obtained positive results with the biopsy urease agar assay, as well as with ELISA and immunoblot analyses, for 100% of both groups of mice (data not shown).

**ELISA.** IgG antibody titers to H. pylori were significantly higher in infected mice than in uninfected controls (P < 0.0001). As detailed in Fig. 1, IgG antibody titers were also higher in infected isogenic control mice than in TNF-R1-deficient mice (P = 0.0049). When the IgG subclass response was examined, significantly higher titers were found for IgG1 (P = 0.0002), IgG2b (P < 0.0001), and IgG3 (P = 0.0008), but not for IgG2a (P = 0.596), in infected isogenic controls than in TNF-R1-deficient mice, as shown in Fig. 2. The use of cytotoxic-positive or cytotoxic-negative H. pylori strains did not influence IgG antibody production in infected TNF-R1-deficient (TNF-R1−/−) mice and isogenic controls.

**Immunoblotting.** All H. pylori-infected mice reacted with specific antigens of H. pylori, whereas the uninfected controls did not react or reacted only with known nontypical and cross-
reacting antigens, like heat shock proteins or flagellin (data not shown). TNF-R1-deficient mice and wild-type mice did not differ in reactivity to specific H. pylori antigens, but the percentage of positive reactions to single antigens was lower in the TNF-R1−/− group (Fig. 3). For individual antigens, statistically significant differences between TNF-R1-deficient mice and wild-type controls were found for the 58-kDa antigen (= Hsp) (P = 0.02742), the 28-kDa antigen (P = 0.00857), and the 25-kDa antigen (P = 0.02248); the values were higher in wild-type mice. Antibodies against the 54-kDa antigen were found only in one wild-type mouse.

No association between the level of the IgG antibody responses in ELISA and immunoblots and the use of cytotoxin-positive or cytotoxin-negative H. pylori strains for infection of mice was found.

**Immunohistochemistry.** Uninfected controls showed significantly smaller numbers of polymorphonuclear leukocytes than infected mice and no infiltration with macrophages and CD4, CD8, and B cells in the gastric mucosa (data not shown). In addition, the gastric mucosa in mice infected with cytotoxin-positive H. pylori strains contained more polymorphonuclear leukocytes than the gastric mucosa in mice infected with cytotoxin-negative H. pylori strains (P < 0.0001) (Fig. 4).

Interestingly, all H. pylori-infected mice showed similar signs of gastritis, and there was no significant difference between isogenic controls and TNF-R1-deficient mice in any of the cell populations analyzed. After H. pylori infection we observed marked infiltration with polymorphonuclear granulocytes (Fig. 5) and found about 90 granulocytes, about 20 macrophages, about 20 B-lymphocytes, about 10 CD4+ cells, and about five CD8+ cells in each field of vision (magnification, ×400).

**Histology.** In the gastric mucosa of uninfected controls no inflammatory reactions could be detected, whereas all infected controls showed morphological changes due to gastritis (P < 0.0001). The accumulation of polymorphonuclear neutrophils (PMN) in the gastric mucosa was greater in mice infected with cytotoxin-positive H. pylori strains than in mice infected with cytotoxin-negative H. pylori strains, as evaluated with a scoring system in which 0 corresponded to low accumulation of PMN, 1 corresponded to low accumulation of PMN, 2 corresponded to intermediate accumulation of PMN, and 3 corresponded to high accumulation of PMN; the means ± standard deviations were 1.4 ± 0.5 and 1.2 ± 0.4, respectively, based on the immunohistochemical results, but the difference was not statistically significant. No significant difference between TNF-R1-deficient mice and isogenic controls was detected when grade and activity of gastritis were examined. In contrast, TNF-R1-deficient mice had significantly fewer lymphoid aggregates in their gastric mucosa than isogenic controls (means ± standard deviations, 0.0 ± 1.0 and 2.0 ± 0.99, respectively [scoring from 0 to 3 as described above]; P = 0.002).

**DISCUSSION**

In our study we used an H. pylori mouse model in which TNF-R1-deficient C57BL/6 mice and isogenic wild-type con-
controls were compared to evaluate the contribution of TNF-R1 in the systemic immune response and gastric inflammation in \textit{H. pylori}-induced gastritis.

Activation of NF-κB, mediated by proteins of the \textit{H. pylori} pathogenicity island, and the resulting increase in TNF-α secretion (22) seem to be critical in \textit{H. pylori}-associated gastritis (45). Increased TNF-α secretion is associated with an increased level of apoptosis (34), independent of expression of the vacuolating cytotoxin (55) or the \textit{cagA} status (50) of \textit{H. pylori}. We therefore hypothesized that there was a reduced mucosal inflammatory response due to the loss of TNF-R1 function. The results of our study, however, demonstrate that TNF-α mediated by the TNF-R1 pathway might support primarily a systemic humoral immune response to \textit{H. pylori}, whereas gastric inflammation in \textit{H. pylori} infection seems to be independent of the function of the TNF-R1 receptor.

Surprisingly, TNF-R1-deficient mice exhibited significantly increased levels of serum IgG antibodies (as determined by ELISA) compared to the levels in wild-type mice at 4 weeks postinoculation (Fig. 1 and 2). In addition, we showed by immunoblotting that the weaker IgG production in TNF-R1-deficient mice cannot be explained by the failure to recognize specific \textit{H. pylori} antigens overall (Fig. 3). Thus, the results of the immunoblot analysis may prove the generally weakened systemic humoral immune response of TNF-R1-deficient mice. All of these findings for the humoral immune response were completely independent of infection with cytotoxin-positive or cytotoxin-negative \textit{H. pylori} strains (data not shown). Beyond that, isogenetic controls had significantly higher IgG antibody titers for IgG1, IgG2b, and IgG3, but not for IgG2a, than TNF-R1-deficient mice. Pasparakis et al. (48, 49) also demonstrated a strongly impaired secondary humoral immune response for all IgG subclass antibodies and IgE to thymus-dependent antigens in TNF-α-deficient mice and in addition defective formation of B-lymphocyte follicles in peripheral lymphoid organs of TNF-R1-deficient mice. Interestingly, we likewise found that the number of lymphoid aggregates was significantly lower in the mucosa of \textit{H. pylori}-infected TNF-R1-deficient mice, but there was no effect on the grade and activity of gastritis. Similar findings were described by Neumann et al. (46) and Matsumoto et al. (43); there was a complete lack of Peyer’s patches in TNF-R1-deficient mice and defective formation of germinal centers in LT-α-deficient mice. Thus, the reduced systemic immune response, together with fewer lymphoid follicles in gastric mucosa of TNF-R1-deficient mice, might lead to the conclusion that signals of TNF-α mediated by the TNF-R1 pathway support a systemic humoral immune response in a more meaningful manner than supposed until now. TNF-R1 may be necessary for activation and stimulation of macrophages for antigen presentation to CD4 $^+$ T-cells with consecutive T-cell–B-cell interaction. The different results for
the IgG2a subclass response in TNF-α- and TNF-R1-deficient mice could be due to a decisive role of TNF-R2.

In the immunohistochemistry analysis, uninfected controls also showed some neutrophil infiltration in their gastric mucosa, possibly because of nonspecific reactions to various environmental antigens, but there was significantly less infiltration (P < 0.0001) than in H. pylori-infected mice (Fig. 5). However, mucosal infiltration with mononuclear cells and markers of chronic inflammation, particularly CD4+ T-helper cells (15), dominated by the Th1 type of inflammation (4, 14), and morphological changes for gastritis in histopathological analyses were found only in H. pylori-infected mice. Likewise, no difference were found between isogenetic controls and TNF-R1-deficient mice in any of the immunohistochemically analyzed different cell populations (Fig. 5) and in the histological grade of gastritis (data not shown). A possible explanation for this might be that TNF-α effects overlap with the effects of various other cytokines, such as IL-1 (35), so that the loss of one mediator does not result in a detectable reduction in acute inflammatory infiltration in the gastric mucosa. H. pylori infection in mice does not reach its peak before 8 weeks postinoculation (36). However, gastric mucosa in mice infected with cytotoxin-positive H. pylori strains in the cell culture assay for vacuolating activity showed higher accumulation of polymorphonuclear leukocytes than gastric mucosa in mice infected with cytotoxin-negative strains (Fig. 4). Interestingly, as shown in Table 1, we found that H. pylori isolates that were cytotoxin negative in the cell culture assay revealed a genotype for vacA that is indicative of cytotoxin activity (s1m1 and s1m2). In addition, although these strains tested positive for IL-8 induction in AGS cells, they were not associated with severe infiltration of polymorphonuclear cells, whereas one strain that was cytotoxin positive did not induce IL-8 but showed significant cellular infiltration. These findings suggest that expression of cytotoxic activity is related to severe gastric inflammation independent of a strain’s capacity to induce IL-8 via the pathogenicity island. Reduced activity of gastritis due to infection with cytotoxin-negative H. pylori strains was reported also by Marchetti et al. (40), as well as Crabtree and Farmery (10). On the other hand, Eaton et al. demonstrated that there is no relationship among the presence of the cag pathogenicity island and IL-8 induction, and neutrophilic gastritis (18).

In other animal models using cytokine knockout mice for analysis of the immunopathogenesis of H. pylori infection, it was shown that in contrast to TNF-α signals mediated by the TNF-R1 pathway, IFN-γ plays an important role in the induction of gastric inflammation caused by H. pylori infection. IFN-γ knockout mice get colonized but do not show any inflammatory signs after H. pylori infection (31, 52, 54). IL-4, in contrast, seems not to be critical in H. pylori infection (1, 8, 39), whereas it plays an important role in Helicobacter felis infection of mice (44), and Smythies et al. (53) found stronger gastric inflammation in IL-4-deficient mice. Chen et al. observed significantly reduced colonization of the gastric mucosa in IL-10-deficient mice so that IL-10 seemed to be an inhibitor of the protective immune response to H. pylori infection (9).

Thus, further investigations will be necessary to clarify the importance of TNF-α in pathogenesis and modulation of inflammatory processes following H. pylori infection leading to H. pylori-associated gastroduodenal disease. The effects mediated by TNF-R2 could be also of particular interest because they might be involved in the activation of IgG2a. In accordance with other results, our findings lead to the conclusion that TNF-α mediated by the TNF-R1 pathway is critical in maturation of the systemic humoral immune response, with formation of primary B-cell follicles, B-cell activation, and production of IgG antibodies (at least IgG1, IgG2b, and IgG3).

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