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The CD4⁺ T Cell-Mediated IFN-γ Response to Helicobacter Infection Is Essential for Clearance and Determines Gastric Cancer Risk¹

Ayca Sayi,²* Esther Kohler,²* Iris Hitzler,²* Isabelle Arnold,⁎ Reto Schwendener,⁎ Hubert Rehrauer,† and Anne Müller³*

Chronic infection with the bacterial pathogen Helicobacter pylori is a risk factor for the development of gastric cancer, yet remains asymptomatic in the majority of individuals. We report here that the C57BL/6 mouse model of experimental infection with the closely related Helicobacter felis recapitulates this wide range in host susceptibility. Although the majority of infected animals develop premalignant lesions such as gastric atrophy, compensatory epithelial hyperplasia, and intestinal metaplasia, a subset of mice is completely protected from preneoplasia. Protection is associated with a failure to mount an IFN-γ response to the infection and with a concomitant high Helicobacter burden. Using a vaccine model as well as primary infection and adoptive transfer models, we demonstrate that IFN-γ, secreted predominantly by CD4⁺/CD25⁻ effector T₉₂ cells, is essential for Helicobacter clearance, but at the same time mediates the formation of preneoplastic lesions. We further provide evidence that IFN-γ triggers a common transcriptional program in murine gastric epithelial cells in vitro and in vivo and induces their preferential transformation to the hyperplastic phenotype. In summary, our data suggest a dual role for IFN-γ in Helicobacter pathogenesis that could be the basis for the differential susceptibility to H. pylori-induced gastric pathology in the human population. The Journal of Immunology, 2009, 182: 7085–7101.

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In humans, the H. pylori-infected gastric mucosa is characterized by high levels of proinflammatory cytokines including IL-8, IL-1β, TNF-α, and IL-6 (24, 25). The signature cytokines of Th1-polarized cellular immune responses, IFN-γ, IL-12, and IL-18, are also present at elevated levels (26, 27). Despite the production of large amounts of both types of cytokines and the efficient recruitment of neutrophil granulocytes, macrophages, and various lymphocyte populations to the site of infection, the immune response of the host is typically inadequate to clear the infection. Rather, above normal production of inflammatory cytokines, for example due to polymorphisms in their promoter regions, has been linked to an increased risk of gastric cancer (25, 28, 29).

In this study, we demonstrate that IFN-γ plays a key (dual) role in the response to Helicobacter infection. Using experimental infection models in wild-type and various knockout strains as well as adoptive transfer and vaccination models, we show that IFN-γ produced by CD4+ CD25− effector cells is crucial for the control of Helicobacter infection on the one hand, and induces preneoplastic changes of the gastric mucosa on the other. We further show that even genetically virtually identical animals differ in their ability to mount strong IFN-γ responses to the infection and that the extent of the IFN-γ response determines gastric cancer risk.

Materials and Methods

Mice and bacterial infections

All mice (C57BL/6, IFN-γ−/−, B6, TCR-β−/− B6, and Rag-1−/− B6) were purchased from Charles River Laboratories and bred at a University of Zurich specific pathogen-free facility. Mice were housed in individually ventilated cages and infected with Helicobacter at 5–6 wk of age. All animal experiments were performed in accordance with institutional policies and have been reviewed and approved by the cantonal veterinary office.

The Helicobacter strains used in this study were H. pylori Sydney strain 1 (SS1, originally obtained from A. Lee, University of New South Wales, Sydney, Australia (30)) and H. felis C51 (ATCC 49179). H. pylori was grown on solid medium on horse blood agar containing 4% Columbia agar base (Oxoid; Basingstoke), 5% defibrinated horse blood (HemoStatLabs), 0.2% β-cyclodextrin, 5 μg/ml trimethoprim, 8 μg/ml amphotericin B, 10 μg/ml vancomycin, 5 μg/ml cefusulon, and 2.5 μl polymyxin B sulfate (all from Sigma-Aldrich) at 37°C for 2 days under microaerobic conditions. For liquid culture, H. pylori was grown in Brucella broth (Difco) containing 10% FBS (Life Technologies) with shaking in a microaerobic atmosphere at 37°C. Before mouse infections, bacteria were inoculated from frozen stocks onto horse blood plates, incubated for 2 days, and then expanded onto fresh plates and grown for 24 h. Bacteria harvested from these plates were used to inoculate overnight liquid cultures. Cultures were routinely assessed by light microscopy for contamination, morphology, and motility. Infections were performed by oral gavage with 108 bacteria in 100 μl of suspension in Brucella broth. H. felis was grown similarly, except that all antibiotics except for trimethoprim and amphotericin were omitted due to polymorphisms in their promoter regions, has been linked to an increased risk of gastric cancer (28, 29, 29).

Preparation of gastric tissues and assessment of colonization and pathology

Mice were killed at 2 wk, 1 mo, or 3 mo after infection by CO2 inhalation; the stomachs were retrieved and cleaned and the forestomach was removed. The remaining piece containing the corpus and antrum was opened along the lesser curvature and spread out in the form of a trapeze. The tissue was then dissected longitudinally (i.e., from the forestomach/corpus junction down to the antrum/duodenal junction) into six equal, parallel pieces that comprise close to identical proportions of antral and corpus tissue. Of every stomach, the same section (i.e., the right-most, left-most etc.) was designated for the same downstream processing (DNA extraction, histology, etc.) to minimize sampling error. For quantitative assessment of H. pylori colonization, one section of each stomach was transferred to a tube containing Brucella broth and homogenized with an Ultra Turrax homogenizer (IKA Scientific). Serial dilutions were plated on horse blood plates to determine bacterial loads. For quantitative PCR-based assessment of either H. felis or H. pylori colonization, another sixth of each stomach was subjected to genomic DNA isolation using a DNeasy blood and tissue kit (Qiagen). Genomic whole stomach DNA served as a template for PCR using primers directed against urease and flagelin genes. Primer sequences and PCR conditions are listed in Table I. For quantitative assessment of colonization according to a protocol described by Stoico et al. (33), 80 ng of extracted DNA was used for real-time PCR (LightCycler; Roche) with the LightCycler 480 SYBR Green I master kit (Roche). Standards were made by sequential 10-fold dilutions of purified H. pylori DNA producing a range from 500,000 to 5 copies per reaction. This was based on the premise that 2 fg of H. felis chromosomal DNA is equivalent to one copy of the H. felis genome (33). Each sample was analyzed in triplicate. For histology, two additional stomach sections of six-sixth each were either fixed in 10% neutral-buffered formalin before paraffin embedding or were embedded in cold OCT medium for cryosectioning. Consecutive paraffin sections were stained with HE, elastin and basic acid-Schiff, and Alcian blue for grading of histopathological changes. One to two longitudinal sections per mouse spanning the length of the stomach from the forestomach/corpus junction to the antrum/duodenal junction were scored with regard to four histopathological parameters (chronic inflammation, gastric atrophy, intestinal metaplasia, and mucus pit cell/epithelial hyperplasia) based on the features described in the updated Sydney classification (34). We attribute points on a scale of 0–6 as described by Chen et al. (35). Specifically, the definition of scores was as follows for the four parameters evaluated. Scores for chronic inflammation were: 0, none; 1, some infiltrates; 2, mild (few aggregates in submucosa and mucosa); 3, moderate (several aggregates in submucosa and mucosa); 4, marked (many big aggregates in submucosa and mucosa); 5, nearly the entire mucosa contains a dense infiltrate; and 6, entire mucosa contains a dense infiltrate. Scores for atrophy were: 0, none; 1, foci where a few gastric glands are lost or replaced; 2, small areas in which gastric glands have disappeared or been replaced; 3, <25% of gastric glands lost or replaced; 4, 25–50% of gastric glands lost or replaced; 5, >50% of gastric glands lost or replaced; and 6, only a few small areas of gastric differentiated glands remaining. For intestinal metaplasia, the scores were: 0, none; 1, only one crypt replaced by intestinal epithelium; 2, one focal area (one to four crypts) replaced; 3, two separate foci with metaplasia; 4, multiple foci; 5, >50% of gastric epithelium not replaced by i.e., and 6, only a few small areas of gastric epithelium are not replaced by intestinal epithelium. For hyperplasia, the scores were: 0,
none; 1, single glands (next to infiltrate); 2, one focal area/one to four crypts (mild); 3, one to three foci; 4, multiple foci; 5, >50% of glands affected; and 6, only few small nonhyperplastic areas.

**Immunohistochemistry**

For staining of cells in the S phase (actively proliferating cells) by BrdU incorporation, animals received 10 μl of 10 mM BrdU in body weight i.p. 60 min before sacrifice. Paraffin sections of 4 μm were stained with anti-BrdU Ab according to instructions provided with an In Situ Cell Proliferation Kit (Roche) and counterstained with a proliferating cell nuclear Ag-specific biotinylated Ab (PCNA; Abcam, Cambridge, MA). Detection was either performed histochemically with HRP-coupled streptavidin (Jackson ImmunoResearch Laboratories) and diaminobenzidine substrate (Research Genetics/Invitrogen) or with Alexa Fluor 594-coupled streptavidin, which binds to the abundant biotin found in this cell type.

Crypt sections were mounted in 4',6-diamidino-2-phenylindole-containing mounting medium to visualize nuclei in blue (Vector Laboratories). For staining of cells in the S phase (actively proliferating cells) by BrdU incorporation, animals received 10 μl of 10 mM BrdU in body weight i.p. 60 min before sacrifice. Paraffin sections of 4 μm were stained with anti-BrdU Ab according to instructions provided with an In Situ Cell Proliferation Kit (Roche) and counterstained with a proliferating cell nuclear Ag-specific biotinylated Ab (PCNA; Abcam, Cambridge, MA). Detection was either performed histochemically with HRP-coupled streptavidin (Jackson ImmunoResearch Laboratories) and diaminobenzidine substrate (Research Genetics/Invitrogen) or with Alexa Fluor 594-coupled streptavidin, which binds to the abundant biotin found in this cell type. Cryosections were mounted in 4',6-diamidino-2-phenylindole-containing mounting medium to visualize nuclei in blue (Vector Laboratories).

**Semiquantitative and quantitative assessment of gastric cytokine responses**

For conventional and real-time RT-PCR of IFN-γ, IFN-inducible protein 10 (IP-10), MIP-2, and GAPDH, total RNA was isolated from one-sixth of every stomach (antrum and corpus) using RNaseasy Mini columns (Qiagen). In brief, 1.5 μg of total RNA was used for first-strand cDNA synthesis with Superscript Reverse Transcriptase III (Invitrogen). The resulting cDNA served as a template for PCR (for conditions and primer sequences, see Table I). IFN-γ-specific real-time PCR (LightCycler; Roche) was performed with a LightCycler 480 SYBR Green I master kit (Roche). Absolute values of IFN-γ expression were normalized to GAPDH expression. For quantitative assessment of IFN-γ gastric protein levels, an IFN-γ ELISA (Quantikine Immunoassay System; R&D Systems) was performed using gastric mucosal extracts according to the manufacturer’s instructions.

**Gene expression profiling using Affymetrix GeneChips and data analysis**

For transcriptional profiling analysis, total RNA was isolated from homogenized samples of scraped mucosa from one-sixth of every stomach (antrum and corpus) using TRIzol reagent (Invitrogen). After the extraction procedure, the RNA was cleaned up by a RNeasy Mini Kit (Qiagen) and RNA integrity was verified by capillary gel electrophoresis using a Bioanalyzer (Agilent Technologies). Reverse transcription was performed with 2 μg of total RNA and the obtained cDNA was subjected to synthesis and biotin labeling of cRNA using Affymetrix GeneChip One-Cycle Target Labeling and Control Reagents according to the manufacturer’s protocol. Ten micrograms of the cRNA sample was hybridized for 16 h at 45°C to Affymetrix GeneChip Mouse Genome arrays 430.2, which contain ~45,000 probes representing the entire mouse genome. After hybridization, the arrays were washed and stained with streptavidin-conjugated PE in a GeneChip Fluidics Station 400 (Affymetrix) and were scanned with a Hewlett-Packard Scanner. Raw gene expression data generated by the GeneChip Operating Software (Affymetrix) were normalized for all probe sets on the array using the Robust Multichip Average method. The expression values were imported into the Resolver application (Rosetta Biosoftware) for hierarchical clustering. Sample clustering was performed based on the present genes only, with a gene being called present if its average expression in the study was above 25. We used the correlation of the log 2 expression values as the measure of similarity of samples and Ward’s minimum variance rule for the merging of cluster branches. The correlation coefficient between each probe set and the IFNG gene (represented by the probe set 1425947_at) was also computed using the log 2 expression values. Although we computed the correlation coefficient for all probe sets, we report only those probe sets with an average expression above 25. Probe sets that did not meet this criterion were considered absent. The differential expression of genes upon IFN-γ treatment was assessed in immortalized primary cells. We report the magnitude of the differential expression in the conditions “average IFN-γ-treated” and “untreated” as the average difference of the log 2 values in the two conditions. The differential expression is only reported for probe sets that were declared present (average expression above 25) in one of the two conditions.

**Neutrophil quantification by myeloperoxidase activity assay**

The myeloperoxidase assay was adapted from Mota et al. (36). One-sixth of every stomach was homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (H5882; Sigma-Aldrich). Homogenates were then subjected to four cycles of freezing in liquid nitrogen and thawing at room temperature. After a subsequent centrifugation step at 4°C for 20 min, the supernatants were collected and their protein content was quantified. Forty micrograms of total protein was analyzed for myeloperoxidase activity in a final reaction volume of 300 μl containing 65 mM potassium phosphate/0.5% hexadecyl trimethyl ammonium bromide, 1.6 mM tetramethylbenzidine (T2885; Sigma-Aldrich), and 0.3 mM H₂O₂. Absorbance was measured photometrically at 655 nm.

**Statistics**

Most statistical analysis was performed by Student’s t test or Mann-Whitney U test using GraphPad Prism software, as indicated in the figure legends. For computing the strength and the significance of the statistical dependence of colonization levels and IFN-γ expression, we used Pearson’s correlation coefficient. Calculating the statistical dependence of IFN-γ expression on each of the histological categorizations (chronic inflammation, atrophy, metaplasia, hyperplasia) required using a test for conditional independence described by Hothorn et al. (37).

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**Table I. Primers and PCR conditions used in this study**

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<tr>
<td>Rv:</td>
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Fw, Forward; Rv, reverse.

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*Abbreviations used in this paper: PCNA, proliferating cell nuclear Ag; IP-10, IFN-γ-inducible protein 10.*
Results
Chronical infection of C57BL/6 mice with H. felis results in gastric inflammation and preneoplastic pathology

To assess the effects of chronic H. felis infection of male C57BL/6 mice on the gastric mucosa, 20 mice were infected and monitored in a temporal manner with respect to colonization levels and gastric pathology. Since H. felis does not readily form colonies, colonization was examined by visual inspection of Giemsa-stained sections and by PCR using two different sets of H. felis-specific primers targeting the flaB and ureB genes. The primer combination amplifying parts of the flaB gene was further used for quantitative PCR to determine the absolute number of H. felis bacteria per stomach as previously described (33). Quantitative histopathological analysis was performed on H&E-, Giemsa-, periodic acid-Schiff-, and Alcian blue-stained tissue sections based on the features described in the updated Sydney classification (34), with a quantitative scoring system as devised by Chen et al. (35). At 3 mo after infection, the bacteria were readily detectable in histological sections (data not shown) and by PCR (Fig. 1A), with bacterial densities ranging from $10^4$ to $10^6$ bacteria per stomach. Extensive infiltration of the infected mucosa and submucosa by various immune cell populations was observed (H&E staining, Fig. 1B, top middle, right, and bottom panels), which is consistent with a diagnosis of chronic active gastritis. H&E staining further revealed cells that histologically resemble intestinal goblet cells (Fig. 1B, top middle panel) in several mice at this early time point; these cells are strongly stained also by Alcian blue (which stains neutral, sulfated mucopolysaccharides; Fig. 1D). The appearance of intestinal-like cells is accompanied by the partial or complete loss of terminally differentiated gastric cells of the parietal and chief cell lineages, thereby fulfilling the criteria for the diagnosis of gastric atrophy or atrophic gastritis (Fig. 1B, top middle panel). Alcian blue-positive metaplasia is mostly found in the proximal third of the corpus, i.e., close to the forestomach junction, at this early time point; the distal corpus is populated by a rapidly proliferating, PCNA-positive and BrdU-incorporating cell type.
FIGURE 2. Gastric IFN-γ production and CD4+ T cell infiltration patterns are indicative of colonization levels and pathology. A, IFN-γ ELISA revealing gastric IFN-γ production in uninfected (uninf) and infected mice 3, 6, 9, and 12 mo after infection. The animals shown are the same as in Fig. 1. B, Gastric IFN-γ transcript levels as determined by conventional (bottom panels) and real-time (upper panels) RT-PCR are shown for each animal of the time course, with GAPDH levels serving as loading controls and for normalization of real-time IFN-γ expression. Mice marked with a red asterisk have lower IFN-γ transcript levels than the other members of their groups. The color code is the same in A and B and also the same as in Fig. 1. C, Mice with severe pathology are characterized by CD4+ T cells (in green) infiltrating diffusely into the lamina propria (e.g., mouse 3.4, left panel). In contrast, the mouse with little or no epithelial changes displays clusters of CD4+ T cells that resemble lymphoid follicles (mouse 3.5, right panel). The absence of parietal cells (orange) indicates gastric atrophy.

(Fig. 1C, lower panel) that morphologically resembles the progenitor of surface mucus (pit) cells. In an uninfected age-matched stomach, PCNA-positive cells (i.e., gastric stem cells or their immediate progeny) reside exclusively in a narrow band of mucosa termed the isthmus, at a density of two to four cells per gland (Fig. 1C, upper panel). In contrast, in chronically infected mice, the entire gland is sometimes found to be PCNA and BrdU positive from the base to the tip. The overrepresentation of this PCNA-positive, rapidly cycling progenitor cell is consistent with a diagnosis of compensatory epithelial hyperproliferation, also termed foveolar hyperplasia or mucus pit cell hyperplasia. Overall, at 3 mo after infection, the predominant histopathological lesions observed are moderate to marked atrophy, moderate epithelial hyperplasia, and evidence of beginning intestinal metaplasia in the proximal part of the corpus (Fig. 1B, lower panel), all of which are recognized precursor lesions of gastric cancer. Although metaplasia and hyperplasia usually affect different regions of the corpus within the same infected animal, both types of lesions can sometimes be found in the same gland (Fig. 1D, lower panel). In these cases, their localization in the gland relative to each other suggests that hyperplastic cells arise from progenitors migrating from the isthmus toward the stomach lumen (i.e., surface mucus cell progenitors), whereas metaplastic cells arise from progenitors of those lineages that differentiate as they migrate toward the base of the gland, i.e., parietal or chief cells.

At the later time points of the study (6, 9, and 12 mo postinfection), bacterial colonization decreased progressively in the majority of mice (Fig. 1A). This decrease in bacterial densities was accompanied by a slight overall increase in the surface area of the corpus affected by marked pathology (Fig. 1B, lower panel), but progression to carcinoma was not observed in the time frame of our study.

Notably, however, we found that in two of the four time points, several animals did not fit into the overall pattern (i.e., animals 3.5, and 9.5., Fig. 1, A and B). These mice were colonized at very high levels (>105 bacteria/stomach), but had minor or no evidence of neoplastic epithelial changes (Fig. 1B, bottom panel). Atrophy was not detected; instead, the gastric mucosa of the mice was characterized by aggregates of lymphocytes confined to few, very restricted areas. The surrounding mucosa was normal with respect to its cellular composition. These exceptional mice therefore differed substantially from their littermates in that they were protected from gastric pathology despite harboring high bacterial loads.

The two types of responses to Helicobacter infection differ with respect to IFN-γ production

Because TH1-polarized T cells and their signature cytokine IFN-γ had been implicated before in H. pylori-induced gastritis (38), we hypothesized that this cytokine might be differentially regulated in mice differing with respect to gastric pathology. We analyzed local gastric IFN-γ expression in all of the mice of the time course shown in Fig. 1 by ELISA (Fig. 2A) as well as conventional and real-time RT-PCR (Fig. 2B). Strong IFN-γ production was detected only in those mice that had successfully reduced bacterial colonization and had significant associated immunopathology. In contrast, the exceptional mice 3.5 and 9.5 of the time course study, bacterial colonization decreased progressively in the majority of mice (marked by red asterisks, Fig. 2, A and B) failed to mount a measurable IFN-γ response, as determined at both the RNA and protein levels. The other striking difference between the two groups of
FIGURE 3. Gastric IFN-γ production is inversely correlated with Helicobacter colonization and determines gastric preneoplastic pathology. Eighteen mice were infected with H. felis for 3 mo. A, H. felis colonization levels as determined by flaB real-time PCR are shown in triplicate with errors bars above the gel photographs for flaB and ureB PCR for all 18 mice (numbered). B, Normalized gastric IFN-γ expression as determined by real-time PCR is shown for the same mice as in A. C, Histopathology scores for the four parameters indicated in the legend are shown for all 18 mice. D, Normalized IFN-γ expression is plotted as a function of H. felis colonization (top panel) as well as the scores for the four parameters (lower panels; each dot represents one mouse). Colonization and IFN-γ expression are anticorrelated with a Pearson’s correlation coefficient of $-0.95$ and an associated $p$ value of $9.78^{-10}$. The $p$ values indicating the significance of the correlation of IFN-γ expression with the histopathological parameters were calculated as described in Materials and Methods and are also shown.
mice was their pattern of gastric infiltration by CD4⁺ T cells (Fig. 2C). Whereas these cells were found to be confined to large mucosal aggregates in highly colonized mice (Fig. 2C, right panel), they were more diffusely distributed in the group of mice characterized by low bacterial counts and severe epithelial pathology (Fig. 2C, left panel). In this group, the T cells infiltrated the entire lamina propria from the base to the tip of the gland in small groups or as single cells, suggesting that this type of CD4⁺ T cell mediates bacterial clearance and induces epithelial changes. Parietal cells (in orange, Fig. 2C) were almost completely absent from the stomachs infiltrated in this manner, but are found in normal numbers in the highly colonized mice. Interestingly, the effects of diffusely infiltrating T cells often are extremely localized, i.e., hyperplasia and metaplasia are found only in glands directly adjacent to these infiltrates (data not shown). Overall, the differences between mice with or without pathology suggest that a soluble factor secreted by lamina propria-infiltrating T cells, possibly IFN-γ, induces the preneoplastic changes characteristic of *H. felis* infected C57BL/6 mice.

To assess the association of IFN-γ expression and gastric pathology on the one hand and the possible inverse relationship of IFN-γ levels with *Helicobacter* colonization on the other in a more representative group of animals, we analyzed 18 additional C57BL/6 mice infected with *H. felis* for 3 mo with respect to colonization (Fig. 3A), IFN-γ expression (Fig. 3B), and gastric histopathology (Fig. 3C). Interestingly, we found that colonization and IFN-γ expression were indeed anticorrelated, with a Pearson’s correlation coefficient of −0.95 and an associated *p* value of 9.78 x 10⁻¹⁰ (Fig. 3D). IFN-γ levels were further significantly associated with chronic inflammation, atrophy, and epithelial hyperplasia (*p* values of 0.0196, 0.0204, and 0.0344) and less closely linked to the appearance of metaplasia (*p* = 0.177; Fig. 3D). From these calculations, we conclude that local IFN-γ expression is indeed a useful indicator of a successful reduction of bacterial burden, as...
FIGURE 5. IFN-γ is crucial for the efficient reduction of H. pylori burden and for the development of H. felis-induced premalignant lesions. A and B, Wild-type (WT) C57BL/6 mice (C) and IFN-γ−/− B6 mice (Δ; four to seven mice per group) were infected with H. pylori S81. Colonization was assessed by colony counting (A) and H. pylori-specific PCR using ureB-directed primers (B) at 2 wk, 1 mo, and 3 mo after infection. Counts and PCR results are shown along with p values indicating significance of the findings as determined by the Mann-Whitney U test (A). C, Giemsa-stained sections are shown for representative C57BL/6 wild-type and IFN-γ−/− mice infected for 3 mo with H. felis along with uninfected controls. D, Histopathology scores for four histopathological parameters (as indicated) for a total of 30 wild-type and 18 IFN-γ−/− mice included in three independent studies. Values of p as calculated by Student’s t test indicate the statistical significance of the differences. Thin bars in A and D indicate the means.

well as a valid predictor of gastric premalignant changes triggered by Helicobacter infection.

Gene expression profiling reveals distinct transcriptional signatures in nonatrophic inflamed vs predominantly hyperplastic vs metaplastic gastric mucosa

To assess whether the histopathological differences between the two groups of mice responding differentially to Helicobacter infection translate into transcriptionally evident differences, we performed gene expression profiling using Affymetrix Genechips. RNA was purified from scraped mucosal samples of an independent set of H. felis-infected mice that were chosen because they had developed uniform pathology of either of the three following types rather than a mixture of all three: 1) symptoms of gastritis, but no epithelial changes; 2) atrophic gastritis accompanied by intestinal hyperplasia only; or 3) atrophic gastritis accompanied by intestinal metaplasia only. An uninfected control group was also included in the analysis, as were two groups of mice that lacked mature T and B cells due to a deletion mutation in the rag-1 gene (Rag-1−/−) and that were either experimentally infected or served as Rag-1−/− uninfected controls. Infected Rag-1−/− mice showed no signs of gastritis despite high colonization levels. An unsupervised hierarchical clustering approach, which deliberately does not take into account the histopathological information we had on the mice, revealed the segregation of inflamed, but nonatrophic (i.e., no epithelial changes) samples from those characterized by hyperplasia or metaplasia (Fig. 4A). Interestingly, the latter two were also significantly different from each other to drive their segregation into two distinct branches of the dendrogram tree. All control samples are found in a separate branch. These cluster along with the samples of infected as well as uninfected Rag-1−/− mice, reflecting the complete lack of gastric inflammation in this genetic background (Fig. 4A). Indeed, the transcriptional changes between infected and uninfected Rag-1−/− mice affected only a small number of genes and were thus insufficient to drive clustering into distinct groups. In conclusion, the clustering of histologically similar samples into distinct branches of the dendrogram tree suggests distinct pathogenetic pathways for hyperplastic and metaplastic lineages and provides transcriptional signatures of these lesions (publicly accessible under http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13873) that can now be mined for clues to their cellular and pathogenetic origin.

Strikingly, the expression of IFN-γ was strongest in the hyperplastic group as judged by the signal intensities of the corresponding probe sets on the array (Fig. 4B). As expected, nonatrophic gastritis samples did not express IFN-γ (Fig. 4B), confirming our previous observations (Figs. 2 and 3). Interestingly, the metaplastic samples also showed significantly less IFN-γ production than hyperplastic samples, suggesting that hyperplasia is the dominant
Lesion induced by local IFN-γ production (Fig. 4B). This was confirmed by IFN-γ-specific conventional RT-PCR as well as real-time RT-PCR performed on the same set of animals (Fig. 4C). Real-time and conventional RT-PCR designed to amplify a known target gene of the IFN-γ signaling pathway, the chemokine IP-10 (also known under the name CXCL-10) was also performed and revealed a virtually identical pattern of expression (Fig. 4C). In summary, we conclude from the two independent experiments described in Figs. 3 and 4 that local IFN-γ production is significantly associated with the induction of hyperplastic, but not metaplastic lesions as a result of the infection, again suggesting that the pathogenic origins of these two premalignant lineages are probably distinct.

Aside from the known IFN-γ target IP-10, the expression patterns of numerous genes correlate with the expression of IFN-γ in our data set (for a list of all genes represented on the array and their distinct genetic origins of these two premalignant lineages are probably different). In an effort to identify genes that are not only correlated, but induced directly by this cytokine, we generated an immortalized cell line from primary murine gastric epithelial cells and treated these cells with increasing concentrations of recombinant murine IFN-γ for 24 h. mRNA extracted from these cells was then subjected to transcriptional profiling analysis using Genechips. All genes on the array were then analyzed for their correlation with IFN-γ on the one hand (first data set, dark blue spots in Fig. 4D) and their induction by IFN-γ on the other (second data set, green spots in Fig. 4D). Turquoise spots indicate representation in both data sets. Interestingly, the vast majority of all 286 genes found to be strongly induced by IFN-γ in vitro were also correlated with IFN-γ in vivo (red spots), suggesting that these genes are induced by IFN-γ in vivo (for a list of these genes, see supplemental Table II).

IFN-γ−/− mice are colonized more heavily and have less severe Helicobacter-induced pathology than wild-type mice

Since IFN-γ production is correlated with both a reduction in bacterial burden and induction of preneoplastic epithelial changes, we assessed both in an IFN-γ-deficient background. For quantification of bacterial colonization, C57BL/6 wild-type and IFN-γ−/− mice were infected with H. pylori strain SS1, a mouse-adapted patient isolate that readily forms colonies and can also be subjected to H. pylori-specific semiquantitative PCR using a primer combination amplifying the urease subunit B gene (ureB) from gastric genomic DNA. IFN-γ−/− mice were colonized more heavily than wild type at 4 and 12 wk after infection, and this difference was significant at both time points (Fig. 5, A and B). In contrast, the colonization levels were reversed at 2 wk after infection, suggesting that the defect of the IFN-γ−/− strain with respect to clearance becomes evident only after an adaptive immune response starts to take effect. These findings suggest that IFN-γ is critical for the control of Helicobacter infection during the adaptive phase of the immune response.

Interestingly, IFN-γ−/− mice were further protected from preneoplastic changes induced by 3 mo of H. felis infection in three independent studies comparing them to wild-type mice (Fig. 5, C and D). They were significantly less likely than wild-type mice to develop gastritis, atrophy, hyperplasia, or metaplasia (with p values of 0.0002, 0.0004, 0.0001, and 0.045, respectively). This observation suggests that the preneoplastic phenotype induced by Helicobacter infection in the C57BL/6 background depends on IFN-γ production.

FIGURE 6. IFN-γ is induced upon and required for efficient vaccination against H. pylori (H.p.). Vaccines were immunized four times in weekly intervals with 1 mg of H. pylori sonicate in conjunction with 10 μg of cholera toxin and challenged with H. pylori strain SS1 2 wk after the last immunization. Colonization was determined 2 wk (A and B) or 7 wk (C) after challenge. A, IFN-γ and IP-10 levels were assessed by conventional (lower panels) and real-time (upper panels) RT-PCR for a group of immunized (imm.H.p.inf.) and challenged (inf.) wild-type (WT) C57BL/6 mice, a group of immunized, but uninfected (imm. control) mice and a group of control-infected mice (inf. control), respectively. GAPDH levels were calculated using the Mann-Whitney U test. Thin bars indicate the means. n.s., Not significant differences for three of the parameters scored; metaplasia was not observed in any of the mice. Thin bars indicate the means. n.s., Not significant.

IFN-γ−/− mice are less protected than wild-type mice upon immunization with a whole cell sonicate H. pylori vaccine

Helicobacter cannot be cleared spontaneously in mice or humans. We were therefore interested in testing the relevance of IFN-γ in a true clearance model. For this purpose, we used a vaccine regimen consisting of four consecutive weekly doses of 1 mg of H.
Adoptive transfer of CD4<sup>+</sup>CD25<sup>-</sup> T cells into immunodeficient mice induces *Helicobacter* clearance, strong local IFN-γ and MIP-2 production, and gastric preneoplastic lesions. A, Immunomagnetically purified CD4<sup>+</sup>CD25<sup>-</sup> T cells from the spleens of *H. felis*-infected wild-type donors were adoptively transferred into 6-wk-old Rag-1<sup>−/−</sup> mice (300,000 cells/mouse), which were subsequently infected with *H. felis* (inf.) or remained uninfected (uninf.) for the duration of the experiment (1 mo). gDNA was isolated from every stomach and used for conventional PCR.
pylori SS1 sonicate plus 10 μg of cholera toxin followed by challenge with the same strain 2 wk after administration of the last dose. This regimen leads to a significant reduction in bacterial burden by two orders of magnitude, from $10^7$ to $10^5$ CFU/stomach to $10^{-1}$–$10^4$ CFU/stomach (Fig. 6B) in most wild-type mice. Immunized/challenged wild-type mice produce IFN-γ at significantly higher levels than immunized controls or mice from a control group that was infected without prior immunization, as assessed by conventional and real-time RT-PCR (Fig. 6A). This is further reflected in the transcript levels of IP-10, a downstream mediator of IFN-γ (Fig. 6A). In contrast to the successful clearance of H. pylori SS1 from stomachs of immunized wild-type mice, IFN-γ–/– mice receiving the same vaccine were significantly less capable of clearing the infection (Fig. 6B; $p = 0.0014$), implying that a strong IFN-γ response is crucial for vaccination-induced protection.

IFN-γ is known to act on numerous immune and nonimmune cell populations. Best understood is its effect on macrophages, which are rendered highly bactericidal and are induced to efficiently present Ag in the context of MHC class II upon exposure which are rendered highly bactericidal and are induced to efficiently activate APC. Neither treatment influenced colonization levels in a significant manner (data not shown), ruling out an important contribution of the two cell types in this experimental infection model.

To assess the functional contribution of CD4+ T cells to Helicobacter control and to Helicobacter-induced immunopathology, we used an adoptive transfer model of purified CD4+CD25+ T cells into immunodeficient hosts. Rag-1–/– and TCR-β–/– mice were selected to complement the immunocompetent mice in the previous experiments. Rag-1–/– mice are rendered highly bactericidal and are induced to efficiently activate APC. Neither treatment influenced colonization levels in a significant manner (data not shown), ruling out an important contribution of the two cell types in this experimental infection model.

Multiple cell types are known to produce IFN-γ during the early, innate, and the adaptive phases of the immune response to invading pathogens. NK cells have been shown to be an important early source of IFN-γ for example during Legionella pneumophila infection (42). NK cells as well as subsets of CD4+ and CD8+ T cells are possible sources of IFN-γ during the adaptive phase of the immune response. To assess the role of NK and NKT cells during H. pylori infection, we depleted them in vivo using a mAb directed against their shared NK1.1 surface marker in the course of a 12-day experimental infection. Alternatively, NK cells were selectively activated in a separate group of mice by i.p. injection of α-galactosylceramide, a known ligand of the semi-invariant Vα14 TCR that is recognized in the context of CD1d molecules on APCs. Neither treatment influenced colonization levels in a significant manner (data not shown), ruling out an important contribution of the two cell types in this experimental infection model.

Vaccination with H. pylori sonicate and cholera toxin reduces the bacterial burden, but does not eliminate the infection entirely (Fig. 6B). The residual colonization is of concern, as it may foster a chronic immune response in the stomach that is more virulent than the response to the natural infection. To assess the pathology that is triggered by a vaccine-induced response over and above what is induced by infection with the same strain alone, vaccinated/challenged mice were compared with an infected-only group 7 wk after infection (Fig. 6C). The vaccinated group scored significantly higher with respect to inflammation, atrophy, and hyperplasia, while intestinal metaplasia was not detected at this point in time (Fig. 6C). Although vaccine-induced gastric pathology may be transient at least with a weakly immunogenic challenge strain that is triggered by a vaccine-induced response over and above the infection (Fig. 6B), negative effects are observed as early as 4 wk after infection compared with infected controls that had not received cells. Clearance was accompanied by strong gastric IFN-γ responses (assessed by conventional and real-time RT-PCR; Fig. 7, A and B, compare light gray and black background).
bars) and the rapid appearance of premalignant lesions in the stomach (Fig. 7, C, E, and F, inf. + CD4\(^{-}\) \_inf). Both elevated gastric IFN-\(\gamma\) and pathology was strictly dependent on Helicobacter infection of the recipient, as cells from an infected donor had no effect in an uninfected recipient (Fig. 7, A, C, and E, uninf. + CD4\(^{+}\) \_inf). Furthermore, cells isolated from an uninfected donor failed to clear Helicobacter infection in the recipient (Fig. 7B) and were also ineffective in inducing gastric pathology (Fig. 7, C and F, inf. + CD4\(^{+}\) \_uninf) or IFN-\(\gamma\) responses (Fig. 7B). In conclusion, CD4\(^{+}\) CD25\(^{+}\) T cell-induced clearance and concomitant pathology depends on infection both on the part of the donor and on the part of the recipient, suggesting that the prior priming and correct homing of transferred CD4\(^{+}\) CD25\(^{+}\) T cells is crucial for both phenotypes. Indeed, staining for the CD4 surface marker reveals massive infiltration of these cells into the (infected) gastric mucosa, where their patterns of lamina propria colonization are strikingly similar to those observed in wild-type mice with severe pathology (Figs. 7D and 2C). Interestingly, production of the murine functional IL-8 analog MIP-2 is independent of infection on the part of the donor (but not the recipient, Fig. 7, A and B), as adoptive transfer of cells from an uninfected donor can trigger MIP-2 expression at similar levels as those from an infected donor. In contrast to the strong effects that adoptive transfer of CD4\(^{+}\) cells has on immunodeficient recipients, we have not observed any such effects upon transfer of purified CD8\(^{+}\) T cell populations, even when much higher numbers of cells were transferred (up to 2 \(\times\) 10\(^{6}\) cells). This result suggests that CD8\(^{+}\) T cells do not contribute to Helicobacter control or gastric pathology in a measurable way in this model.

IFN-\(\gamma\) production by CD4\(^{+}\) CD25\(^{+}\) effector T cells is essential for efficient clearance of Helicobacter

The control of Helicobacter by CD4\(^{+}\) CD25\(^{+}\) effector T cells in the adoptive transfer model is accompanied by the production of large quantities of local IFN-\(\gamma\) (Fig. 7, A and B). To assess the contribution of effector T cell-derived IFN-\(\gamma\) to bacterial clearance and concomitant immunopathology, we adoptively transferred wild-type or IFN-\(\gamma^{-/-}\) CD4\(^{+}\) CD25\(^{+}\) effector T cells from infected donors into immunodeficient Helicobacter-infected hosts. Whereas the recipients of wild-type cells efficiently cleared the infection, the recipients of IFN-\(\gamma^{-/-}\) cells were still heavily colonized 4 wk later (Fig. 8A showing flaB- and ureB-specific PCRs and quantitative flaB PCR performed on gastric genomic DNA), suggesting that IFN-\(\gamma\) production by effector T cells is essential for clearance. Indeed, IFN-\(\gamma\) expression in gastric tissue of IFN-\(\gamma^{-/-}\) cell recipients was minimal (Fig. 8A, middle panel), implying that CD4\(^{+}\) CD25\(^{+}\) effector T cells are the main producers of the cytokine in this setting. In contrast, the neutrophil chemoattractant MIP-2 was detectable in moderate amounts also in the gastric mucosa of IFN-\(\gamma^{-/-}\) cell recipients, suggesting that it is produced (or induced) also by IFN-\(\gamma^{-/-}\) T cells (Fig. 8A). This observation led us to quantify the levels of neutrophil infiltration in all groups of mice by an assay measuring the activity of myeloperoxidase, an enzyme produced in large quantities by neutrophils and, to a lesser extent, monocytes and macrophages. Gastric neutrophil infiltration proved to be clearly dependent on adoptively transferred CD4\(^{+}\) CD25\(^{+}\) effector T cells, as there was only minor infiltration due to infection alone (Fig. 8B). However, neutrophil infiltration was virtually identical in the two groups that had received wild-type or IFN-\(\gamma^{-/-}\) T cells, suggesting that IFN-\(\gamma\) does not affect neutrophil recruitment, but may rather act by modulating neutrophil phagocytic or microbicidal activity, survival, or other characteristics.

We further assessed the role of effector T cell-derived IFN-\(\gamma\) in Helicobacter-associated gastric pathology as a function of

**FIGURE 8.** IFN-\(\gamma\) produced by adoptively transferred CD4\(^{+}\) CD25\(^{+}\) T cells is essential for efficient clearance of Helicobacter. Six-week-old immunodeficient TCR-\(\beta^{-/-}\) mice received 350,000 immunomagnetically purified CD4\(^{+}\) CD25\(^{+}\) T cells from infected wild-type (WT) or IFN-\(\gamma^{-/-}\) donors as indicated. The recipients were infected (inf.) with \(H.\) felis 1 day later and were sacrificed 1 mo later. Infected TCR-\(\beta^{-/-}\) mice that had not received cells served as controls. All tissues were analyzed as described in the legend to Fig. 7. A. Colonization and transcript levels as determined by conventional and real-time PCR or RT-PCR. Only mice that have received wild-type CD4\(^{+}\) CD25\(^{+}\) T cells are able to clear the infection and launch a strong IFN-\(\gamma\) response. Asterisks indicate values that were below the detection limit. Values of \(p\) were calculated by the Mann-Whitney \(U\) test. B. Myeloperoxidase assay reflecting the influx of neutrophils and, to a lesser extent, monocytes and macrophages into the gastric mucosa. Duplicate readings were performed for extracts of every mouse and averages as well as SDs are plotted. A representative myeloperoxidase assay of three is shown. The animals plotted are the same as in A; three uninfected mice and two additional infected mice are also included. Values of \(p\) were calculated using Student’s \(t\) test. C. Histopathological analysis of the study shown in A and B (lower panel, 350,000 cells transferred) as well as an independent study using fewer cells (upper panel, 50,000 cells transferred). Group sizes ranged from four to seven mice. Values of \(p\) were calculated using Student’s \(t\) test. Thin bars in C indicate the means. The color code is the same in A–C. Two representative experiments of three are shown. n.s., Not significant.
the number of transferred cells. In an experiment in which we adoptively transferred only 50,000 cells into immunodeficient mice, we saw a significant difference in all four histopathological parameters when comparing recipients of wild-type vs IFN-γ−− cells (Fig. 8C, top panel). In an independent transfer experiment with 350,000 cells, the differences were significant for atrophy and hyperplasia scores, but less clear for metaplasia (Fig. 8C, bottom panel). This result suggests that induction of gastric pathology, especially manifesting as atrophy accompanied by compensatory hyperplasia, largely depends on IFN-γ. The induction of metaplasia, in contrast, seems to be linked less clearly with IFN-γ, a finding that is in line with the less pronounced expression of this cytokine in predominantly metaplastic compared with hyperplastic tissues (Fig. 4, B and C) and the less compelling statistical link between IFN-γ expression and metaplasia compared with hyperplasia scores in wild-type mice (Fig. 3D).

**Neutrophil granulocytes are essential for clearance of Helicobacter**

Neither *H. felis* nor *H. pylori* are cleared spontaneously from experimentally infected mice; therefore, nonvaccine-induced clearance models are not readily available. Our model of clearance/reduction of *H. felis* upon adoptive transfer of CD4+CD25− T cells provides a system in which effector mechanisms involved in efficient *Helicobacter* clearance can be studied. Because neutrophils have been implied in phagocytic removal of other mucosal pathogens, we depleted them for the duration of the experiment by treating the mice three times weekly with doses of 250 μg of an Ab targeting the neutrophil-specific surface marker GR-1. Depletion of neutrophils prevented clearance in all but one of five mice (Fig. 9A; the exceptional mouse later turned out not to have responded to the treatment, as neutrophil numbers were normal in the stomach and spleen) compared with a control group in which all five mice had cleared the infection. Interestingly, the gastric IFN-γ production indicative of T cell infiltration was also blocked in all four mice that had failed to clear due to successful Ab treatment (Fig. 9A), suggesting that neutrophils contribute to clearance of *Helicobacter* at least in part by recruiting more effector cells to the stomach. This reduced gastric infiltration of T cells is reflected also in a significant protection from preneoplasia (Fig. 9B and C). To rule out that the GR-1 Ab acts directly on activated T cells and depletes them from the gastric mucosa, we monitored gastric T cell levels before and after treatment in immunocompetent mice with preexisting lesions. In this scenario, we did not observe unwanted effects of the Ab treatment on T cells, suggesting that the Ab affects neutrophils only (data not shown). In conclusion, neutrophils play an important role during clearance of *Helicobacter* by facilitating T lymphocyte recruitment and possibly also by phagocytic removal of luminal bacteria.

**Discussion**

In this study, we demonstrate a crucial role of IFN-γ for the control of *Helicobacter* infection and for induction of preneoplastic changes of the infected gastric mucosa. This dual role is evident in an experimental murine host that is capable of reducing the bacterial burden upon the onset of an adaptive immune response (but not capable of complete spontaneous clearance) and that therefore is also susceptible to ensuing immunopathology. We show that the extent of the IFN-γ response differs among genetically virtually identical animals (littermates) and that it is not only predictive of *Helicobacter* control and resulting gastric pathology, but is also causally associated with both. In addition to contributing to the
spontaneous reduction in bacterial burden in experimentally infected mice, a strong IFN-γ response additionally is characteristic of and required for protection induced by an *H. pylori* vaccine. Our data confirm and extend several early studies that have proposed a role for T\textsubscript{H}1 cells (38, 44), and IFN-γ in particular, in the control of *H. pylori* infection upon vaccination (45) or primary infection (46). Several previous studies also noted reduced gastritis in infected IFN-γ−/− compared with wild-type mice (38, 44, 45), but did not assess preneoplastic epithelial pathology because it does not readily form after infection with *H. pylori* (as opposed to the more virulent *H. felis*). In conflict with our data and those of Akhian et al. (45), two previous studies have not found a conclusive role for IFN-γ in vaccine-induced protection (38, 47), despite elucidating the contribution of IL-12, another T\textsubscript{H}1 signature cytokine, to *H. pylori* clearance (47). Experimental differences between the four studies that have assessed the role of IFN-γ in protection so far include the challenge strain used, the time-to-sacrifice after challenge, the overall level of protection achieved by vaccination, and, perhaps most importantly, the gender of the vaccinated mice. We have observed clear differences in vaccine-induced protection in female and male wild-type mice (females are typically significantly better protected), as well as in spontaneous clearance models (i.e., IL-10−/− mice; A. Sayi and A. Müller, unpublished observation). Although the significance of this gender difference remains unclear, we suspect that it might provide an explanation for the conflicting results of the four studies. We should also stress that although the differences in protection between wild-type and IFN-γ−/− animals are clearly significant in our hands (*p* = 0.0014), the defect in clearance of the IFN-γ−/− strain is much less pronounced than the defect of MHCII−/− or CD4−/− strains lacking CD4+ T cells entirely (which are colonized as well as unvaccinated controls; I. Hitzler and A. Müller, unpublished data).

In the vaccine model, the successful control of *Helicobacter* colonization upon immunization is accompanied by considerable gastritis and mild atrophy at the antrum/corpus junction (Fig. 6). This so-called “postimmunization” gastritis is of concern in *Helicobacter* vaccine development (41), as none of the published vaccination approaches achieves sterile immunity. Indeed, we did not observe a regression of vaccine-induced pathology even 7 wk after challenge (i.e., 5 wk after a significant reduction in bacterial loads; Fig. 6), confirming published observations (41). We have shown previously in a long-term vaccine study that *H. felis*-induced MALT lymphoma formation in BALB/c mice is prevented by vaccination (48). Similarly, Sutton et al. (41) have demonstrated that at least the mild pathology triggered by *H. pylori* SS1 infection in C57BL/6 mice is not exacerbated upon vaccination in the long run. Nevertheless, the issue of whether vaccination and challenge with more immunogenic strains (better mimicking the human-pathogen interaction) will induce unacceptable preneoplastic pathology in susceptible (e.g., C57BL/6) hosts because the bacteria are reduced but not eliminated remains unsolved and certainly requires future attention if *Helicobacter* vaccination is to become a reality. In any case, our data argue that the distinction between “pathogenic T cells” and “protective T cells” may not be correct and that the protective (vaccine-induced) vs natural responses to the infection are quantitatively rather than qualitatively different (as discussed in Ref. 49).

Multiple cell types are capable of producing IFN-γ during both the innate and adaptive phases of the immune response. By depletion of NK and NKT cells from experimentally infected mice, we have ruled out a crucial contribution of these two cell types in the control of *Helicobacter* infection. Rather, we observed that CD4+CD25− effector T cells produce large amounts of IFN-γ when adoptively transferred into immunodeficient recipients (Rag-1−/− or TCR-β−/−). In these adaptive transfer models, the recipients clear or reduce the infection and develop severe premalignant pathology, as was also documented recently by Lee et al. (50); IFN-γ is essential for both effects since cells from an IFN-γ−/− background fail to eliminate the infection and are less potent than wild-type cells in initiating gastric pathology (Fig. 8). IFN-γ production and CD4+CD25− effector cell-mediated pathology (as well as clearance) depend on *Helicobacter* infection on the part of the donor and on the part of the recipient (Fig. 7). In addition to NK-, NKT- and T\textsubscript{H}1-polarized CD4+ cells, CD8+ T cells are known to be important producers of IFN-γ during the adaptive phase of an immune response and have been implicated previously in *Helicobacter*-induced gastritis (51). We thus tested their possible contribution to *Helicobacter* clearance and associated immunopathology in our adoptive transfer model. However, we found no evidence for gastric IFN-γ production, or for a reduction of bacterial burden, or for gastric pathology in CD8+ T cell recipients, thereby ruling out an important effector function of CD8+ T cells at least in this model.

IFN-γ is best known for its effects on macrophages and dendritic cells (DC), which are induced to present Ag in the context of MHC class II on exposure (39). Depletion of macrophages with liposome-encapsulated chlorodeoxuridine (a bishophosphate toxin; data not shown) did not reduce vaccine efficacy, suggesting that macrophages are dispensable for the effector phase of *Helicobacter* clearance. The presence of IFN-γ during the TLR-dependent activation of DC has been shown to promote the production of IL-12, a cytokine which drives further T\textsubscript{H}1 differentiation and amplifies the production of IFN-γ, thereby creating a positive feedback loop. According to two recent reports, DC residing in Peyer’s patches of the small intestine prime a *Helicobacter*-specific T\textsubscript{H}1-polarized immune response upon exposure to the coelomic form of *H. pylori* and direct migration of effector T cells to the gastric mucosa (52, 53). However, the role of DC in *Helicobacter* clearance upon vaccination remains poorly understood, mostly due to the lack of appropriate tools.

Interestingly, multiple lineages of gastric epithelial cells respond to IFN-γ exposure. Kang et al. (54) found that the human gastric cell line NCI-N87 acquired mucous neck cell traits upon treatment with IFN-γ such as secretion of mucus and expression of the mucin MUC6, TFF2, and pepsinogen II. Infusion of IFN-γ into wild-type mice was further shown to induce expansion of the mucous neck cell compartment in vivo, probably by triggering the deregulated proliferation of this cell type (54). In a study investigating a rare progenitor cell type of the antral gland by lineage tracing, Qiao et al. (55) observed that these cells have multilineage potential (i.e., they give rise to all lineages of the antral gland) and that they multiply in response to IFN-γ. In our own previous study investigating the response of the three terminally differentiated gastric epithelial lineages to *Helicobacter* infection in vivo using a laser capture approach, we found that the mucus–producing pit cell responds strongly to infection (56) by up-regulating numerous known target genes of the IFN-γ signaling pathway. We extended these findings in the current study by treating an immortalized gastric murine epithelial cell line with IFN-γ. Virtually all of the most strongly IFN-γ-induced genes in this line were coregulated with IFN-γ in preneoplastic gastric lesions, suggesting their induction by IFN-γ also in vivo. It is interesting to note in this context that predominantly hyperplastic, but not metaplastic, mucosa was characterized by high IFN-γ production (Fig. 4); this finding possibly suggests that only the former cell type expands as a result of IFN-γ exposure, whereas metaplasia may arise by an alternative mechanism. Additional evidence also points in this direction: metaplasia was the only one of the four histopathological...
parameters evaluated that did not show a significant association with IFN-γ expression in a large group of wild-type mice with various degrees of pathology (Fig. 3D). It was also the only lesion that appeared in immunodeficient recipients of IFN-γ−/− CD4+ effector cells (Fig. 8C). Concluding these findings, it is tempting to speculate that IFN-γ produced in the setting of chronic inflammation in the stomach acts directly on one or more epithelial lineages to induce their deregulated proliferation and transformation to premalignant, hyperplastic phenotypes. Whether epithelial cells are also involved in clearance of the bacteria is currently unknown.

Indeed, the downstream effector mechanisms leading to elimination of the bacteria from the infected stomach are not well understood, neither in spontaneous nor vaccine-induced clearance models. Whereas Abs seem to be dispensable (57), neutrophil granulocytes (58, 59) and mast cells (60) have been implied in Helicobacter clearance. Neutrophils in particular were found to be important in a spontaneous clearance model of H. felis infection in IL-10−/− mice (58), in a vaccine-induced clearance model (59), and in our CD4+ T cell-mediated clearance model in immunodeficient mice (Fig. 9), suggesting a common downstream effector mechanism involving this cell type.

The CXC chemokine IL-8 (CXCL-8) and its functional murine counterpart MIP-2 are well known to induce neutrophil attraction, activation, and transendothelial migration. IL-8 is strongly induced in human gastric cancer cells such as the commonly used AGS cell line upon coculture with H. pylori (61–63), and this signal is generally assumed to initiate the acute (neutrophil-dominated) inflammation in the early stages of Helicobacter infection. Similarly, we observed that H. pylori infection triggers the production of MIP-2 in primary as well as immortalized murine gastric cells in vitro (I. Arnold and A. Müller, unpublished data). In vivo, IL-8 has been shown to be strongly induced in the infected gastric human mucosa and its levels are correlated positively with pathology (64). In accordance with these findings, we measured high levels of MIP-2 transcript in infected mice and we detected the presence of neutrophils in the gastric mucosa histologically and by ELISA (data not shown for wild-type mice). Neutrophils are sometimes found luminaly and in close proximity to Helicobacter organisms (data not shown). Interestingly however, we observed only minor MIP-2 induction (Figs. 7–9) and neutrophil infiltration in mice lacking either T cells or T and B cells. Adoptive transfer of CD4+ effector populations restores MIP-2 signals (Figs. 7–9) and neutrophil infiltration (Fig. 8) in these strains. Whether MIP-2 is produced by the T effector cells themselves in this scenario or its increased production by epithelial cells is triggered by lymphocyte-derived signals is currently not known. Either way, efficient gastric MIP-2 production and neutrophil recruitment depends on CD4+ T cell infiltration in conjunction with infection (Fig. 7), but is independent of concomitant IFN-γ production (Fig. 8). In conclusion, our data are consistent with the following scenario: the initial contact of bacteria with gastric epithelial cells triggers moderate production of MIP-2. This instigates the modest infiltration and activation of neutrophils, which is itself a prerequisite for the subsequent recruitment of Helicobacter-specific T cells to the site of infection. These cells then generate the strong MIP-2 signals that are required to attract more neutrophils, which in turn accomplish clearance or reduction of the infection. IFN-γ secretion by CD4+ T helper cells is crucial for clearance. Although being dispensable for the recruitment of neutrophils in our as well as other model systems (65), IFN-γ is known to induce neutrophil phagocytosis (66), microbialid activity (67), and survival (68). We propose that CD4+ T cell-derived IFN-γ must act locally on neutrophils to activate their anti-Helicobacter properties. In addition (or alternatively) to IFN-γ, another proinflammatory cytokine, IL-17, has recently been assigned an important function in the mucosal response to Helicobacter infection, in particular in neutrophil recruitment. IL-17 levels are increased in the human-infected mucosa (69) as well as in vaccinated mice (59), and this seems to initiate acute inflammation in the mouse model (70). However, a conclusive functional role for IL-17 in Helicobacter control has not yet been found; on the contrary, IL-17−/− mice seem to be less colonized than wild-type mice (70). Data on vaccine studies in IL-17−/− mice are not yet available, but will hopefully clarify soon whether IL-17 is important in vaccine-induced protection.

Being the signature cytokine of Th1-biased Th responses, IFN-γ is efficiently down-regulated by Th2 cytokines such as IL-4. Indeed, the BALB/c strain of mice that is known to predominantly mount Th2-biased responses to infectious agents is completely protected from developing the preneoplastic lesions typically observed in the C57BL/6 background. Experimental polarization of systemic responses to Helicobacter by concurrent helminth infection was further shown to alleviate gastric premalignant pathology (71), lending considerable support to this model. In fact, an epidemiological study from Colombia looking at Th2-polarized responses to Helicobacter and concurrent helmint infections in children suggested that populations with low gastric cancer risk had significantly higher rates of helminths than high-risk populations (72). Apart from environmental factors affecting Th1/Th2 balance, disease outcome upon chronic infection may be influenced by polymorphisms affecting IFN-γ signaling, for instance in the IFN-γ receptor chain 1 (IFNGR) or the IFNG gene itself (25, 73–75). Overall, both animal experimental and epidemiological evidence seems to increasingly imply the (T cell-driven) immune response to the infection as a driving force behind gastric preneoplasia. Our finding that Rag-1−/− and TCR-β−/− are completely protected from premalignant pathology is in line with this assumption. Although there is obviously a clear contribution of Helicobacter virulence factors (such as the type IV secretion substrate CagA) to gastric cancer risk (76), one may speculate that such factors act as immune modulators (e.g., by disrupting the epithelial barrier and thereby exacerbating inflammation (77)) rather than as true bacterial oncoproteins. Finally, additional, as yet unknown, factors seem to affect the type of immune response launched against the infection, as illustrated by the heterogeneity of responses observed in mice of the same gender and same age, infected for the same time with the same strain (Figs. 1 and 3). Although superinfections with other bacteria could be a contributing factor (although this may not be very likely for mice inhabiting the same cage for many months), we favor an explanation factoring in the “stress” component. We speculate that in groups of mice housed together (especially males, which were used mostly in our studies), the highest ranking male(s) might be the least stressed and, as a result, least susceptible to Helicobacter-induced gastritis and pathology.

In conclusion, our results confirm and extend experimental and epidemiological data suggesting that IFN-γ plays an important role in the control of Helicobacter infection. At the same time, CD4+ T cell-derived IFN-γ provides the key stimulus for development of gastric premalignant lesions that can progress to gastric cancer.

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