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Interferon-γ inhibits gastric carcinogenesis by inducing epithelial cell autophagy and T cell apoptosis

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Abstract

Interferon-gamma (IFN-γ) mediates responses to bacterial infection and autoimmune disease but it is also an important tumor suppressor. IFN-γ 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 pro-inflammatory role during infection, we found that IFN-γ overexpression failed to induce gastritis and instead inhibited gastric carcinogenesis induced by IL-1β and/or Helicobacter infection. Th1 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. Lastly, 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.

Keywords

IFN-γ; gastric inflammation; gastric carcinogenesis; immune response; autophagy; apoptosis; progenitor cell; H. felis

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#These authors contributed equally to this work
The authors declare that they have no conflict of interest.
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. IFN-γ 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 extra-cellular bacteria. IFN-γ primes mononuclear phagocytes for production of monokines, and in concert with TNF-α can augment the bacteriostatic activity of phagocytes. IL-12 produced by monocytes can further polarize towards a Th1 response resulting in additional IFN-γ production.

_Helicobacter pylori_ represents one of the world’s most common chronic bacterial infection, and IFN-γ has been shown to be upregulated in the stomach of humans and mice infected with _Helicobacter sp._ (2, 3). _H. pylori_ survives 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. Pro-inflammatory IL-1β, TNF-α and IL-10 genotypes are associated with an increased risk for 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 pre-neoplasia (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 Interleukin-17-producing Th17 cells (30). 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 mycobacterium from macrophages through autophagy (14). Autophagy is a highly conserved, bulk degradation system 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 anti-tumor 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 considered debatable. Indeed, while 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 GM-CSF 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 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 1,060bp fragments of the mouse H⁺/K⁺-ATPase β subunit promoter (8) and the 450bp 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 × SJL F2 hybrid zygotes. A total of 5 positive founders were obtained and backcrossed to C57BL/6J mice. A high IFN-γ expressing line (Line 54) and a low FN-γ-expressing line (Line 32) were selected for further study. IFN-γ−/− (B6.129S7-Ifng<sup>tm1Ts</sup>/J) mice were purchased from Jackson Laboratories.

**Helicobacter felis infection and detection**

*H. felis* infection was performed 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. The bacteria were Gram-negative and had spiral rod morphology and they expressed urease, catalase, and oxidase. Warthin-Starry and 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 U/day) (22) (R&D) was diluted in 2% BSA and PBS buffer and loaded into Alzet™ 15 days 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/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, San Diego, CA). Tissues were sonicated in medium containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Absorbance was measured at 450 nm by a Multiscan MC reader, and the samples were analyzed by DELTA SOFT II software (BioMetallics, Inc., Princeton, NJ).

**Cell Lines**

The human gastric cancer cell line MKN28 was obtained from the Riken Cell Bank (Tsukuba, Japan), which was established from a moderately differentiated adenocarcinoma. These cells were maintained in DMEM supplemented with 10% FCS (Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Inc., Grand Island, NY).

**Quantitative and semi-quantitative polymerase chain reaction**

Reverse transcription was performed using the SuperScript III System. Quantitative real-time PCR was performed with a 3-step method using the Bio-Rad iCycler real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and QuantiTect SYBR Green PCR (Qiagen, Valencia, CA). 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 GFP–LC3 construct (Cell Biolabs, Inc. San Diego, Ca) and then treated with 20ng IFN-γ for 48 hours. Cells were harvested and protein was extracted. GFP–LC3 was detected by Western blot using an anti-LC3 antibody (Abcam, Cambridge, MA).

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 tissues slides were stained with an In Situ Apoptosis Detection Kit (Chemicon, Billerica, MA) according to the manufacturer’s instructions. The apoptotic index was calculated by assessing the mean of the total number of TUNEL-positive cells per field at 10 different locations of the stomach section under light microscopy (×400).

Analysis of Autophagy

The gastric cancer cell line MNK-28 was transfected with GFP-LC3 plasmids in the presence or absence of 20ng IFN-γ for 48 hours. Autophagy was detected by visualizing punctuate dots in cells under fluorescence microscopy. MKN-28-GFP-LC3 stable transfectants were transfected with Beclin 1 siRNA and control siRNA (Santa Cruz, CA) for 6 hours. The transfectants were cultured in the presence or absence of 20ng 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 Blot 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 from human genomic DNA (Promega) using synthetic primers (23). MKN-28 cells (1.0×10^5 cells/12-well) were transfected with 1.5 μg Beclin 1-luciferase or 1.5μg empty reporter vector DNA using the FuGene HD transfection reagent (Promega). To control for background luciferase activity, 0.05μg/well of a Renilla luciferase reporter vector DNA, driven by a minimally active thymidine kinase promoter (pRLTK; Promega), was cotransfected. After 6 hours, the transfected cells were cultured either with or without 20ng/ml IFN-γ for 24-hours. Luciferase activity was determined as previously described (23). All assays were performed in triplicate and repeated three times.

Immunohistochemical staining

Paraffin sections fixed in 10% formalin were incubated with primary antibodies: Ki-67 (DAKO), Dclk1 or DCAMKL1 (Abgent), F4/80 (Santa Cruz), LC3 (Abcam, Cambridge, MA) and control rat IgG2a. Biotinylated secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and ABC avidin-biotin–DAB detection kit (Vector Labs) were used for detection and visualization according to supplied protocol.

Single cell preparation and FACS 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 1mM DTT, 1mM EDTA, 5% FBS in PBS at 37° and filtered through a 40μm nylon mesh strainer and then resuspended in Dulbecco’s PBS (D-PBS)\textsuperscript{24}. For splenic single cell suspensions, spleens were disaggregated.
in cold Hanks balanced salt solution. Erythrocytes were removed by hypotonic lysis. Splenocytes were resuspended in Dulbecco’s PBS (D-PBS). Single cell suspensions were stained with fluorescent labeled anti-CD45, CD3, CD19, CD8, CD4, CD11b, Ly-6G, c-kit, FLK-1, and Scal-1 antibodies (BD Pharmingen, Franklin Lakes, New Jersey) and detected using a LSAlII flow cytometer (BD Bioscience, San Jose, California). Data were analyzed by FlowJo7 software (Tree Star, Inc, Ashland, OR).

Statistics
Data are present 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, where 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 (Figure 1A). We identified two lines of IFN-γ transgenic mice (Line 54 - high and Line 32 - low) (Figure 1B) that showed elevated levels of mouse IFN-γ (line 32: 2 fold and line 54 3.5 fold) specifically in the stomach by RT-PCR and ELISA assays (Supplementary Figure 1). 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) (Figure 1C). IFN-γ expression in the H⁺/K⁺-ATPase-IFN-γ mice (line 54, 3.5 fold) was comparable to 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 (Figure 1D) and these mice had near normal histopathology scores (Supplementary Table I). Consistent with these observations, mRNA expression levels of the pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) were not significantly different between H⁺/K⁺-ATPase-IFN-γ and WT mice (data not shown).

IFN-γ inhibits the development of H. felis- or IL-1β-induced gastric dysplasia
Since IFN-γ overexpression induced minimal gastritis in the mouse stomach, we next examined its role in the setting of H. felis infection, since 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 (Figure 2A-C). Histopathology scores were significantly lower in H. felis-infected H⁺/K⁺-ATPase-IFN-γ mice compared to H. felis-infected WT mice (Supplementary Table I, Figure 2C, D). No IFN-γ transgenic mice developed dysplasia (Figure 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 (Figure 2E) and qPCR (Figure 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 demonstrated inflammation induced gastric carcinogenesis (8). IL-1β transgenic mice were crossed to H⁺/K⁺-ATPase-IFNγ mice and were followed for 12–14 months with or without H. felis infection. IL-1β;IFN-γ mice developed less severe hyperplasia, chronic inflammation, atrophy, metaplasia and dysplasia, compared to their IL-1β littermates (Supplementary Table 1). No IL-1β;IFN-γ mice developed dysplasia (Figure 3C and Supplementary Table 1)
while *H. felis*-infected IL-1β mice progressed rapidly to metaplasia and high-grade dysplasia. These findings again indicating that overexpression of IFN-γ inhibits inflammation-dependent carcinogenesis.

**IFN-γ inhibits gastric epithelial cell proliferation and Dclk1+ 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 to WT mice (Figure 3A). Consistently, the number of Ki-67+ cells in *H. felis*-infected WT mice were much lower in comparison to infected WT mice (Figure 3B, top panel), and proliferation rates were also lower in IL-1β;IFN-γ mice compared to IL-1β mice without (Figure 3A) and with *H. felis* (Figure 3B) infection, demonstrating that both *H. felis*- and IL-1β-induced cell proliferation could be inhibited by overexpression of IFN-γ.

Doublecortin 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 (Figure 3A). Dclk-1+ cells were markedly elevated in *H. felis*-infected WT mice (Figure 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 to WT mice (Figure 3A), and even further increased in *H. felis*-infected IL-1β mice compared to *H. felis*-infected WT mice (Figure 3B). The increase in Dclk-1+ cells was inhibited by overexpression of IFN-γ (Figure 3B). IFN-γ also inhibited IL-1β-induced inflammation, cell proliferation, and Dclk1+ cell expansion in very young IL-1β;IFN-γ mice (3 month old) in the absence or presence of *H. felis* infection (Supplementary Table 1, Supplementary Figure 2A, B). Overall, these results suggest that inflammatory signals such as IL-1β and *H. felis* infection can stimulate cell proliferation and Dclk1+ 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 pro-inflammatory 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 (Figure 4A, Supplementary Figure 3A). However, in the setting of *H. felis* infection or IL-1β overexpression, the mean number of apoptotic epithelial cells per high power field and cleaved-Caspase-3 stained cells, were markedly decreased by IFN-γ overexpression (Figure 4B and Supplementary Figure 3B). 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. 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 to WT mice, with the biggest increase observed in the setting of *H. felis* infection (Figure 4C) and in IL-1β mice compared to double transgenic IL-1β;IFN-γ mice (Supplementary Figure 4A). T cell apoptosis correlated with an abrogated Th1 immune response, since the expression of pro-inflammatory cytokines (IL-1β), TNF-α and IL-6 was significantly decreased in *H. felis*- infected H^+/K^+ATPase-IFNγ compared to *H. felis*-infected WT mice (Figure 4D). While expression of Th2 cytokines (IL-4 and IL-10) was not significantly different between IFN-γ and WT mice without *H. felis* infection (Supplementary Figure 4B), IL-10 was significantly upregulated in IFN-γ mice after *H. felis* infection.
infection. Furthermore, Th17 related cytokines (IL-17A and IL-17F) were significantly
upregulated in IL-1β mice and H. felis-infected WT mice compared to uninfected WT mice,
but downregulated by IFN-γ overexpression (Figure 4E). IL-12 and IL-23 stimulate either
Th1 or Th17 immune responses through T-cell activation, and we observed a down-
regulation of IL-12, and even more so of IL-23, in association with IFN-γ overexpression
(Figure E), indicating that IFNγ overexpression leads to a reduction of the Th17 response.
Moreover, macrophages were diminished in IFN-γ mice compared to WT mice, in H. felis
infected IFN-γ mice compared to H. felis infected WT mice, and in IL-1β/IFNγ mice
compared to IL-1β mice (Supplementary Figure 4C), indicating that IFN-γ in our model
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, qPCR showed upregulated FAS expression in the
gastric mucosa (Supplementary Figure 5C). The gene expression of IFN-γ downstream
target genes TRAIL and XAF1, was also slightly increased (1.5–3 fold) in H+/K+-ATPase-
IFN-γ compared to WT mice (Supplementary Figure 5A 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 homolog of
yeast Atg8, were significantly increased in the stomachs of H+/K+-ATPase-IFN-γ mice
compared to WT mice (Figure 5A). With H. felis infection, inducing similar tissue-levels of
IFN-γ, the number of LC3-II positive cells was also increased in WT mice (Figure 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 to WT mice, with or
without H. felis infection (Figure 5B) and were higher in IL-1β/IFN-γ than in the IL-1β mice
(Figure 5C, Supplementary Figure 6A). 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 (Figure 5D) in contrast to IL-1β mice where 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 (20ng/ml) for 24h or 48h, which resulted in characteristic
morphologic changes of autophagy. The percentage of GFP-LC3-II dots (Figure 5E and G)
and protein expression (Figure 5F), was significantly increased in IFN-γ treated cells,
compared to 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 (Figure 6B) and protein
(Figure 6A) levels in the stomachs of mice with or without H. felis infection. IHC staining
localized Beclin-1 protein to epithelial cells (Figure 6A and Supplementary Figure 6A) and
overexpression of IL-1β correlated with inhibited expression of Beclin-1 in the stomach
(Supplementary Figure 6A and B). Beclin-1 upregulation correlated with the induction of
autophagy in our mouse model. Indeed, knockdown of Beclin 1 expression by siRNA in
MKN-28 cells reduced IFN-γ-induced autophagy (Figure 6C-E), confirming a direct
mediation of Beclin-1 in IFN-γ-induced autophagy. Moreover, Beclin 1 appeared to be a direct target of IFN-γ, since IFN-γ activated Beclin 1 promoter activity (Figure 6F). Using the transcriptional regulatory element database (http://rulai.cshl.edu/TRED), we located Gamma interferon 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 1000bp of the Beclin1 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**

While 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 Figure 7A) and serum (data not shown) in all infused mice. Two weeks post-cessation 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 Figure 7B). The short-term IFN-γ infusion did result in reduced cell proliferation (Ki-67 staining) (Figure 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 Figure 7B), 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 (Figure 7A and B), induced autophagy in gastric epithelial cells (Figure 7A and D), and upregulated the expression of Beclin-1 (Figure 7E). Finally, IFN-γ infusion inhibited the production of IL-1β, TNF-α and IL-6 in all treated mice (Supplementary Figure 7C).

**DISCUSSION**

While 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 pre-neoplasia (3). Here, we show that IFN-γ overexpression is able to suppress gastric cancer in models of IL-1β- and H. felis-dependent carcinogenesis. Our studies involving both transgenic overexpression and exogenous infusion demonstrate that IFN-γ is able to 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 (I) induces CD4 T cell apoptosis, resulting in reduced Th1 and Th17 cytokine responses, and (II) induces autophagy in gastric epithelial cells through upregulation of Beclin-1. Finally, (III) 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 appeared to be Fas-dependent, confirming prior reports (35, 36). 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 (37). IFN-γ can inhibit the development of Th17 cells, and recent studies have pointed to Th17 cells as playing 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, 38), while inhibition of the Th17 response prevents gastric dysplasia (39). Interestingly, macrophages, representing the innate immune response, also appeared 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.

Since 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 appears 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 IFN-γ’s role in protection from pathogens, since *Helicobacter* can occasionally be found intracellularly (40), and published reports have indicated that autophagy represents an important mechanism for elimination of intracellular pathogens (29) independent of apoptosis (41). This new mechanism should be considered in Helicobacter infection, especially since 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). While Nod1 and Nod2 have been shown to comprise potential intracellular signals for triggering autophagy and bacterial elimination (42), 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 (43). 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 to uninfected IL-1β mice. Moreover, the switch to autophagy was associated with decreased epithelial cell apoptosis, a critical factor in gastric carcinogenesis (44).

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, 45). Beclin-1 expression was upregulated by IFN-γ overexpression or infusion and was directly associated with the induction of autophagy in gastric epithelial cells. Computer-based searches were able to identify interferon-responsive GAS elements in the Beclin-1 promoter, suggesting binding motifs for direct regulation through IFN-γ activated STAT1, as shown previously (46). Since 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 (47), 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 DCAMKL1, was strongly associated with inflammation-related carcinogenesis and preceded the development of gastric cancer. Although formal lineage tracing has not been reported, Dclk1 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 (48).

In summary, overexpression of IFN-γ inhibits H. felis- and IL-1β-dependent gastric inflammation and carcinoma. While previous studies have shown that tissue-specific IFN-γ transgenic mice developed severe chronic inflammation, including chronic active hepatitis (49), no IFN-γ transgenic mouse model has yet demonstrated development of carcinoma (49). 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.

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References


Figure 1. H/K-ATPase IFN-γ mice fail to develop spontaneous gastric metaplasia

(A) The IFN-γ construct contains the mouse H+/K+-ATPase-s subunit gene and murine IFN-γ cDNA. (B) ELISA determined murine IFN-γ in stomachs of 4-month-old H+/K+-ATPase-IFN-γ mice and control mice. Data are mean ± SD of 10 mice. (C) Gene expression levels of mouse IP-10 and Mig mRNA in the stomach. (D) Representative macroscopic and histologic appearance of IFN-γ transgenic mouse stomach at 24-month
Figure 2. Overexpression of IFN-γ alleviates development of *H. felis*-induced gastric inflammation and neoplasia

Representative photomicrographs of *H. felis* infected H+/K+-ATPase-IFN-γ mouse stomach at (A) 12 months and (B) 24 months. (C) Representative macroscopic pictures of *H. felis* infected H+/K+-ATPase-IFN-γ and WT mice at 12 months (D) Histopathologic scores for stomachs of indicated mice (data represent the mean ± SD of 20 mice (*p< 0.05, WT vs. IFN-γ mice (both lines)). (E) Overexpression of IFN-γ does not affect the colonization of *H. felis* in the stomach. Stomach sections from H+/K+-ATPase-IFN-γ and WT mice infected for 2 months were stained with H&E. Arrow and insert indicate *H. felis* localization in the lumen of gastric glands. (F) Quantification of *H. felis* DNA in the stomach from indicated mice by PCR. The data represent the mean ± SD of 5 mice.
Figure 3. Overexpression of IFN-γ inhibits IL-1β-induced gastric inflammation, cell proliferation and progenitor cell expansion

12-month-old uninfected mice (A) and H. felis-infected mice (B) Proliferation index was calculated using the number of positive epithelial cells per total epithelial cell number per gland in 10 different high power fields (×400). The presented data represent the mean ± SD of 5 mice (*p < 0.05). (C) Representative macroscopic pictures of IL-1β mice and IL-1β/H⁺/K⁺-ATPase-IFN-γ stomachs at 12 months.
Figure 4. Overexpression of IFN-γ induces apoptosis of T cells and inhibits the production of pro-inflammatory cytokines

(A) TUNEL staining of stomach sections from 12-month old mice showing percentage of TUNEL+ cells in different locations from each of the groups (n = 5). All data represent the mean ± SD. *P < 0.05. (B) Apoptotic epithelial cells were assessed in H&E stained sections and Caspase-3 stained cells were counted in 10 high power fields and numbers are presented as mean ± SD. *P < 0.05. (C) Immune cells isolated from the indicated mice were stained with PerCP-CD4, APC-CD8, TITC-Annexin V and PI and analyzed by FACS (n=5) to analyze apoptosis. (D-E) mRNA expression levels of cytokines in 12 month-old mice, as indicated.
Figure 5. Overexpression of IFN-γ induces autophagy in gastric epithelial cells in vivo

(A) Immunohistochemical staining for LC3 in stomach sections from 12-month-old mice, as indicated. Representative results from 8 different groups are shown (original magnification, × 200). (B and C) Western blot showing expression of LC3 in the stomach tissue of indicated mice. (D) Accumulation of LC3-II due to increased autophagosome formation after chloroquine treatment (30mg/kg for 10 days intraperitoneal). (E) Gastric cancer MKN-28 cell line was transfected with GFP–LC3 and then treated with 40μg IFN-γ for 24 hours and 48 hours. (original magnification, × 400). (F) IFN-γ increased LC3-II expression in MKN-28 cells after treatment with 40μg IFN-γ for 48 hours. GFP–LC3 was detected by Western blot. (G) GFP–LC3-positive punctate dots per cell. Data are from 100–200 cells from three independent experiments. Data are mean ± SEM, n = 100–200 cells from three independent experiments; * P < 0.01.
Figure 6. Overexpression of IFN-γ induces autophagy via Beclin 1 in gastric cancer cells in vitro

(A) Immunohistochemical staining for Beclin 1 in stomach sections from 12-month-old mice. Representative photomicrographs are shown (original magnification, × 200). (B) mRNA expression of Beclin-1 in the stomach from indicated mice, normalized to GAPDH expression. The data represent the mean ± SD of 5 mice. (*p < 0.001, vs. indicated group of mice). (C) MKN-28 cells were transfected with control and Beclin 1 siRNA for 6 hours and cultured with or without IFN-γ 20ng/ml for another 48 hours. (E) Downregulation of Beclin-1 expression by siRNA decreased IFN-γ-induced autophagy in MKN-28 cells. *p<0.05. (F) IFN-γ activated the promoter activity of Beclin 1 in MKN-28 cotransfected with the indicated Beclin 1-promoter plasmids or empty pGL3 vector and Renilla luciferase vector for 6 h before IFN-γ treatment. Data are shown as means ± SD of 3 independent experiments. *p < 0.05.
Figure 7. IFN-γ infusion inhibits Dclk-1+ cell expansion and induces autophagy

(A) Representative stomach sections from indicated mice infused with IFN-γ for 4 weeks were stained with H&E (first lane), Ki-67 (second lane), DCAMKL-1 (Dclk-1) (third lane) and LC3 (Fourth lane) (original magnification, ×400). (B-D) Quantification of Ki-67-, DCAMKL-1-and LC3-positive cells in indicated mice with and without IFN-γ infusion. The percentage of positive epithelial cells was calculated using the number of positive cells to total cell number per field at 10 different locations in each stomach under light microscopy (×400). The presented data represent the mean ± SD of 5 mice (*p < 0.05, vs. infused mice). (E) mRNA expression levels of Beclin1 from indicated mice was measured by qPCR and normalized to GAPDH expression. The data represent the mean ± SD of 5 mice (*p < 0.01, vs. control group mice).