Chronic inflammatory interferonγ-signaling suppresses hepatocarcinogenesis in mice by sensitizing hepatocytes for apoptosis

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Abstract

Chronic liver inflammation is a critical component of hepatocarcinogenesis. Indeed, inflammatory mediators are believed to promote liver cancer by upholding compensatory proliferation of hepatocytes in response to tissue damage. However, inflammation can also mediate the depletion of malignant cells, but the difference between tumor-suppressive and tumor-promoting inflammation is not defined at the molecular level. Here, we analyzed the role of the major inflammatory mediator interferon-γ (IFNγ) in chemical hepatocarcinogenesis of transgenic mice that over-express IFNγ in the liver; these mice manifest severe chronic inflammatory liver damage and lasting compensatory regeneration. We found that chronic exposure to IFNγ suppressed chemical hepatocarcinogenesis, despite overt liver injury. Indeed, IFNγ-transgenic mice had significantly fewer and significantly less advanced malignant lesions than non-transgenic mice. This tumor-suppressive effect of IFNγ seemed to be mediated in part by its known immune activating function, indicated by infiltration of IFNγ-transgenic livers with CD8 T cells, NKT and NK cells. However, IFNγ seemed to prevent carcinogenesis also by activating the cell-intrinsic p53 tumor suppressor pathway. Indeed, exposure to IFNγ in vivo or in vitro was associated with accumulation of p53 in hepatocytes and the sensitization of hepatocytes to apoptosis induced by genotoxic stress. The IFNγ-induced increase in apoptosis of hepatocytes seemed to be p53-dependent. Thus, chronic inflammation dominated by IFNγ may prevent hepatocarcinogenesis, despite continued inflammatory liver injury and regeneration. Therefore, the carcinogenic potential of inflammation seems to be determined by type and composition of its mediators, and manipulating the type of chronic inflammation may serve the prevention of cancer.
Introduction

Chronic inflammation of tissue is believed to predispose to the formation of cancer\textsuperscript{1}. Hepatocellular carcinoma (HCC), which is one of the five most common and most lethal tumors worldwide\textsuperscript{2,3}, may serve as a paradigm for inflammation-induced cancer. Indeed, HCC develops most frequently on grounds of cirrhosis after years of chronic hepatitis, independent of what had caused this condition\textsuperscript{2,3}. However, how chronic inflammation is linked to carcinogenesis at the molecular level is not entirely clear. Recent findings indicate that inflammatory mediators, which cause tissue injury, may promote carcinogenesis by inducing compensatory proliferation and regeneration\textsuperscript{4,5}, and the chronic stimulation of aberrant cells with inflammatory mediators, such as interleukin-6\textsuperscript{6,7} or TNF$\alpha$\textsuperscript{8} was found to promote carcinogenesis.

A major inflammatory pathway that may modulate carcinogenesis is the IFN$\gamma$/STAT1 pathway\textsuperscript{9}. However, the role of IFN$\gamma$, which is secreted by inflammatory cells, in carcinogenesis is not clear. On the one hand, IFN$\gamma$ signaling has been shown to suppress carcinogenesis in vivo, since a lack of IFN$\gamma$ receptors or STAT1 molecules, which are the major signal transducers of IFN$\gamma$, promotes carcinogenesis\textsuperscript{10-13}. It is believed that the anti-carcinogenic activity of IFN$\gamma$ is mediated mainly by enhancing tumor cell immunogenicity and cancer immunosurveillance\textsuperscript{12}. On the other hand, however, sustained STAT1-signaling was reported to be associated with the development of liver cancer\textsuperscript{14}. Indeed, a lack of or silencing of SOCS-1, which is an endogenous inhibitor of the IFN$\gamma$/STAT1 pathway\textsuperscript{15}, was found to promote IFN$\gamma$-dependent carcinogenesis\textsuperscript{16-18}. However, SOCS-1 also interferes with other cytokine pathways, notably with the interleukin-6/STAT3 pathway; therefore, the exact role of sustained IFN$\gamma$-signaling in hepatocarcinogenesis is not established.
To explore the role of IFNγ in hepatocarcinogenesis, we used the model of IFNγ-transgenic mice that over-express IFNγ in the liver under control of the human serum amyloid P component gene promoter19. These mice manifest significant hepatic IFNγ levels, life-long chronic hepatitis, continuous liver injury and consecutive chronic regeneration19; it is believed that such chronic state of regenerative hepatocyte proliferation under inflammatory stress constitutes a major risk factor for hepatocarcinogenesis4,5. Since these mice are not prone to spontaneous liver cancer, we induced chemical hepatocarcinogenesis by application of diethylnitrosamine (DEN) and phenobarbital4.

Despite chronic inflammation and sustained IFNγ signaling, the IFNγ-transgenic mice were resistant to chemically induced liver cancer. This protection from liver cancer was associated with IFNγ-induced accumulation of the p53 molecule and the sensitization of damaged hepatocytes to apoptosis. Our findings indicate that 1) the activation the p53 pathway and apoptosis of damaged hepatocytes by IFNγ may be a relevant mechanism that limits hepatocarcinogenesis, and 2) chronic inflammation dominated by IFNγ can protect from liver cancer, despite continued compensatory regeneration of inflammatory liver damage. Thus, chronic inflammation need not be tumorigenic and the carcinogenic potential of hepatic inflammation may be determined by the type of its mediators rather than by mere chronicity.
Materials and Methods

Mice

SAP-IFNγ transgenic or non-transgenic C57BL/6 mice were bred and kept in a specific pathogen free animal facility at the Johannes Gutenberg-University Mainz, Germany or at the University Medical Centre Hamburg-Eppendorf, Germany. The experiments were approved by the institutional animal experimentation committee. Liver carcinogenesis was induced in male mice by neonatal application of DEN (5mg/kg at day ten after birth) and subsequently promoted by phenobarbital (0.05% in drinking water). p53 -/- mice for the preparation of p53 -/- hepatocytes were obtained from the Jackson Laboratory (Bar Harbor, ME, USA).

Histology

At the indicated time-points, mice were sacrificed and the livers were fixed in 4% neutral-buffered formaldehyde, embedded in paraffin, sectioned at 2 μm and stained with hematoxylin/eosin. At least six representative sections of each liver were evaluated by a senior hepatopathologist in a blinded fashion (P.S.). Tumor burden was determined as the number of lesions per cm² and as total lesion area (%). TUNEL assay was performed with the in situ cell death detection kit (Roche, Mannheim, Germany); cells were counterstained with Hoechst 33258 nuclear dye (Invitrogen, Darmstadt, Germany). Immunohistological staining was performed with antibody to p53, cleaved caspase 3 (Cell Signaling, Danvers, MA, USA), or p21 (Santa Cruz, Santa Cruz, Ca, USA).
Western Blot

Whole cell lysates were prepared by lysis in Hepes buffered saline with 1% Triton X-100 containing proteinase/phosphatase-inhibitors. Liver lysates were prepared from snap frozen tissue in T-Per (Pierce, Rockford, IL, USA) with proteinase/phosphatase-inhibitors. Protein was separated in 12% SDS-polyacrylamide gels, and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After incubation with 5% dry non-fat milk, membranes were probed with primary antibodies and appropriate HRP-conjugated secondary antibodies (all from Cell Signaling except antibodies to actin, p53, p21, Cyclin D1, PCNA (Santa Cruz). Detection was performed using an ECL kit (Roth, Karlsruhe, Germany).

Cell culture

Primary hepatocytes were isolated as described and stimulated with 10ng/ml recombinant mouse IFNγ (Peprotech, Rocky Hill, NJ, USA), 30 or 60μM cisplatin, 200μM DEN (both from Sigma, Munich, Germany), or 50μM pifithrin-a (Calbiochem, Darmstadt, Germany) as indicated. Cell survival was measured by adding 0.5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) for 2 hours. The supernatant was removed, the tetrazolium dye dissolved in DMSO and absorbance was measured at 570nm. Results shown are means of quadruple experiments. Alternatively, cell survival was determined by counting alive and dead cells after trypan blue staining in at least 6 microscope fields per cell culture sample.

Analysis of intrahepatic infiltrates

Non-parenchymal liver cells from perfused mouse livers were prepared as described and the included hepatic inflammatory cells were then stained with...
fluorescence-labeled antibody to CD3, CD4, CD8, CD56, or NK1.1 (BD Biosciences; Heidelberg, Germany) and analyzed by flow cytometry.
Results

Sustained activation of STAT1 suppresses chemical hepatocarcinogenesis

Liver protein extracts of 6 or 12 weeks old non-transgenic or IFN\(\gamma\)-transgenic mice were assessed by Western Blot for activity of the IFN\(\gamma\)/STAT1 pathway and markers of regenerative hepatocyte proliferation (Figure 1A). The non-transgenic mice did not show STAT1 phosphorylation at the age of 6 or 12 weeks. In contrast, IFN\(\gamma\)-transgenic mice showed continued up-regulation and activation of STAT1 both at 6 and 12 weeks of age. The degree of STAT3 activation was considerably higher in the IFN\(\gamma\)-transgenic mice than in non-transgenic mice, both at 6 or 12 weeks of age. Furthermore, the lysates from IFN\(\gamma\)-transgenic livers, taken at 6 or 12 weeks of age, contained higher amounts of the proliferation markers PCNA and cyclinD1 than those of non-transgenic mice. These findings indicated that IFN\(\gamma\)-signaling in the transgenic mice was continuously active and not silenced, and that the IFN\(\gamma\)-transgenic livers were characterized by continued regenerative proliferation.

To confirm these in vivo findings, we assessed in vitro the response of primary hepatocytes from non-transgenic mice to stimulation by IFN\(\gamma\) (Figure 1B). IFN\(\gamma\) induced the rapid up-regulation and activation of STAT1 and STAT3 within 1 hour. STAT3 activation was silenced after 12 hours; STAT1 activation, in contrast, continued for at least 48 hours. These findings are in agreement with the continuously activated IFN\(\gamma\)-signaling found in vivo (shown in Figure 1A).

We then induced chemical hepatocarcinogenesis in non-transgenic or IFN\(\gamma\)-transgenic mice by neonatal application of DEN, which was subsequently promoted by application of phenobarbital in drinking water. Cancer development in DEN-treated mice was assessed histologically after 40 weeks, since by then two mice in the group of non-transgenic mice already had died from liver cancer. The livers of
IFN-γ-transgenic mice (n=22) had significantly fewer (Figure 2A; P<0.0001) and smaller lesions (Figure 2B; P<0.0001) than those of the non-transgenic mice (n=14). Representative histological sections of IFN-γ-transgenic mice (Figure 2C) show mild portal inflammation and mild perivenular fatty change, but lack of premalignant or malignant changes. Representative histological sections of non-transgenic mice (Figure 2D) show a premalignant eosinophilic (dysplastic) nodule without signs of cytological atypia, increased proliferation or invasive growth. Moreover, carcinogenesis seemed to be delayed in the IFN-γ-transgenic mice compared to the non-transgenic mice (Table 1). Indeed, significantly fewer IFN-γ-transgenic mice had pre-malignant dysplastic nodules (Table 1; P=0.0017), and significantly fewer IFN-γ-transgenic mice had malignant lesions (HCC; Table 1; P=0.0002), with an odds ratio of 25 for the non-transgenic mice vs. IFN-γ-transgenic mice to develop liver cancer (95% confidence interval: 3.89 - 160.57). Therefore, activation of the IFN-γ/STAT1 pathway in IFN-γ-transgenic mice seemed to protect from chemical hepatocarcinogenesis, despite continuous liver injury and regeneration.

*IFN*γ*-induced regulation of p53 activity*

The question then was how IFN-γ induced protection from chemical hepatocarcinogenesis. Since IFN-γ has been shown to increase MHC expression and the immunogenicity of aberrant cells, IFN-γ may have indirectly activated cancer immunosurveillance by tumor-reactive lymphocytes11,12. Indeed, there was a strong and significant accumulation of both CD8 T cells and NKT cells in the livers of IFN-γ-transgenic mice, with an increase of the CD8 T cell percentage among the intrahepatic lymphocytes from 11% in wild-type mice to 32% in IFN-γ-transgenic mice (P=0.0002), and an increase in the percentage of NKT cells from 4% in wild-type
mice to 24% in IFNγ-transgenic mice (P=0.0002). To a lesser degree, there was also an increase in NK cells in the livers of IFNγ-transgenic mice (2% in wild-type mice vs. 4% in IFNγ-transgenic mice; P=0.0002), whereas CD4 T cells did not accumulate (11% in wild-type mice vs. 9% in IFNγ-transgenic mice; P=0.3282). Thus, it was possible that IFNγ-induced protection from malignancy was related to indirect activation of immune cells.

However, it has been observed in vitro that IFNγ signaling in hepatocytes can induce cell cycle arrest or apoptosis22,23. Thus, it was also possible that IFNγ-induced protection from malignancy was related to direct effects of IFNγ on hepatocytes. Indeed, it has been reported that activated STAT1 can regulate p53 activation24,25; the p53 molecule functions to inhibit the growth of aberrant cells by inducing cell cycle arrest or apoptosis in response to various stimuli26. Thus, IFNγ-signaling may have induced p53 activation and p53-dependent apoptosis of DEN-treated hepatocytes. To address this possibility, we stimulated primary hepatocytes from non-transgenic mice with IFNγ. Exposure to IFNγ induced both the accumulation of p53 and its activation, indicated by up-regulation of p21 and cleavage of caspase 3 (Figure 3A). We then incubated primary hepatocytes from non-transgenic mice, which had or had not been stimulated with IFNγ, with DEN or Cisplatin (Figure 3B); this application of genotoxic stress reduced the expression of p21, a marker of growth arrest, and induced cleavage of caspase 3, indicating apoptosis. These findings indicated that IFNγ-signaling induced p53 activation in hepatocytes and additional genotoxic stress shifted p53 activity from predominant growth arrest to predominant apoptosis.

IFNγ-induced p53 accumulation and apoptosis in vivo
To confirm these in vitro observations, we assessed the amounts of p53 and of p53-induced p21 and cleaved caspase-3 in liver extracts from DEN-treated or untreated non-transgenic or IFNγ-transgenic mice (Figure 4A). Indeed, IFNγ-transgenic mice had accumulated p53 irrespective of DEN-treatment; non-transgenic mice, in contrast did not exhibit p53 accumulation. DEN exposure itself did not appear to modulate STAT1 or STAT3 activation (Figure 4A). The STAT1-induced p53 accumulation in IFNγ-transgenic mice was associated with elevated p53 activity, as indicated by the induction of the p21 molecule and cleavage of caspase-3 (Figure 4A). IFNγ-induced p53 activity seemed to be modulated by DEN: DEN-treated mice displayed higher amounts of cleaved caspase-3, indicative of apoptosis, whereas untreated mice showed higher levels of p21, indicative of growth arrest. Thus, IFNγ-signaling may induce p53 accumulation and activation, and the additional presence of genotoxic stress may shift p53 activity from predominant growth arrest to apoptosis.

To confirm that apoptosis of hepatocytes was induced by IFNγ-signaling in combination with genotoxic stress, we performed TUNEL assays on liver sections of non-transgenic and IFNγ-transgenic mice 6 days after treatment with DEN (Figure 4B). Hepatocytes of IFNγ-transgenic mice displayed significantly higher rates of apoptosis than those of non-transgenic mice (4B, lower panel; \(P<0.0001\)); moreover, exposure to DEN significantly further increased the incidence of apoptosis in hepatocytes of IFNγ-transgenic mice (\(P<0.0001\)). A representative TUNEL stain is shown in Figure 5A.

To further confirm that IFNγ-signaling had induced p53 activation in hepatocytes in vivo, we treated non-transgenic or IFNγ-transgenic mice with DEN and obtained the livers after 6 days. We then stained liver sections by histochemistry for p53 (Figure 5B), p21 (Figure 5C) and cleaved caspase 3 (Figure 5D). In contrast to non-
transgenic hepatocytes, hepatocytes of IFNγ-transgenic mice displayed considerable accumulation of p53 and cleavage of caspase 3, notably when treated with DEN. Moreover, there was considerable expression of p21 in IFNγ-transgenic hepatocytes, which was reduced after DEN treatment; non-transgenic hepatocytes did not express p21. These in vivo findings are in full agreement with the in vitro findings of Figure 3.

To further substantiate these in vivo findings, we analyzed the survival of primary hepatocytes from non-transgenic mice in vitro, after stimulation with IFNγ and in response to DNA damage induction by treatment with DEN (Figure 6A, white columns) or cisplatin (Figure 6B, white columns). In concordance with the in vivo findings, IFNγ and genotoxic stress synergistically reduced the survival of hepatocytes (P<0.05). The induced hepatocyte death seemed to be p53-dependent, since it was prevented by the p53-inhibitor pifithrin-a27 (Figures 6A and 6B; black columns). To further test the p53-dependence of the induced hepatocyte death, we analyzed in vitro the survival of primary hepatocytes from p53-/- mice or wild-type mice in response to incubation with IFNγ and DEN (Figure 6C) or cisplatin (Figure 6D). In agreement with the findings above, IFNγ and genotoxic stress synergistically reduced the survival of wild-type hepatocytes (white columns; P<0.02), but not of p53-/- hepatocytes (black columns; P>0.5). These findings indicated that IFNγ/STAT1-signaling to hepatocytes may suppress liver cancer by p53-dependent inhibition of the outgrowth of premalignant cells.
Discussion

Inflammation has both the capacity to foster or to inhibit carcinogenesis, by either promoting the growth or the depletion of malignant cells\textsuperscript{28}. Whether inflammation is rather tumor-promoting or tumor-suppressive seems to depend on the cell type and cytokine composition of the tumor-associated inflammatory reaction\textsuperscript{28}. The role of IFN\textsubscript{γ} in this respect has been controversial; both tumor-promoting and tumor-suppressive functions of IFN\textsubscript{γ} have been reported\textsuperscript{11,18}. On the one hand, IFN\textsubscript{γ} may function as a tumor-promoter by stimulating the release of inflammatory mediators that may advance carcinogenesis, such as TNFα, reactive oxygen species or nitric oxide\textsuperscript{28,29}; on the other hand, IFN\textsubscript{γ} may function as a tumor-suppressor by enhancing tumor cell immunogenicity and cancer immunosurveillance\textsuperscript{12}. The role of IFN\textsubscript{γ} in hepatocarcinogenesis is further complicated by its anti-fibrotic effects, which may also influence carcinogenesis. Indeed, a liver cancer model that is associated with severe liver fibrosis showed a mild reduction in lesion numbers in mice lacking the IFN\textsubscript{γ} receptor\textsuperscript{30}.

To clarify the role of IFN\textsubscript{γ} in hepatocarcinogenesis, we used liver-specific IFN\textsubscript{γ}-transgenic mice; the livers of these mice are marked by continuous inflammation, injury and compensatory regeneration in the absence of fibrosis. Regenerating hepatocytes under chronic inflammatory stress are believed to be prone to malignant transformation\textsuperscript{4,5}. However, despite chronic inflammation and regeneration, the IFN\textsubscript{γ}-transgenic mice were significantly less susceptible to chemical hepatocarcinogenesis (Figure 2 and Table 1); the odds ratio of non-transgenic mice vs. IFN\textsubscript{γ}-transgenic mice to develop liver cancer was 25. This is in agreement with the earlier finding that IFN\textsubscript{γ}-insensitive mice, which either were IFN\textsubscript{γ}-receptor deficient or STAT1 deficient, exhibited increased development of chemically-induced skin cancer\textsuperscript{10}. In skin cancer,
IFNγ seemed to act by increasing tumor immune surveillance by lymphocytes\textsuperscript{10,11}. We find the livers of IFNγ-transgenic mice strongly infiltrated mainly by CD8 T cells and NKT cells and to a lesser degree by CD4 T cells and NK cells. At least CD8 T cells and NK cells are capable of depleting malignant cells; indeed, it has been reported that IFNγ-driven accumulation of NK cells in established tumors seems to correlate with survival\textsuperscript{31}. Thus, it is conceivable that the protection from malignancy mediated by IFNγ was related to IFNγ-enhanced cytotoxicity of immune cells.

However, in addition to its indirect effects on malignant cells by activating cancer immune surveillance, we find that IFNγ could also directly induce depletion of premalignant cells by activating the cell-intrinsic p53 tumor suppressor pathway. Indeed, we find that IFNγ signaling to damaged hepatocytes seemed to modify the acute damage response by activation of the p53 pathway, resulting in sensitization to apoptosis in response to genotoxic stress (Figures 3, 4 and 5). Although damage-induced cell death of hepatocytes seemed to depend only partly on p53, there was a consistent increase in cell death induced by IFNγ, and this increase was consistently lost in the absence of p53 activity (Figure 6). Thus, our findings indicate a tumor-suppressive role of IFNγ at early stages of carcinogenesis by the induction of apoptosis in damaged cells. Since lymphocytes are the most important physiological source of IFNγ in non-transgenic mice and in humans, it is possible that IFNγ-secretion is an important cancer-preventing activity of lymphocytes independent of their cytolytic activity.

It has been reported that IFNγ may promote colorectal carcinogenesis in SOCS1-deficient mice, which manifest hyperactivation of the STAT1 pathway, by inducing expression of cyclooxygenase-2 and inducible nitric oxide synthase\textsuperscript{18}. Indeed, colorectal carcinogenesis in SOCS1-deficient mice could be abrogated by
blockade of IFNγ. Since silencing of the SOCS1 gene by methylation has been observed in liver cancer, a lack of SOCS1 activity and STAT1 hyperactivity may also promote hepatocarcinogenesis. Indeed, SOCS1+/- mice are more susceptible to chemical hepatocarcinogenesis than SOCS1+/+ mice, suggesting that STAT1 hyperactivation due to a lack of SOCS1 activity may promote carcinogenesis. However, this observation is in seeming contrast to our finding that chronic STAT1 activation in IFNγ-transgenic mice was associated with suppressed hepatocarcinogenesis. One explanation for this divergence could be that SOCS-1 does not only interfere with the STAT1 pathway but also with other cytokine pathways, such as the STAT3 pathway; therefore, the tumor-promoting effect of SOCS1-deficiency may depend on STAT3 rather than STAT1 signals. Indeed, the STAT3 pathway has been identified as a critical driver of carcinogenesis, and tumor progression may be determined by the balance of STAT1 and STAT3 signals. Although IFNγ stimulated both, STAT1 and STAT3 (Figure 1B), the STAT1 pathway in hepatocytes stimulated with IFNγ seemed to be continuously activated, whereas the STAT3 signal seemed to be rather transient (Figure 1B).

Taken together, our findings indicate that chronic inflammation and compensatory regeneration are not necessarily oncogenic, and that the type of inflammation, notably the proportion of IFNγ-secreting lymphocytes in chronic inflammatory tissue infiltrates may determine the carcinogenic potential of chronic inflammation. Indeed, one may speculate that these findings could explain the clinical observation that some chronic inflammatory conditions, such as viral hepatitis, confer a high risk of progression to cancer, while in other inflammatory conditions, such as autoimmune hepatitis, progression to cancer is uncommon. Our findings further indicate that inflammatory IFNγ/STAT1-signaling to damaged cells, in addition to
indirectly activating cancer immune surveillance\textsuperscript{12}, may also directly activate the cell-intrinsic p53 tumor suppressor pathway. Our findings seem to indicate that the IFNγ-promoted apoptosis of hepatocytes can be an efficient barrier to hepatocarcinogenesis.

\textit{Acknowledgements:}

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References


Table 1: Incidence of pre-malignant and malignant lesions in non-transgenic or IFN-γ transgenic mice.

<table>
<thead>
<tr>
<th>Histological stage</th>
<th>Non-transgenic mice (n=14)</th>
<th>IFN-γ transgenic mice (n=22)</th>
<th>significance (P)</th>
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<td>Dysplastic foci (small cell dysplastic foci)</td>
<td>13 / 14 (93%)</td>
<td>16 / 22 (73%)</td>
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<td>Dysplastic nodules (‘adenomas’)</td>
<td>11 / 14 (79%)</td>
<td>5 / 22 (23%)</td>
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<td>HCC</td>
<td>10 / 14 (71%)</td>
<td>2 / 22 (9%)</td>
<td>0.0002</td>
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</tbody>
</table>
Figure legends

Figure 1: Sustained STAT1 signaling and regenerative proliferation in IFNγ-transgenic mice.

A) The livers of 6 or 12 weeks old IFNγ-transgenic or non-transgenic mice were analysed for activity of the STAT1 and STAT3 signaling pathways and markers of regenerative proliferation (cyclin D1, PCNA). In contrast to non-transgenic mice, the STAT1 pathway was continuously active and not silenced in IFNγ-transgenic mice. Moreover, IFNγ-transgenic livers were marked by continued regenerative proliferation.

B) Primary non-transgenic hepatocytes were stimulated with IFNγ, which induced continued STAT1 activation for at least 48 hours; in contrast, STAT3-activation, which was also induced by IFNγ, was silenced after 6 hours.

Figure 2: Sustained activation of the IFNγ/STAT1 pathway suppresses chemical hepatocarcinogenesis.

In IFNγ-transgenic or non-transgenic mice, chemical hepatocarcinogenesis was induced with diethylnitrosamine and cancer development was assessed by histology after 40 weeks. IFNγ-transgenic mice had significantly fewer (A) and smaller (B) hepatic lesions than non-transgenic mice (P<0.0001). Representative liver histology of IFNγ-transgenic mice (C) shows mild portal inflammation and mild perivenular fatty change, but lack of premalignant or malignant changes. Representative liver histology of non-transgenic mice (D) shows a premalignant eosinophilic (dysplastic) nodule.
Figure 3: IFNγ-induced p53-activation in primary hepatocytes

Cell extracts of primary non-transgenic hepatocytes after IFNγ-treatment in vitro were assessed by Western blot. A) Cell extracts were derived from primary non-transgenic hepatocytes that were stimulated for indicated time with IFNγ. IFNγ-stimulation seemed to induce accumulation of p53 and its activation, indicated by increased expression of p21 and cleavage of caspase-3. B) Cell extracts were derived from primary non-transgenic hepatocytes that were stimulated with IFNγ and the DNA-damaging agents Cisplatin or diethylnitrosamine (DEN), as indicated. Damaged primary hepatocytes incubated with IFNγ showed reduced p21 expression and increased cleavage of caspase-3.

Figure 4: IFNγ-induced activation of p53 pathway in diethylnitrosamine (DEN)-treated mice

Non-transgenic or IFNγ-transgenic mice were treated with DEN in vivo and, after six days, liver extracts (A) or histological liver sections (B) were obtained and analyzed. A) Liver extracts were analyzed by Western blot. In contrast to non-transgenic mice, the livers of IFNγ-transgenic mice exhibited marked p53 accumulation, independent of whether the mice had been treated with DEN or not. Activation of p53 in the IFNγ-transgenic mice was indicated by expression of p21 and cleavage of caspase 3; previous treatment with DEN reduced p21 expression and increased caspase 3 cleavage. B) Histological liver sections were quantified for apoptosis of hepatocytes by TUNEL assay, indicating that IFNγ induced apoptosis of hepatocytes, and exposure to DEN further increased apoptosis rates of IFNγ-exposed hepatocytes.
Figure 5: Histological analysis of IFNγ-induced p53-activity and apoptosis of hepatocytes after diethylnitrosamine (DEN)-treatment in vivo.

Six days after treatment of non-transgenic or IFNγ-transgenic mice with DEN in vivo, liver sections were assessed for apoptosis of hepatocytes by TUNEL assay (A), p53 accumulation (B), p21 expression (C) and cleavage of caspase-3 (D). Shown are representative findings. A) Apoptotic hepatocytes stained by the TUNEL assay appear in green; the nuclei are counterstained in blue with Hoechst 33258. IFNγ seemed to induce apoptosis of hepatocytes; exposure to DEN further increased apoptosis rates of IFNγ-exposed hepatocytes. B) Hepatocytes in IFNγ-transgenic mice showed accumulation of p53, notably after previous DEN treatment in vivo. C) Hepatocytes of IFNγ-transgenic mice showed increased expression of p21, which was reduced after previous DEN treatment in vivo. D) Hepatocytes in IFNγ-transgenic mice showed increased cleavage of caspase-3, notably after previous DEN treatment in vivo.

Figure 6: IFNγ-induced increase of apoptosis in damaged hepatocytes in vitro is partly p53-dependent.

Primary hepatocytes were treated with IFNγ, diethylnitrosamine (DEN) or cisplatin, as indicated and survival was assessed; the role of p53 was probed by incubating wild-type hepatocytes with the p53-inhibitor pifithrin-a (A, B) or by using p53-deficient hepatocytes (C, D). Stimulation with IFNγ reduced the survival of primary wild-type hepatocytes (white columns) after exposure to DEN (A) or Cisplatin (B); inhibition of p53 with pifithrin-a (black columns) prevented apoptotic cell loss in response to combined stimulation with IFNγ and DEN (A) or IFNγ and Cisplatin (B). Stimulation with IFNγ reduced the survival of primary non-transgenic hepatocytes (white
columns) after exposure to DEN (C) or Cisplatin (D); p53-deficient hepatocytes (black columns) resisted IFNγ-induced sensitization to apoptosis by DEN (C) or Cisplatin (D). An asterisk indicates statistical significance (P<0.05); the lack of significance is indicated by ‘n.s.’ (not significant).
Figure 2

A

B

C

D

lesions per cm$^2$

lesion area (%)

median
50% percentile
90% percentile

non-

interferon $\gamma$-transgenic

lesion

non-

interferon $\gamma$-transgenic

C

D

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