Germ-Line p53-targeted Disruption Inhibits Helicobacter-induced Premalignant Lesions and Invasive Gastric Carcinoma through Down-Regulation of Th1 Proinflammatory Responses

James G. Fox,2 Barbara J. Sheppard, Charles A. Dangler,3 Mark T. Whary, Melanie Ihrig, and Timothy C. Wang

Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 [J. F. G., B. J. S., C. A. D., M. T. W., M. F.], and University of Massachusetts Medical Center, Worcester, Massachusetts 01605 [T. C. W.]

ABSTRACT

p53 is a tumor suppressor gene that is mutated in many human malignancies, including gastric cancer. It remains unclear why patients with germ-line p53 mutations (i.e., Li-Fraumeni syndrome) are not at increased risk for gastric adenocarcinoma, despite the fact that they show a high rate of many other tumors. Furthermore, the precise relationship between germ-line p53 mutations and the response to chronic bacterial infections (such as Helicobacter spp.) has not been investigated. To assess the role of germ-line p53 deletions in modulating the progression to gastric cancer, p53+/− and wild-type (WT) C37BL/6 mice were infected with H. felis. The gastric pathology and immune response in these two groups of mice were analyzed for up to 15 months postinfection. The gastric fundus and antrum were evaluated independently using a 0 – 4 scale to score inflammation, parietal and chief cell loss, mucus metaplasia, and helicobacter colonization. Nonparametric statistical analysis was performed to determine the effects of p53+/−, infection status, and postinoculation (p.i.) time on inflammation, preneoplastic changes, invasive lesions, and helicobacter colonization. mRNA expression for γIFN, interleukin (IL)-1, IL-10, and IL-4 was quantified by PCR. Sera were also evaluated for H. felis antibody by ELISA. Antral inflammation increased significantly with time in infected mice. There was a significant, protective effect on the development of preneoplastic fundic lesions and invasive carcinoma attributable to the deletion of one p53 allele (P < 0.05). Submucosal invasive foci were observed in 9 of 11 WT-infected mice ranging from 13 to 15 months p.i.; invasion of adjacent submucosal blood vessels by glandular epithelia also was present in 5 of these mice. None of these lesions were observed in 33 p53+/− mice, infected or not, at any time p.i. p53+/− mice had significantly higher helicobacter colonization consistent with a Th2 host response. In sera from WT mice, IgG2a, considered a proinflammatory Th1 response, continued to rise throughout the 15-month study (P < 0.004). In contrast, IgG2a levels of the p53+/− mice were 50–60% lower than those of the WT mice at each time point (P range, <0.012 to 0.002) and did not progress in magnitude between 12 and 15 months of chronic H. felis infection (P = 0.167). mRNA levels for γIFN and IL-1 were significantly upregulated in WT mice infected with H. felis (P < 0.05) but were slightly elevated or were at background levels in p53+/− mice. IL-10 and IL-4 mRNA expression was not significantly different from control samples. Our results support the hypothesis that germ-line deletion of one p53 allele results in a down-regulated Th1 response to gastric helicobacter infection, possibly because of Th-cell senescence, which may indirectly protect against the development of gastric cancer and other epithelial-derived neoplasms associated with chronic inflammation.

INTRODUCTION

As a transcription factor, p53 has clearly defined roles in the regulation of proliferation, apoptosis, and genomic repair. In addition, p53 controls the onset of cellular senescence, a process that limits the number of times that a cell can potentially divide. Thus, it is clear that p53 has a number of different properties that can contribute to the suppression of tumorigenesis, and the p53 gene is frequently deleted or mutated during the process of neoplasia. Mutations in the p53 gene are among the most common genetic alterations in human tumors, with estimations that more than 50% of human cancers contain mutations in this gene (1). Despite extensive databases cataloguing these mutations in multiple cell types, the precise function of p53 has not been fully elucidated from cell type to cell type. Gastric cancer is among the leading causes of cancer-related deaths in the world. Although declining in the United States, gastric cancer remains the second leading cause of cancer-related mortality and the 14th leading cause of death worldwide. As is the case for many other tumors, p53 appears to be the most commonly mutated tumor suppressor gene in gastric cancer. p53 mutations have been detected in over 60% of advanced gastric cancers, as well as in intestinal metaplasia (38%), gastric dysplasia (60%), and early gastric cancer; and these targeted mutations are associated with a generally worse prognosis (2–7).

Although p53 mutations are common in most sporadic gastric cancers, gastric cancer does not seem to be significantly increased in patients with germ-line p53 mutations (Li-Fraumeni syndrome). Most large studies have noted only a limited spectrum of tumors in patients with germ-line p53 mutations. Excess incidence of tumors in these patients is generally confined to six cancer types, breast carcinomas, soft tissue sarcomas, osteosarcomas, leukemia, brain tumors, and adrenocortical carcinomas, whereas additional component tumors have failed to be implicated (8). Given the likely importance of p53 mutations in gastric cancer progression, the absence of gastric tumors as part of the Li-Fraumeni syndrome remains puzzling. Unlike most other tumors, gastric cancer is characterized by a strong association with chronic Helicobacter pylori infection. This bacterial pathogen induces gastric cancer, primarily by inducing chronic, persistent inflammatory response that accelerates remodeling of the gastric epithelium and glandular loss (gastric atrophy) followed by metaplasia, dysplasia, and progression to gastric cancer (9, 10). The development of neoplasia has in fact been associated with a number of chronic inflammatory conditions, although the precise relationship between inflammation and tumor development remains largely obscure at the molecular level.

In a previous study, we infected WT4 and p53+/− hemizygous knockout mice with Helicobacter felis and followed the mice for up to 1 year postinoculation (11). These studies demonstrated that H. felis infection of p53+/− mice resulted in increased but not statistically significant gastric epithelial proliferation (bromodeoxyuridine labeling) when compared with infected WT control mice. Similar degrees of inflammation and colonization were observed in the two helicobacter-infected groups, and after 1 year of observation, progression to gastric cancer was not found (11). However, the possibility that...
neoplasia might develop after a longer observation period could not be excluded.

To test this possibility, we undertook a 15-month study of \textit{H. felis}-infected p53\textsuperscript{+/−} mice. Surprisingly, this study revealed that p53 mutations seem to protect against the development of atrophy and cancer. This protection appears to be associated with down-regulation of immune responsiveness, particularly Th1-associated pathology secondary to \textit{H. felis} infection.

**MATERIALS AND METHODS**

**Animals.** Forty-six (25 female, 21 male) 3–4-month-old hemizygous TSG-p53\textsuperscript{−/−} mice, deficient in one of the p53 genes, were obtained from Taconic. The TSG-p53 mice were generated in 129-derived embryonic stem cells (A31) and have been backcrossed onto a C57BL/6 background. Thirty 12-week-old C57BL/6 (15 male, 15 female) mice were also obtained from Taconic as controls. The mice were all maintained in an Association for Assessment and Accreditation of Laboratory Care, International-approved facility under barrier conditions on 5% lysed horse blood agar. The bacteria were harvested and stored at −70°C. Prior to use, aliquots were thawed, analyzed for motility, and cultured for evidence of aerobic or anaerobic bacterial contamination.

**Experimental Infection.** Of the 46 TSG-p53 mice, 33 (15 male, 18 female) were inoculated with \textit{H. felis} and 13 (6 male and 7 female) as controls. Of the 30 wild-type C57BL/6 mice, 20 (10 male, 10 female) were inoculated with \textit{H. felis} and 10 (5 male and 5 female) were controls. Brain heart infusion broth containing \textasciitilde 10\textsuperscript{10} colony-forming units of \textit{H. felis} per ml was used as inoculum. The inocula (0.5 ml) were delivered by gastric intubation to each test mouse three times at 2-day intervals by using a sterile paraffin-embedded莪 dalle 小管组织 seçeneğini altı ve üstün bağışıklık ürolojisi, as follows: none (score of 0); sparsely colonized glands (score of 1 or 2); and those containing dense aggregates of bacteria (score of 3 or 4).

**Statistical Evaluation.** Histological scores were statistically analyzed using nonparametric Wilcoxon rank-sum (\(P < 0.05\)) testing to determine significant differences among groups. ELISA values were analyzed using Student’s \(t\) tests. Correlation between the humoral immune response and the assessment of lesions and number of \textit{H. felis} organisms was performed using a Bonferroni correction.

**RESULTS**

Pathology. Uninfected WT and p53\textsuperscript{+/−} mice had no significant gastritis with median gastric lesion scores of zero. \textit{H. felis}-infected mice demonstrated many significant differences by strain within the lesion categories of inflammation, fundic gland atrophy, glandular hyperplasia with mucoid metaplasia, gastric cancer, and colonization levels (Fig. 1). Atrophy and lymphoid follicle development scores for the fundus of uninfected mice of both strains were zero. At 8–11.5 months p.i. but not at 12–15 months p.i., \textit{H. felis}-infected C57BL/6 mice had greater fundic lymphoplasmacytic infiltration (\(P = 0.0062\)) and submucosal lymphoid follicle formation (\(P = 0.0146\)) than did p53\textsuperscript{+/−} mice (Fig. 2, A and B). There were no

Table 1 Longitudinal study of \textit{H. felis}-infected p53\textsuperscript{−/−} mice and WT C57BL/6 mice\textsuperscript{a}

<table>
<thead>
<tr>
<th>Infection status at age</th>
<th>H. felis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>8–11.5 mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. felis</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>p53\textsuperscript{+/−}</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Total (n)</td>
<td>14</td>
<td>6</td>
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\(\textsuperscript{a}\) Several mice in the p53\textsuperscript{+/−} groups died or were killed because of lymphoid tumors and were not included in the final analysis.

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significant differences in antral lymphoplasmacytic infiltration or lymphoid follicle development between WT and p53<sup>−/−</sup> mice at the early time point. At the later time point, WT mice had significantly greater lymphoplasmacytic inflammation than did the p53<sup>−/−</sup> mice (P = 0.0469). There were also no significant differences between the infected groups in granulocytic infiltrates within the fundus or antrum at either time point.

At 12–15 months p.i., H. felis-infected C57BL/6 mice had significantly greater fundic atrophy of parietal and chief cells (P = 0.0477; Fig. 2, C and D). Infected mice of both genotypes experienced fundic hyperplasia; however, C57BL/6 mice consistently demonstrated extensive areas of remarkably abnormal mucosa that were not observed in p53<sup>−/−</sup> mice. Areas with the most severe hyperplasia and metaplastic alterations in C57BL/6 mice often formed multiple cystic chambers with extension into the submucosa (Fig. 2I). The degree of loss of the normal mucosal architecture and cellular atypia associated with these areas was suggestive of invasive carcinoma; however, diverticulosis or gastritis cystica profunda of noncarcinomatous lesions could not be ruled out based on morphology alone. Five of the nine cases with invasion into the submucosal stroma also demonstrated well-demarcated intravascular cellular aggregates within adjacent submucosal vessels (Fig. 2G). The epithelial character of the intravascular cellular aggregates was verified by positive immunohistochemical identification using a pancytokeratin antibody (Fig. 2H), which confirmed that these foci represented extension of the abnormal mucosal tissue into the adjacent vasculature. p53<sup>−/−</sup> mice did not develop preneoplastic lesions or lesions characterized by invasion into the submucosal stroma or vasculature.

Colonization of the pylorus and fundus at 8–11.5 months p.i. was similar for both WT and p53<sup>−/−</sup> mice. At 12–15 months p.i., however, the difference in colonization of H. felis within pyloric glands was significant (P < 0.019) with greater colonization in the p53<sup>−/−</sup> mice (Fig. 2, E and F). Colonization of the fundic glands (P = 0.0480) also differed significantly at this later time point.

Serum IgG2a and IgG1 Humoral Responses to H. felis. Anti-H. felis serum IgG2a and IgG1 from infected mice significantly increased to above control levels by 2 months p.i. (P < 0.006; Fig. 3). In sera from WT C57BL/6 mice, the IgG2a response, which is
Fig. 2. Comparison of gastric lesions in infected C57BL/6 and p53−/− mice. A and B, 8–11.5-month p.i. fundic lymphoplasmacytic inflammation and follicle formation are on average greater in C57BL/6 (A, ×40; inset, detail showing predominately lymphoplasmacytic infiltrates at ×400) than that in p53−/−, for which one of the most severely affected region is illustrated (B, ×100). Glandular atrophy with atypical hyperplasia and metaplasia also are present to varying degrees (C and D). C and D, within the fundic glands, atrophy of parietal and chief cells and the associated hyperplasia with mucoid metaplasia are greater in C57BL/6 (C, ×100; inset, detail showing preservation of parietal cells at ×400). E and F, 12–15-month p.i. antral glands have much more extensive colonization by silver-positive H. felis in p53−/− (E, Warthin-Starry, ×200; inset, detail showing dense colonization of glands with H. felis at ×1000) than C57BL/6 (F, Warthin-Starry, ×200; inset, detail showing negligible colonization at ×1000). G and H, 12–15-month p.i. invasion of markedly abnormal mucosal tissue into the submucosa including extension into endothelium-lined spaces was observed only in C57BL/6, shown on H&E-stained sections (G, ×100; inset, detail showing tumor cell focus within a dilated erythrocyte-filled submucosal blood vessel at ×400) and on immunohistochemical sections labeled with pancytokeratin antibody verifying epithelial character of the intravascular foci (H, ×100; insets, detail showing pancytokeratin Ab-labeled tumor cell focus within a dilated erythrocyte-filled submucosal blood vessel at ×400). I, 12–15-month p.i. submucosal extension of the markedly abnormal mucosal tissue associated with the formation of multiple dilated cystic spaces was observed only in C57BL/6 (×40).
C57BL/6 mice were 29-fold higher (95% CI, 27.8–30.2) compared with that observed in WT C57BL/6 mice at 2 months p.i. The serum IgG1 response of p53+/− mice was significantly higher and increased over time, compared with that in the p53−/− mice (P < 0.02–0.002). The serum IgG1 response in WT mice was similar to the IgG1 response of p53+/− mice at 2 months p.i. but was 40–50% higher in WT mice at later time points (P < 0.02–0.003).

The IgG2a response of the H. felis-infected WT and p53+/− mice was positively correlated with the extent of fundic mononuclear cell infiltrates (r² = 0.603; P = 0.005) and the degree of atrophy in the fundus (r² = −0.52; P = 0.02) and was negatively correlated with the number of H. felis colonizing the antrum (r² = −0.61; P = 0.004). The IgG1 response was not significantly correlated with any pathology assessment or the number of H. felis organisms.

Cytokine mRNA Expression in Gastric Samples. Proinflammatory γIFN and IL-1 mRNA expression in H. felis-infected C57BL/6 mice were both significantly higher than in p53+/− H. felis-infected mice (P < 0.05). γIFN mRNA levels in H. felis-infected C57BL/6 mice were 29-fold higher (95% CI, 27.8–30.2) compared with uninfected controls and were 4.4-fold higher (95% CI, 3.5–5.2) in p53+/− H. felis-infected mice compared with uninfected controls. IL-1 mRNA expression levels in H. felis-infected C57BL/6 were 3.5-fold higher (95% CI, 3.0–4.1) and were at background levels in p53+/− H. felis-infected mice (0.72 fold; 95% CI, 0.2–1.3) when compared with control samples. mRNA expression for the Th2-associated anti-inflammatorcy cytokines of IL-4 and IL-10 were only slightly elevated or were unchanged, respectively, in H. felis-infected C57BL/6 or p53−/− mice compared with controls, without statistical significance (data not shown).

**Discussion**

This study describes for the first time the occurrence of helicobacter-associated gastric adenocarcinomas in WT C57BL/6 mice. We characterized the lesions as early invasive carcinomas based on the consistent identification of markedly abnormal morphology and invasion of pancytokeratin-positive mucosal cells into the submucosal vessels. These gastric tumors are identical to those diagnosed in INS/GAS mice infected with *H. felis* (15). The C5BL/6 tissues also were found to consistently display a much greater degree of the mucosal preneoplastic changes that previous literature has consistently defined as fundic gland atrophy and associated development of hyperplastic glands with a mucoid morphology (11, 13, 14). This finding supports the epidemiological role of *H. pylori* in human gastric cancer and augments experimental studies in gerbils demonstrating that *H. pylori* infection causes gastric adenocarcinoma (17). We also report that the inflammatory response to *H. felis* when one p53 allele is deleted leads to an altered gastritis and inflammatory cytokine pattern and diminished frequency of invasive gastric epithelial lesions, as well as gastric carcinoma and depressed immune responsiveness in TSG p53−/− mice compared with that observed in WT C57BL/6 mice. In a previous study, we reported that *H. felis*-infected p53 hemizygous mutant mice showed a similar degree of chronic atrophic gastritis of the gastric corpus and a trend for increased glandular proliferative response when compared with infected WT mice (11). The finding in the present study that *H. felis*-infected p53−/− mice (up to 15 months p.i.) show significantly less neoplastic progression was unexpected. This finding is contrary to what might have been predicted solely on the basis of the presumed relationship between mutant p53 and cancer in epithelial cells. However, these discrepant observations can be explained by the apparent greater relative depression of a *H. felis*-associated Th1 phenotype in p53−/− mice compared with the greater inflammatory response of WT mice to chronic *H. felis* infection.

A number of observations support the current paradigm that *H. pylori*-mediated progression to gastric atrophy and cancer depends to a large extent on a strong proinflammatory Th1 immune response. In mouse models, chronic infection with *H. felis* in the C57BL/6 background results in an intense Th1 response, as demonstrated using serology for the IgG2a humoral response and reverse transcription-PCR analysis of tissue cytokines, and is associated with marked atrophy of the glandular epithelium (13). In the present study, we show that such atrophy progresses in 80% of infected C57BL/6 mice to invasive lesions with penetration into the submucosa and, in selected cases, with invasion into endothelial lined vessels.

The literature suggests that total IgG systemic antibody responses to gastric helicobacter infections are not predictive of the severity of gastric lesions. However, in our study, the relationship between the Th1 response to *H. felis* infection and the severity of gastritis was supported by the positive correlation between the Th1-associated IgG2a response and the extent of mononuclear inflammatory infiltrate and atrophy in the fundus. In addition, the IgG2a response was significantly negatively correlated with the number of colonizing *H. felis*, as reported previously (13, 18).

Infected WT mice had a high γIFN response, which is known to promote MHC Class I (endogenous) and Class II (exogenous) antigen presentation. Thus, increased γIFN promotes chronic inflammation in the WT mice in response to *H. felis* infection with an associated higher risk of genetic mutations inherent in the hyperplastic response. Increased antigen presentation of helicobacter antigens (Class II) and self-antigens (Class I) would help promote tissue damage and secondary DNA damage from inflammatory mediators (19). This Th1 response to *H. felis* becomes polarized toward a Th2 response when

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**Fig. 3.** A, serum IgG2a response (mean ± SE) to *H. felis* in WT and p53+/− mice (n = 10–20 of each genotype at each time point) over 15 months of experimental infection. The response in WT mice was significantly higher and increased over time, compared with that in the p53−/− mice (P < 0.01–0.002). B, serum IgG1 response (mean ± SE) to *H. felis* in WT and p53+/− mice (n = 10–20 of each genotype at each time point) over 15 months of experimental infection. The response in WT mice was similar to the IgG1 response of p53+/− mice at 2 months p.i. but was 40–50% higher in WT mice at later time points (P < 0.02–0.003).
mice are concurrently infected with the murine nematode *Heligmosomoides polygyrus*. This Th2 polarization is associated with a marked reduction in gastric atrophy despite high *H. felis* colonization (13). There is a general correlation in selected inbred mouse strains between Th1 responses [IL-1, IFN, tumor necrosis factor (TNF)] and progression to gastric atrophy in response to helicobacter infection. In support of this Th1/gastric cancer paradigm, recent reports by El-Omar *et al.* (20) have indicated that the development of atrophy and achlorhydria in human patients is associated with an IL-1β gene polymorphism that would be expected to result in higher levels of IL-1β production.

The finding of attenuated gastric pathology secondary to *H. felis* infection in the p53+/− atrophy may in part be explained by accelerated aging of the immune system in association with the altered p53 status. Induction of p53 expression has been reported to be impaired in activated T cells from elderly individuals (21). An age-related decline in immune function has been associated with an accumulation of memory phenotype T cells, potentially resulting in a Th1/Th2 imbalance. Age-related alterations in production of cytokines include higher IL-4 and IL-10, with lower IL-2 production in aged WT mice (22–24). This Th2 shift, which is associated with senescence, appears to be even more exaggerated in p53 mutant mice. A recent study reported that the accumulation of memory T cells was spontaneously accelerated in p53−/− mice (25). In addition, measurement of induced cytokine production in p53−/− mice showed higher expression of IL-4, IL-6, and IL-10 after stimulation of the T-cell receptor and in response to antigenic stimulation, whereas expression of IL-2 remained unaltered (25). We did not use p53−/− mice in this study because of their limited life span associated with a high incidence of spontaneous tumors, particularly lymphoma (26).

Although a functional decline in activity of the immune system observed with aging might confer an increased risk for certain forms of tumor because of a lack of immune surveillance, our data suggest that immune senescence may actually protect against progression to gastric atrophy and cancer when the lesions are secondary to *Helicobacter* spp. infection. In addition, our data support the hypothesis that cells with a deleted p53 allele may have very different consequences depending on the cell type carrying such mutations. The finding of increased proliferative index at earlier time points in the infected p53−/− mice supports the hypothesis that p53-targeted disruptions in the glandular epithelial compartment may predispose to gastric adenocarcinoma. However, the altered pattern of inflammation and lack of invasive gastric lesions in these animals are consistent with a protective role conferred by a p53 gene deletion in the lymphocyte population.

Thus, with respect to the Li-Fraumeni syndrome, in which p53 mutations are germ-line and present in all cell types, the notion of an “immune phenotype” might explain why certain types of tumors, such as gastric cancer, are not clearly found in excess in such individuals. Our data supports the hypothesis that germ-line p53 mutations do not accelerate tumorigenesis in those tissues in which neo-epigenetic progression is heavily dependent on Th1 inflammatory responses to helicobacter infection and may, in fact, be protective against such tumors.

The association between germ-line mutations in tumor suppressor genes and altered immune responses, noted for p53−/−, may, in fact, represent a more general paradigm that applies to other tumor suppressor genes. For example, in a recent study, we showed that Apc 1638 mice with a targeted disruption of the Apc allele had a similar decrease in epithelial proliferation, immune responsiveness, and inflammation compared with WT mice (18). The finding of higher bacterial and urease scores in the Apc 1638 mice observed at earlier (4.5–7.5 months) time points also suggested a Th2 shift (13, 18). The serum IgG levels of *H. felis* in Apc+/− mice were diminished when compared with those in WT mice as was the Th1 immune response (IgG2a) (18). Thus, alterations in immunological responses to bacterial pathogens affecting the gastric mucosa may be a general phenomenon associated with germ-line mutations in tumor suppressor genes that warrants further study.

In conclusion, our results suggest two distinct roles for p53 within the gastric epithelium. Deletion of one p53 allele leads initially to increased proliferation within the epithelial compartment, which by itself might be associated with a heightened cancer risk. However, also significantly over time, p53 mutations, presumably within the lymphocyte compartment, result in immune senescence and a decreased immune responsiveness, particularly in the progression of gastric lesions. The decrease in inflammation leads to the slowing of progression to gastric atrophy and a decreased progression to cancer. However, further validation of this hypothesis will require segregating the influences of the p53 genotype on the gastric epithelial and immune cell populations, either through application of immune cell reconstitution experiments or targeted gene knockout studies. Although the helicobacter-associated gastric tumors that we have described in this mouse model do not fulfill all of the histological criteria of gastric carcinoma in humans, such as penetration of carcinoma into the muscular layers and serosa with metastasis to regional lymph nodes, our findings will provide a useful *in vivo* model to study mechanisms involved in gastric carcinogenesis.

REFERENCES


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