IFN-γ Inhibits Gastric Carcinogenesis by Inducing Epithelial Cell Autophagy and T-Cell Apoptosis

Shui Ping Tu, Michael Quante, Govind Bhagat, Shigeo Takaishi, Guanglin Cui, Xiang Dong Yang, Sureshkumar Muthuplani, Wataru Shibata, James G. Fox, D. Mark Pritchard, and Timothy C. Wang

Abstract

IFN-γ mediates responses to bacterial infection and autoimmune disease, but it is also an important tumor suppressor. It is upregulated in the gastric mucosa by chronic Helicobacter infection; however, whether it plays a positive or negative role in inflammation-associated gastric carcinogenesis is unexplored. To study this question, we generated an H+/K+-ATPase-IFN-γ transgenic mouse that overexpresses murine IFN-γ in the stomach mucosa. In contrast to the expected proinflammatory role during infection, we found that IFN-γ overexpression failed to induce gastritis and instead inhibited gastric carcinogenesis induced by interleukin-1β (IL-1β) and/or Helicobacter infection. Helper T cell (Th) 1 and Th17 immune responses were inhibited by IFN-γ through Fas induction and apoptosis in CD4+ T cells. IFN-γ also induced autophagy in gastric epithelial cells through increased expression of Beclin-1. Finally, in the gastric epithelium, IFN-γ also inhibited IL-1β- and Helicobacter-induced epithelial apoptosis, proliferation, and Dckl1− cell expansion. Taken together, our results suggest that IFN-γ coordinately inhibits bacterial infection and carcinogenesis in the gastric mucosa by suppressing putative gastric progenitor cell expansion and reducing epithelial cell apoptosis via induction of an autophagic program.

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Introduction

IFN-γ is a cytokine produced primarily by activated CD4+ or CD8+ T cells and natural killer cells and is recognized as an important mediator of innate and adaptive immunity. It induces a variety of immunomodulatory molecules (1) and has been identified as a critical effector in numerous models of inflammatory and autoimmune diseases. Proposed roles of IFN-γ include orchestrating defense responses against intra- and extracellular bacteria. IFN-γ primes mononuclear phagocytes for production of monokines, and in concert with TNF-α, can augment the bacteriostatic activity of phagocytes. Interleukin (IL)-12 produced by monocytes can further polarize toward a helper T-cell (Th) 1 response resulting in additional IFN-γ production.

Helicobacter pylori represents one of the world’s most common chronic bacterial infections, and IFN-γ has been shown to be upregulated in the stomach of humans and mice infected with Helicobacter sp. (2, 3). Helicobacter pylori bacteria survive for decades in the human stomach, occasionally showing intracellular invasion of gastric epithelial cells (4). Chronic infection with Helicobacter represents an important risk factor for gastric cancer (5), and growing evidence suggests that it is the host immune response that is predictive of susceptibility to gastric cancer. Proinflammatory IL-1β, TNF-α, and IL-10 genotypes are associated with an increased risk for developing gastric cancer in the setting of H. pylori infection (6, 7), and overexpression of IL-1β alone can induce gastric cancer in mice (8).

Interestingly, there has been no reported human association between IFN-γ genotypes and gastric cancer risk, and the precise role of IFN-γ in gastric carcinogenesis remains unclear. Although several studies have suggested that neutralization or deletion of IFN-γ protects against the development of gastric atrophy, others could not confirm the importance of IFN-γ in preneoplasia (9, 10). Furthermore, studies of IFN-γ−/− mice suggest a role in protection from H. pylori infection (3, 11), supported by findings that IFN-γ can inhibit the development of IL-17-producing Th17 cells. IL-17 is overexpressed in H. pylori–infected stomachs in mice and human (12), and likely plays a role in the clearance of extracellular pathogens (13).

One way that IFN-γ defends against intracellular pathogens is by activation of immunity-related GTPases (IRG), some of which (Irgm1/LRG-47) have been shown to eliminate mycobacteria from macrophages through autophagy (14). Autophagy is a highly conserved, bulk degradation process in which
portions of cytoplasm are sequestered into autophagosomes. Several studies have linked autophagy to host defense against intracellular bacterial pathogens (15); however, the precise mechanisms remain to be elucidated. In addition, autophagy is thought to protect against cancer, in part, through removal of cellular debris that promotes chronic inflammation (16).

IFN-γ has also been linked to antitumor immunity and, in some models, the immune system plays an active role in suppressing the development of incipient tumors (17). Work from a number of groups has shown that IFN-γ prevents tumor induction (18, 19), although whether IFN-γ suppresses tumorigenesis by stimulation of “cancer immunosurveillance” is debatable. Although IFN-γR–deficient animals are more susceptible to tumorigenesis, immunodeficient Rag–2–deficient mice do not consistently show increased tumor susceptibility (19, 20), and spontaneous tumor development in mice lacking IFN-γ and granulocyte macrophage colony-stimulating factor can be inhibited by treatment with antibiotics (21), suggesting that carcinogenesis in immunodeficient mice may be mediated in part by changes in bacterial flora.

To investigate the potential role of IFN-γ in gastric inflammation and carcinogenesis, we generated a transgenic mouse with stomach-specific overexpression of IFN-γ by using the H+/K+-ATPase promoter. Surprisingly, IFN-γ inhibited the development of IL-1β- and H. felis–induced gastritis and neoplasia by induction of apoptosis, Beclin-1–mediated autophagy, and suppression of potential progenitor cell expansion.

Materials and Methods

Generation of H+/K+-ATPase and IFN-γ transgenic mice

The 1,060-bp fragments of the mouse H+/K+-ATPase β-subunit promoter (8) and the 450-bp fragments of mature secreted form mouse IFN-γ cDNA (Howard Young, NCI) were subcloned together with human growth hormone polyadenylation sequence into pBluescript vector. The transgenic construct was used for pronuclear injection of C57BL/6 mice and backcrossed to C57BL/6J mice. A high IFN-γ hybrid zygotes. A total of 5 positive founders were obtained by using the H+/K+-ATPase promoter. Surprisingly, IFN-γ inhibited the development of IL-1β– and H. felis–induced gastritis and neoplasia by induction of apoptosis, Beclin-1–mediated autophagy, and suppression of potential progenitor cell expansion.

Helicobacter felis infection and detection

Helicobacter felis infection was carried out as previously described (8). Pieces of gastric mucosa were streaked onto a Brucella agar plate, which was incubated at 35°C in a microaerophilic atmosphere until pinhead-sized colonies with a yellow appearance were noted. These gram-negative bacteria had spiral rod morphology and expressed urease, catalase, and oxidase. Warthin–Starry and hematoxylin and eosin (H&E)–stained sections of gastric tissue were examined for the presence of H. felis– and bacteria-associated pathology.

IFN-γ infusion

Recombinant mouse IFN-γ (10,714 units/d; ref. 22; R&D) was diluted in 2% bovine serum albumin (BSA) and PBS and loaded into Alzet 15-day micro-osmotic pumps according to the manufacturer’s instructions. Control pumps contained equivalent volumes of 2% BSA in PBS. Pumps were inserted into male C57BL/6 IFN-γ−/− and IL-1β mice (n = 4 per time point).

Measurement of cytokine levels

The levels of IL-1β, TNF-α, IL-6, and IFN-γ in serum or gastric tissues of transgenic mice were determined using a mouse ELISA kit (BD Biosciences). Tissues were sonicated in medium containing a protease inhibitor cocktail (Roche Applied Science). Absorbance was measured at 450 nm by a Multiscan MC reader, and the samples were analyzed by DeltaSoft software (BioMetallics, Inc.).

Cell lines

The human gastric cancer cell line MKN28, which was established from a moderately differentiated adenocarcinoma, was recently obtained from the Riken Cell Bank, and cells were stored in multiple aliquots in liquid nitrogen and passaged for fewer than 3 months after resuscitation (MKN28 cells were regularly tested by short tandem repeat polymorphism analysis test by Riken). These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Inc.).

Quantitative and semiquantitative PCRs

Reverse transcription was carried out by using the SuperScript III System. Quantitative reverse transcriptase PCR (qRT-PCR) was carried out with a 3-step method by using the Bio-Rad iCycler qRT-PCR detection system (Bio-Rad Laboratories) and Quantitect SYBR Green PCR (Qiagen). The PCR conditions were as follows: 95°C for 3 minutes, followed by 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

Western blot

The gastric cancer MNK-28 cell line was transfected with a green fluorescent protein (GFP)-LC3 construct (Cell Biolabs, Inc.) and then treated with 20 ng IFN-γ for 48 hours. Cells were harvested and protein was extracted. GFP-LC3 was detected by Western blot by using an anti-LC3 antibody (Abcam).

Histopathologic analysis

Sections of stomach and other tissues from transgenic and control mice were fixed in 10% formalin and embedded in paraffin. Histopathologic indices were scored as previously described (8).

Analysis of apoptosis

Deparaffinized, colonic tissue slides were stained with an In Situ Apoptosis Detection kit (Chemicon) according to the manufacturer’s instructions. The apoptotic index was calculated by assessing the mean of the total number of TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling)–positive cells per field at 10 different locations of the stomach section under a light microscope (400×).
Analysis of autophagy

The gastric cancer cell line MNK-28 was transfected with GFP-LC3 plasmids in the presence or absence of 20 ng IFN-γ for 48 hours. Autophagy was detected by visualizing punctuate dots in cells under a fluorescence microscope. MKN-28-GFP-LC3 stable transfectants were transfected with Beclin-1 siRNA and control siRNA (Santa Cruz Biotechnology) for 6 hours. The transfectants were cultured in the presence or absence of 20 ng IFN-γ for 48 hours. Autophagy was assessed by detection of GFP-LC3-II punctuate dots in cells by fluorescence microscopy. Cells were harvested for Western blotting to detect the expression of LC3-II and Beclin-1.

Reporter construct and luciferase assay

A 950-bp fragment of the Beclin-1 promoter was cloned into the pGL3-basic vector through PCR amplification of human genomic DNA (Promega) by using synthetic primers (23). MKN-28 cells (1.0 × 10^5 cells/12-well) were transfected with 1.5 μg Beclin-1-luciferase or 1.5 μg empty reporter vector DNA by using the FuGene HD transfection reagent (Promega). To control for background luciferase activity, 0.05 μg/well of a Renilla luciferase reporter vector DNA, driven by a minimally active thymidine kinase promoter (pBLTK; Promega), was cotransfected. After 6 hours, the transfected cells were cultured with or without 20 ng/mL IFN-γ for 24 hours. Luciferase activity was determined as previously described (23). All assays were conducted in triplicate and repeated 3 times.

Immunohistochemical staining

Paraffin sections fixed in 10% formalin were incubated with the following primary antibodies: Ki-67 (Dako), Dclk-1 or DCAMKL-1 (Abgent), F4/80 (Santa Cruz), LC3 (Abcam), and control rat IgG2a. Biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) and ABC avidin–biotin–DAB (3,3′-diaminobenzidine) detection kit (Vector Labs) were used for detection and visualization according to supplied protocol.

Single-cell preparation and fluorescence-activated cell-sorting analysis

For single-cell suspension preparation from stomach tissue, the mucosa was gently scraped free from the serosa, minced and digested for 1 hour in 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 5% FBS in PBS at 37°C, filtered through a 40-μm nylon mesh strainer, and then resuspended in Dulbecco’s PBS (D-PBS; ref. 24). For splenic single-cell suspensions, spleens were disaggregated in cold Hank’s balanced salt solution. Erythrocytes were removed by hypotonic lysis. Splenocytes were resuspended in D-PBS. Single-cell suspensions were stained with fluorescent labeled anti-CD45, CD3, CD19, CD8, CD4, CD11b, Ly-6G, c-kit, FLK-1, and Sca-1 antibodies (BD Pharmingen) and detected using a LSII flow cytometer (BD Bioscience). Data were analyzed by FlowJo7 software (Tree Star, Inc.).

Statistics

Data are presented as the mean ± SD. The significance of the difference between groups was evaluated with the Student’s t test or ANOVA test; P < 0.05 was considered significant.

Results

H^+/K^+-ATPase-IFN-γ mice fail to develop spontaneous gastritis or metaplasia

To investigate the role of IFN-γ in gastric inflammation and carcinogenesis, we generated H^+/K^+-ATPase-IFN-γ transgenic mice, in which the mouse IFN-γ cDNA (kind gift of Howard A. Young, NCI) was placed downstream of the mouse parietal cell–specific H^+/K^+-ATPase promoter (Fig. 1A). We identified 2 lines of IFN-γ transgenic mice (line 54, high; line 32, low; Fig. 1B) that showed elevated levels of mouse IFN-γ (line 32, 2-fold; line 54, 3.5-fold), specifically in the stomach by qRT-PCR and ELISA assays (Supplementary Fig. S1). IFN-γ bioactivity was confirmed by elevated levels of IFN-γ–regulated genes such as IFN-γ–inducible protein 10 (IP-10) and monokine induced by IFN-γ (Mig, Fig. 1C). IFN-γ expression in the H^+/K^+-ATPase-IFN-γ mice (line 54, 3.5-fold) was comparable with that found in aged H. felis–infected mice, providing an opportunity to study the role of moderate elevation of IFN-γ in the absence of prior inflammation or infection.

Overexpression of IFN-γ in the gastric mucosa did not induce spontaneous atrophic gastritis or dysplasia (Fig. 1D), and these mice had near normal histopathology scores (Supplementary Table S1). Consistent with these observations, mRNA expression levels of the proinflammatory cytokines (IL-6, TNF-α, and IL-1β) were not significantly different between H^+/K^+-ATPase-IFN-γ and wild-type (WT) mice (data not shown).

IFN-γ inhibits the development of H. felis- or IL-1β–induced gastric dysplasia

Because IFN-γ overexpression induced minimal gastritis in the mouse stomach, we next examined its role in the setting of H. felis infection, as H. felis–infected WT mice develop gastric inflammation, atrophy metaplasia, and dysplasia (8). Surprisingly, we found that H. felis–infected H^+/K^+-ATPase-IFN-γ mice developed only mild gastritis, with minimal degrees of atrophy and metaplasia after 12 or 24 months (Fig. 2A–C). Histopathology scores were significantly lower in H. felis–infected H^+/K^+-ATPase-IFN-γ mice compared with H. felis–infected WT mice (Supplementary Table S1, Fig. 2C and D). No IFN-γ transgenic mice developed dysplasia (Fig. 2D), suggesting that elevated levels of IFN-γ inhibit carcinogenesis. Despite previous observations that IFN-γ may reduce H. pylori colonization (3), in our model, there were no differences in colonization when we quantified H. felis infection levels by H&E staining (Fig. 2E) and qRT-PCR (Fig. 2F).

We next examined the effects of IFN-γ overexpression in a second model of gastric cancer, the H^+/K^+-ATPase-IL-1β transgenic mouse model, in which we had shown inflammation-induced gastric carcinogenesis (8). IL-1β transgenic mice were crossed with H^+/K^+-ATPase-IFNγ mice and followed for 12 to 14 months with or without H. felis infection. IL-1β/IFN-γ mice developed less severe hyperplasia, chronic inflammation,
atrophy, metaplasia, and dysplasia compared with their IL-1β littermates (Supplementary Table S1). No IL-1β/IFN-γ mice developed dysplasia (Fig. 3C; Supplementary Table S1), whereas H. felis–infected IL-1β mice progressed rapidly to metaplasia and high-grade dysplasia. These findings again indicate that overexpression of IFN-γ inhibits inflammation-dependent carcinogenesis.

**IFN-γ inhibits gastric epithelial cell proliferation and Dclk-1+ cell expansion**

The development of Helicobacter-dependent gastric cancer is preceded by increased epithelial proliferation. Ki-67+ cells in the stomach were significantly reduced in IFN-γ mice compared with WT mice (Fig. 3A). Consistently, the number of Ki-67+ cells in H. felis–infected H+/K+-ATPase-IFN-γ mice were much lower than those present in infected WT mice (Fig. 3B, top), and proliferation rates were also lower in IL-1β/IFN-γ mice than in IL-1β mice without (Fig. 3A) and with H. felis (Fig. 3B) infection, showing that both H. felis- and IL-1β–induced cell proliferation could be inhibited by overexpression of IFN-γ.

Double cortin and CaM kinase-like-1 (Dclk-1) has been associated with the proliferative zone of the stomach and has been proposed as a putative gastrointestinal progenitor cell marker (24). In the absence of H. felis infection, Dclk-1+ cells were not significantly different between WT and H+/K+-ATPase-IFN-γ mice (Fig. 3A). Dclk-1+ cells were markedly elevated in H. felis–infected WT mice (Fig. 3B), an effect that was abrogated by IFN-γ overexpression. In addition, Dclk-1+ cells were significantly increased in stomachs of uninfected IL-1β mice compared with WT mice (Fig. 3A), and even further increased in H. felis–infected IL-1β mice compared with H. felis–infected WT mice (Fig. 3B). The increase in Dclk-1+ cells was inhibited by overexpression of IFN-γ (Fig. 3B). IFN-γ also inhibited IL-1β–induced inflammation, cell proliferation, and Dclk1+ cell expansion in very young IL-1β/IFN-γ mice (<3 months old) in the absence or presence of H. felis infection (Supplementary Table S1; Supplementary Fig. S2A and B). Overall, these results suggest that inflammatory signals such as IL-1β and H. felis infection can stimulate cell proliferation and Dclk-1+ cell expansion and that IFN-γ inhibits the expansion of these cells.

**IFN-γ overexpression accelerates apoptosis of gastric T lymphocytes and inhibits the production of proinflammatory Th1 and Th17 cytokines**

IFN-γ has been shown to induce CD4+ T-cell apoptosis (25), and CD4+ T cells are required for H. pylori–induced atrophic gastritis (8). In uninfected WT and IFN-γ mice, TUNEL staining showed low levels of gastric epithelial and inflammatory cell apoptosis (Fig. 4A; Supplementary Fig. S3A). However, in the setting of H. felis infection or IL-1β overexpression, the mean number of apoptotic epithelial cells per hpf and cleaved caspase-3–stained cells were markedly decreased by IFN-γ overexpression (Fig. 4B; Supplementary Fig. S3B). The IFN-γ–dependent decrease in epithelial apoptosis showed an inverse correlation with leukocyte apoptosis. In both WT and H+/K+-ATPase-IFN-γ mice, H. felis infection resulted in an increase in leukocyte apoptosis, but there were significantly more apoptotic cells in the H. felis–infected H+/K+-ATPase-IFN-γ mice than in...
the *H. felis*-infected WT mice. Fluorescence-activated cell-sorting (FACS) analysis confirmed this finding and revealed that apoptosis was primarily occurring in gastric CD4+ and CD8+ T cells. An increase in Annexin V staining was noted in both CD4+ and CD8+ T cells in *H+/K+-ATPase-IFN-γ* mice compared with WT mice, with the biggest increase observed in the setting of *H. felis* infection (Fig. 4C) and in IL-1β mice compared with double transgenic IL-1β/IFN-γ mice (Supplementary Fig. S4A). T-cell apoptosis correlated with an abrogated Th1 immune response, because the expression of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) was significantly decreased in *H. felis*-infected *H+/K+-ATPase-IFNγ* compared with *H. felis*-infected WT mice (Fig. 4D).

Although expression of Th2 cytokines (IL-4 and IL-10) was not significantly different between IFN-γ and WT mice without *H. felis* infection (Supplementary Fig. S4B), IL-10 was significantly upregulated in IFN-γ mice after *H. felis* infection. Furthermore, Th17-related cytokines (IL-17A and IL-17F) were significantly upregulated in IL-1β mice and *H. felis*-infected WT mice compared with uninfected WT mice but downregulated by IFN-γ overexpression (Fig. 4E). IL-12 and IL-23 stimulate either Th1 or Th17 immune responses through T-cell activation, and we observed a downregulation of IL12, and even more so of IL-23, in association with IFN-γ overexpression (Fig. 4E), indicating that IFNγ overexpression leads to a reduction of the Th17 response. Moreover, macrophages were diminished in IFN-γ mice compared with WT mice, in *H. felis*-infected IFN-γ mice compared with *H. felis*-infected WT mice, and in IL-1β/IFNγ mice compared with IL-1β mice (Supplementary Fig. S4C), indicating that in our model IFN-γ reduces not only the adaptive immune response but possibly also the innate immune response.

FAS, TRAIL, and XAF1 can all mediate IFN-γ-induced apoptosis (26), in particular, FAS mediates IFN-γ-dependent apoptosis of T cells (27). Indeed, in both *H. felis*-infected and uninfected *H+/K+-ATPase-IFN-γ* mice, qRT-PCR showed upregulated FAS expression in the gastric mucosa (Supplementary Fig. S5C). The gene expression of IFN-γ downstream...
target genes TRAIL and XAF1 was also slightly increased (1.5- to 3-fold) in H+/K+-ATPase-IFN-γ compared with WT mice (Supplementary Fig. S5A and B).

**IFN-γ induces autophagy in gastric epithelial cells through upregulation of Beclin-1**

Current thinking suggests that in early stages of carcinogenesis, autophagy protects from carcinogenesis (28) and IFN-γ can induce autophagy in epithelial cells and immune cells (29). We found that the number of cells expressing LC3-II, the mammalian homologue of yeast Atg8, were significantly increased in the stomachs of H+/K+-ATPase-IFN-γ mice compared with WT mice (Fig. 5A). With *H. felis* infection, inducing similar tissue levels of IFN-γ, the number of LC3-II+ cells was also increased in WT mice (Fig. 5A), confirming that *H. felis* infection and/or the immune response to *Helicobacter* infection may trigger autophagy in gastric epithelial cells (30, 31). Western blot analysis showed that LC3-II levels were increased in H+/K+-ATPase-IFN-γ mice compared with WT mice, with or without *H. felis* infection (Fig. 5B), and were higher in IL-1β/IFN-γ than in the IL-1β mice (Fig. 5C, Supplementary Fig. S6A). Importantly, to confirm that the accumulation of LC3-II was due to increased autophagosome formation, rather than decreased autophagosome degradation, we treated H+/K+-ATPase-IFN-γ mice with chloroquine for 10 days to block lysosomal turnover (32), observing a further increase...
of the LC3 signal in H⁺/K⁺-ATPase–IFN-γ mice (Fig. 5D) in contrast to IL-1β mice in which we did not detect autophagy.

To confirm that IFN-γ can directly induce autophagy in gastric epithelial cells, we transfected gastric cancer cells (MNK-28) with a GFP-LC3 construct. Transfected cells were treated with IFN-γ (20 ng/mL) for 24 or 48 hours, which resulted in characteristic morphologic changes of autophagy. The percentage of GFP-LC3-II dots (Fig. 5E and G) and protein
expression (Fig. 5F) was significantly increased in IFN-γ-treated cells, compared with untreated cells, confirming that IFN-γ can directly induce autophagy in gastric epithelial cells.

Beclin-1 is known to play an important role in the regulation of autophagy (33, 34). Overexpression of IFN-γ upregulated Beclin-1 mRNA expression (Fig. 6B) and protein (Fig. 6A) levels in the stomachs of mice with or without H. felis infection. Immunohistochemical staining localized Beclin-1 protein to epithelial cells (Fig. 6A; Supplementary Fig. S6A), and overexpression of IL-1β correlated with inhibited expression of Beclin-1 in the stomach (Supplementary Fig. S6A and B). Beclin-1 upregulation correlated with the induction of autophagy in our mouse model. Indeed, knockdown of Beclin-1 by siRNA in MKN-28 cells reduced IFN-γ-induced autophagy (Fig. 6C–E), confirming a direct mediation of Beclin-1 in IFN-γ-induced autophagy. Moreover, Beclin-1 seemed to be a direct target of IFN-γ because IFN-γ activated Beclin-1 promoter activity (Fig. 6F). By using the transcriptional regulatory element database (35), we located IFN-γ activation site (GAS) elements, which are short stretches of DNA required for the rapid transcriptional induction of genes in response to IFN-γ. Three GAS elements were located in the first 1,000 bp of the Beclin-1 promoter 5’ of the ATG and 13 GAS elements within the different introns of the Beclin-1 gene (data not shown). These results suggest that IFN-γ induces autophagy in part through upregulation of Beclin-1.

**IFN-γ infusion inhibits progenitor cell expansion and induces autophagy**

Although constitutive overexpression of IFN-γ inhibits proliferation and carcinogenesis and induces autophagy, our
transgenic model does have the possible limitation of lifelong chronic expression that could potentially result in compensatory responses. Thus, we infused IFN-γ into 2-month-old WT B6, IFN-γ-knockout, and IL-1β mice for 4 weeks and confirmed significantly increased levels of IFN-γ in the stomach (Supplementary Fig. S7A) and serum (data not shown) in all infused mice. Two weeks postcessation of IFN-γ infusion, we found that the treatment induced only mild gastritis in both the WT mice and IFN-γ knockout mice (inflammation score <0.5; data not shown). No mice developed gastric atrophy or metaplasia, a finding confirmed by Alcian blue staining (Supplementary Fig. S7B). The short-term IFN-γ infusion did result in reduced cell proliferation (Ki-67 staining; Fig. 7A and C) in all infused mice, consistent with previous reports (8). Moreover, IFN-γ infusion abrogated the development of gastric metaplasia in the IL-1β transgenic mice (Supplementary Fig. S7B), which was confirmed by histopathologic analysis (data not shown), a result that was consistent with previous findings from the double IL-1β;IFN-γ transgenic mice. Interestingly, IFN-γ infusion also reduced the number of Dclk-1+ cells (Fig. 7A and B), induced autophagy in gastric epithelial cells (Fig. 7A and D), and upregulated the expression of Beclin-1 (Fig. 7E). Finally, IFN-γ infusion inhibited the production of IL-1β, TNF-α, and IL-6 in all treated mice (Supplementary Fig. S7C).

Discussion

Although IFN-γ is a signature Th1 cytokine mediating protection from pathogens, its role in cancer surveillance...
and suppression of tumorigenesis is less well understood. It has been suggested that IFN-γ promotes gastric preneoplasia (3). Here, we show that IFN-γ overexpression can suppress gastric cancer in models of IL-1β- and H. felis-dependent carcinogenesis. Our studies involving both transgenic overexpression and exogenous infusion show that IFN-γ can inhibit inflammation-driven carcinogenesis. In addition, we link the cancer-suppressive effect to changes in both immune cells and epithelial cells. Our data suggest that IFN-γ induces CD4 T-cell apoptosis, resulting in reduced Th1 and Th17 cytokine responses, as well as induces autophagy in gastric epithelial cells through upregulation of Beclin-1. Finally, IFN-γ inhibited epithelial cell apoptosis, normalized cell proliferation, and reduced Dclk-1+ putative progenitor cell expansion. Among multiple effects, IFN-γ clearly has an impact on both the stromal and epithelial compartments and the distinct effects on autophagy and progenitor cells seemed to correlate particularly well with suppression of gastric carcinogenesis.

IFN-γ is primarily produced in response to injury or infection and has multiple effects on the immune system such as the previously reported effect on apoptosis of CD8+ and CD4+ T cells (27), a finding confirmed in our models of H. felis- or IL-1β–induced gastritis. Specific IFN-γ–dependent induction of CD4+ T-cell apoptosis may restrain inflammation (26), suggesting that IFN-γ maintains T-cell homeostasis during immune responses. In H. felis–infected IFN-γ mice, apoptosis of CD4+ T cells seemed to be Fas dependent, confirming prior reports (36, 37). Apoptosis of CD4+ T cells resulted in a reduction in Th1 and Th17 immune responses without a major change in Th2 responses, despite elevated IL-10 in H. felis–infected IFN-γ mice, a cytokine milieu that has previously been shown to suppress inflammation-induced cancer in the gut (38). IFN-γ can inhibit the development of Th17 cells, and
recent studies have pointed that Th17 cells play a key role not only in the clearance of extracellular pathogens such as H. felis (13) but also in cancer. IL-17 is overexpressed in H. pylori–infected gastric mucosa in both mice and humans (12, 39), whereas inhibition of the Th17 response prevents gastric dysplasia (40). Interestingly, macrophages, representing the innate immune response, also seemed to be reduced in number, suggesting that IFN-γ inhibits recruitment of macrophages, a potential source of Th1- and Th17-inducing cytokines such as IL-12 and IL-23, which are downregulated in IFN-γ transgenic mice. Because we have shown that IFN-γ can affect adaptive and innate immune responses in H. felis–induced and in IL-1β–induced gastric carcinogenesis, it is tempting to speculate that the changes in the cytokine profile are due to either directly expression of IFN-γ or to missing recruitment of tumor-promoting immune cells, such as immature myeloid cells, which contribute to the promotion of carcinogenesis in the IL-1β model of gastric cancer (8).

Although IFN-γ is clearly able to promote T-cell apoptosis and potentially affect innate immunity in the stomach, it seems to have a different effect on the epithelium, inhibiting gastric epithelial apoptosis while promoting autophagy during early stages of inflammation-associated gastric carcinogenesis. The induction of autophagy seems highly relevant to the role of IFN-γ in protection from pathogens, because Helicobacter can occasionally be found intracellularly (41), and published reports have indicated that autophagy represents an important mechanism for elimination of intracellular pathogens (29) independent of apoptosis (42). This new mechanism should be considered in Helicobacter infection, especially because recent studies have shown that IFN-γ can enhance the degradation of Mycobacterium tuberculosis and Rickettsia conorii by induction of autophagy in infected cells (29, 33). Although Nod1 and Nod2 have been shown to comprise potential intracellular signals for triggering autophagy and bacterial elimination (43), the induction of IFN-γ may represent a second pathway.

Autophagy may have arisen during evolution as a response to infection or starvation stress, but it has also been associated with protection from neoplasia at early stages of carcinogenesis (44). Thus, while overexpression of IL-1β correlated with reduced autophagy and increased apoptosis of epithelial cells, overexpression of IFN-γ, in contrast, increased autophagy, reduced epithelial cell apoptosis, and inhibited progression to cancer. Interestingly, H. felis infection itself could induce some degree of autophagy in the stomach as suggested by recent reports (30, 31), as well as by elevated levels of IFN-γ, thus explaining the somewhat slower progression to cancer in H. felis–infected WT mice compared with uninfected IL-1β mice. Moreover, the switch to autophagy was associated with decreased epithelial cell apoptosis, a critical factor in gastric carcinogenesis (45).

Our data suggest that IFN-γ–induced autophagy involves upregulation of Beclin-1, a haploinsufficient tumor suppressor and a key regulator of autophagosome formation (34, 46). Beclin-1 expression was upregulated by IFN-γ overexpression or infusions and was directly associated with the induction of autophagy in gastric epithelial cells. Computer-based searches could identify interferon-responsive GAS elements in the Beclin-1 promoter, suggesting binding motifs for direct regulation through IFN-γ–activated STAT1, as shown previously (47). Because downregulation of Beclin-1 expression reduced IFN-γ–induced autophagy, consistent with previous reports on the effect of IFN-γ on phagosome–lysosome fusion in infected murine macrophages (48), our findings suggest that IFN-γ–induced phagosome maturation may be responsible for the autophagic response.

The expansion of cells expressing Dclk-1, a putative progenitor cell marker also known as DCAMKL-1, was strongly associated with inflammation-related carcinogenesis and preceded the development of gastric cancer. Although formal lineage tracing has not been reported, Dclk-1 is expressed in the progenitor zone of the stomach and intestine and has been shown to be upregulated in response to carcinogen treatment in the colon. Notably, overexpression or infusion of IFN-γ reduces both cell proliferation and the number of Dclk-1 cells and therefore raises the possibility of a direct effect of IFN-γ on gastric progenitor cells. These results are consistent with previous report that IFN-γ can inhibit hematopoietic stem or progenitor cell expansion (49).

In summary, overexpression of IFN-γ inhibits H. felis- and IL-1β–dependent gastric inflammation and carcinoma. Although previous studies have shown that tissue-specific IFN-γ transgenic mice developed severe chronic inflammation, including chronic active hepatitis (50), no IFN-γ transgenic mouse model has yet shown development of carcinoma (50). Our data suggest that rather than contributing to the rejection or destruction of incipient tumors, IFN-γ mediates a switch from epithelial apoptosis to Beclin-1–mediated epithelial cell autophagy early in the process of inflammation–induced carcinogenesis. Decreased epithelial cell apoptosis reduces the need for cell and tissue replacement, leads to decreased progenitor or stem cell proliferation, and reduces inflammation. In addition, IFN-γ induces CD4+ T-cell apoptosis, resulting in decreased Th1 and Th17 immune responses and less epithelial stress that might decrease genetic alterations. The overall tumor suppressor function of the immune system seems indeed critically dependent on the actions of IFN-γ, and greater attention should be given in the future to the potential role of IFN-γ in cancer prevention.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

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