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Interleukin-22 Induces Hepatic Stellate Cell Senescence and Restricts Liver Fibrosis

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Abstract

Interleukin-22 (IL-22) is known to play a key role in promoting antimicrobial immunity, inflammation, and tissue repair at barrier surfaces by binding to the receptors IL-10R2 and IL-22R1. IL-22R1 is generally thought to be expressed exclusively in epithelial cells. In this study, we identified high levels of IL-10R2 and IL-22R1 expression on hepatic stellate cells (HSCs), the predominant cell type involved in liver fibrogenesis in response to liver damage. *In vitro* treatment with IL-22 induced the activation of signal transducer and activator of transcription 3 (STAT3) in primary mouse and human HSCs. IL-22 administration prevented HSC apoptosis *in vitro* and *in vivo*, but surprisingly, the overexpression of IL-22 via either gene targeting (IL-22 transgenic mice) or exogenous administration of adenovirus expressing IL-22 reduced liver fibrosis and accelerated the resolution of liver fibrosis during recovery. Furthermore, IL-22 overexpression or treatment increased the number of senescence-associated β -galactosidase-positive HSCs and decreased α -smooth muscle actin expression in fibrotic livers *in vivo* and cultured HSCs *in vitro*. Deletion of STAT3 prevented IL-22-induced HSC senescence *in vitro*, whereas the overexpression of a constitutively activated form of STAT3 promoted HSC senescence via p53- and p21-dependent pathways. Finally, IL-22 treatment upregulated suppressor of cytokine signaling 3 expression in HSCs. Immunoprecipitation analyses revealed that SOCS3 bound p53 and subsequently increased the expression of p53 and its target genes, contributing to IL-22-mediated HSC senescence.

Conclusion—IL-22 induces the senescence of HSCs, which express both IL-10R2 and IL-22R1, thereby ameliorating liver fibrogenesis. The anti-fibrotic effect of IL-22 is likely mediated via the induction of HSC senescence in addition to the previously discovered hepatoprotective functions of IL-22.

Keywords

p53; SOCS3; IL-22R; liver repair

Microbial infection activates the innate and adaptive immune responses, which in turn control infection and promote tissue repair. For example, bacterial infection results in the activation of different immune cells that produce interleukin-22 (IL-22), which plays an important role in controlling bacterial infection via the upregulation of anti-microbial proteins. IL-22 also promotes tissue repair by upregulating a variety of genes expressed in

epithelial cells such as hepatocytes.¹⁻³ The action of IL-22 is mediated by binding to the receptors IL-10R2 and IL-22R1, which activates signal transducer and activator of transcription 3 (STAT3).¹⁻³ IL-10R2 is ubiquitously expressed, whereas IL-22R1 is believed to be expressed exclusively in the epithelial cells of various organs.¹⁻³ In the liver, hepatocytes express IL-22R1 and IL-10R2. By ligating these receptors in a heterodimer, IL-22 promotes hepatocyte survival and proliferation, resulting in liver repair.^{4, 5} However, the effect of IL-22 on liver fibrogenesis remains unknown.

Liver fibrosis is a consequence of chronic liver injury and is characterized with an accumulation of extracellular matrix proteins and activation of hepatic stellate cells (HSCs).⁶⁻⁸ Following liver injury, HSCs become activated, express α -smooth muscle actin (α -SMA), and produce large amounts of collagen.⁶⁻⁸ There has been tremendous progress in discovering the regulatory mechanisms that control the activation of HSCs during liver fibrogenesis, including inflammatory cells (e.g., Kupffer cells, NK cells), growth factors, cytokines, and chemokines.⁶⁻⁸ Additionally, the senescence of activated HSCs is also an important step in limiting the fibrogenic response to tissue damage.^{9, 10} After becoming senescence, activated HSCs stop proliferation and express reduced levels of extracellular matrix components but increased levels of extracellular matrix-degrading enzymes.^{9, 10} Deletion of the important cell cycle regulator p53 reduces HSC senescence leading to extensive liver fibrosis.⁹ Moreover, many cytokines, such as IL-6 and IL-8, and their downstream signaling molecules STAT5 and SOCS1 have been shown to promote cellular senescence in many cell types;^{11, 12} however, their roles in HSC senescence have not been reported. In the current study, we demonstrate for the first time that HSCs express high levels of IL-10R2 and IL-22R1. Furthermore, we provided evidence suggesting that IL-22 induces HSC senescence via the activation of STAT3, SOCS3, and p53 pathways, thereby inhibiting liver fibrosis.

Materials and Methods

Animal models

C57BL/6 mice and SOCS3^{flx/flx} mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The IL-22 transgenic (IL-22TG) mice and hepatocyte-specific STAT3 knockout (STAT3^{Hep-/-}) mice were described previously.¹³ To induce hepatic fibrosis, mice were treated intraperitoneally (i.p.) with 2 ml/kg body weight of 10% carbon tetrachloride (CCl₄) (Sigma, St. Louis, MO) for 8 weeks. Animals were sacrificed at 1d or 5d after the last injection. All animal experiments were approved by the NIAAA Animal Care and Use Committee.

Analysis of HSC senescence

HSC senescence in fibrotic livers or in cultured HSCs was determined by the detection of SA- β -gal (senescence-associated β -galactosidase) activity using an SA- β -gal staining kit (Cell Signaling, Danvers, MA). Briefly, frozen liver sections or adherent cells were fixed with 0.5% glutaraldehyde in PBS for 15 min, washed with PBS containing 1 mM MgCl₂, and stained overnight in PBS containing 1 mM MgCl₂, 1 mg/ml X-Gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide. Sections were counterstained with eosin. The SA- β -gal-positive areas were measured in at least three low-power (X100) microscope fields using the Image-Pro Plus Version 6.0 software.

Statistical Analysis

Data are expressed as the mean $\pm$ SEM (n=6-10). To compare values between two groups, Student's *t*-test was used. A *P*-value < 0.05 was considered significant. Most of the

experiments were repeated in three or four independent trials with similar results, and representative images are included in the paper.

All other materials and methods are described in the supporting information.

Results

IL-22 predominately activates STAT3 in HSCs that express high levels of IL-22R1 and IL-10R2

As illustrated in Fig. 1A, IL-22R1 mRNA expression was detected in quiescent and activated mouse HSCs, and these levels were comparable to IL-22R1 mRNA levels in hepatocytes. IL-22R1 mRNA expression increased further following treatment with IL-22 in cultured HSCs (Fig. 1B). Expression of IL-10R2 mRNA, which is also required for IL-22 signaling, was detected in HSCs as well as in hepatocytes and Kupffer cells (Fig. 1A). Additionally, western blot analyses revealed the expression of IL-22R1 protein in primary mouse HSCs, which was slightly increased after IL-22 treatment (Fig. 1C). Fluorescence-activated cell sorting (FACS) analyses detected IL-22R1 protein expression on the surface of primary mouse HSCs, and comparable expression levels were observed in HSCs from WT and IL-22TG mice (supporting Figs. S1a-b). Finally, expression of IL-22R1 and IL-10R2 mRNA was also detected in primary human HSCs from three human donors and in the human HSC cell line LX2 (Fig. 1D).

The effects of IL-22 on the signaling pathways in HSCs are shown in Fig. 1E. IL-22 exposure significantly activated STAT3 in all samples with peak effects observed at 30-60 min. Activated STAT3 levels returned to basal levels by 120 min. IL-22 also induced ERK1/2 activation in primary mouse HSCs and to a lesser extent in human HSCs and LX2 cells. Furthermore, IL-22-dependent STAT3 activation in HSCs was further confirmed by immunostaining for pSTAT3 in the nuclei of HSCs (supporting Figs. S1c-d).

IL-22 inhibits HSC apoptosis without affecting HSC proliferation *in vitro*

IL-22 has been shown to promote hepatocyte survival and proliferation;⁴ therefore, we examined the potential anti-apoptotic and mitogenic effects of IL-22 on HSCs. The nuclear morphology of HSCs revealed a significant increase in apoptosis following a 4-h incubation with cycloheximide (CHX) that was markedly reduced in IL-22 pre-treated HSCs (Fig. 2A and supporting Fig. S2). The anti-apoptotic function of IL-22 in HSCs was further demonstrated by a reduction in CHX-mediated induction of caspase 3/7 activity and cleaved caspase-3 expression in HSCs following IL-22 treatment (Figs. 2A-B). Furthermore, Fig. 2C shows that serum and platelet-derived growth factor (PDGF) but not IL-22 treatment increased BrdU incorporation in HSCs (Fig. 2C), indicating that IL-22 does not affect HSC proliferation. Finally, expression of the anti-apoptotic proteins such as pSTAT3 and Bcl-2 was markedly increased, while expression of the mitogenic protein cyclin D1 was slightly elevated in HSCs after IL-22 exposure (Fig. 2D).

IL-22TG mice are resistant to chronic CCl₄ treatment-induced liver fibrosis but not liver injury

Our results support a model in which IL-22 protects against HSC apoptosis *in vitro*; therefore, we examined whether IL-22 also prevents HSC apoptosis *in vivo*. Although hepatic and serum IL-22 levels were not significantly elevated in CCl₄-treated mice, hepatic IL-22 levels were markedly increased in viral hepatitis patients.¹³⁻¹⁵ To identify the effect of high IL-22 levels on liver fibrosis, we used CCl₄ to induce liver fibrosis in IL-22TG mice, which overexpress IL-22 in the liver¹³ and mimic the elevated IL-22 levels associated with viral hepatitis. Both WT and IL-22TG mice were treated with CCl₄ for 8 weeks (supporting

Fig. S3a), and then the mice were sacrificed and the extent of HSC apoptosis and liver fibrosis was analyzed. Figs. 3A-B show that HSC apoptosis (α -SMA⁺TUNEL⁺) decreased in the liver of IL-22TG mice when compared with WT mice. This suggests that IL-22 promotes HSC survival *in vivo*, which led us to hypothesize that IL-22 may exacerbate liver fibrosis. However, to our surprise, the degree of liver fibrosis was lower in IL-22TG mice when compared to WT mice, as shown in Figs. 3A-C and supporting Fig. S3b. Following CCl₄ treatment, we observed reduced areas of α -SMA and Sirius red staining as well as a reduction in the expression of α -SMA protein and collagen mRNA in IL-22TG mice when compared with WT mice. Finally, the percentage of 5d/1d Sirius red areas was significantly lower in the IL-22TG mice when compared with the WT mice during the fibrosis resolution stage (supporting Fig. S3c), indicating that the IL-22TG mice resolved hepatic fibrosis much faster than the WT mice.

Next, we investigated whether the observed reduction in liver fibrosis in IL-22TG mice was due to the hepatoprotective effects of IL-22.⁴ Although IL-22TG mice were completely resistant to Con A-induced liver injury,¹³ surprisingly, serum ALT levels were comparable between IL-22TG and WT mice following CCl₄ treatment (Fig. S3d). In addition, expression of hepatic Cyp2E1, a key enzyme responsible for the metabolism of CCl₄ in the liver, was not upregulated in IL-22TG mice (supporting Fig. S3e). These results suggest that the decreased hepatic fibrosis observed in the IL-22TG mice was neither caused by a reduction in CCl₄ metabolism nor by liver injury.

To further understand the mechanisms underlying the reduction in fibrosis in the IL-22TG mice, HSC senescence, a key step in limiting liver fibrosis,^{9, 10} was examined. As illustrated in Fig. 3D, more SA- β -Gal⁺ cells accumulated in the fibrotic scar tissue of liver sections from CCl₄-treated IL-22TG mice when compared with liver tissues from WT mice. Serial co-immunostaining using α -SMA antibody with SA- β -Gal staining or with another senescence marker HMGA1¹⁶ showed that the expression of SA- β -Gal and HMGA1 co-localized with α -SMA (supporting Fig. S3f). These results indicate that IL-22TG mice have a higher number of senescent HSCs when compared with WT mice following chronic CCl₄ treatment.

Administration of IL-22 adenovirus (Ad-IL-22) accelerates spontaneous liver fibrosis resolution

To examine the effect of IL-22 on liver fibrosis resolution, CCl₄-treated mice were challenged with Ad-IL-22 or control Ad-GFP adenovirus immediately following the final CCl₄ injection (supporting Fig. S4a). Figs. 4A-B show that the number of apoptotic HSCs was lower in Ad-IL-22-treated mice when compared with Ad-GFP-treated mice. Despite reduced HSC apoptosis, liver fibrosis resolution was faster in the Ad-IL-22-treated mice than in the WT mice as demonstrated by lower levels of α -SMA expression, Sirius red staining, α -SMA protein, and collagen mRNA in the Ad-IL-22-treated mice 5 d post-CCl₄ treatment (Figs. 4A-C, supporting Fig. S4b).

Immunohistochemistry staining revealed an increase in the number of SA- β -Gal⁺ cells in the livers of Ad-IL-22-treated mice than in the livers of Ad-GFP-treated mice, and these cells tended to reside within fibrotic scar tissue (Fig. 4D). Moreover, the administration of Ad-IL-22 upregulated the expression of matrix metalloproteinase-9 (MMP9) and pro-inflammatory genes but downregulated the TIMP expression (supporting Fig. S4c), which is consistent with a senescence-associated secretory phenotype.⁹ Finally, we also isolated HSCs and performed SA- β -Gal staining *in vitro* to further confirm that IL-22 promotes HSC senescence. The data in supporting Fig. S4d show that approximately 60% of HSCs from the Ad-IL-22-treated mice were positive for SA- β -Gal staining, while only 20% of HSCs from the Ad-GFP-treated mice were positive.

Because liver regeneration affects liver fibrosis,¹⁷ we examined hepatocyte proliferation in CCl₄-treated mice following IL-22 administration. As shown in supporting Fig. S4f, Ad-IL-22 injection for 5d markedly increased hepatocyte proliferation, whereas such treatment for 24h resulted in no differences. To further determine whether the hepatoprotective and mitogenic functions of IL-22, which are mediated via the activation of STAT3 in hepatocytes,⁴ also contribute to the IL-22-mediated inhibition of liver fibrosis, we used hepatocyte-specific STAT3 knockout mice (STAT3^{Hep-/-}). As shown in supporting Figs. S4f-g, treatment with Ad-IL-22 ameliorated liver fibrosis in STAT3^{Hep-/-} mice, but the degree of inhibition was lower than in WT mice. This suggests that IL-22 inhibits liver fibrosis via hepatocyte STAT3-dependent and STAT3-independent mechanisms.

IL-22 promotes activated HSC senescence and inhibits HSC activation *in vitro*

Next, we used cultured HSCs to test whether IL-22 has a direct effect on HSC activation and senescence. IL-22 exposure decreased α -SMA and collagen-(I) mRNA and protein levels in HSCs cultured for 4d (D4) or 7d (D7) (Figs. 5A-B) suggesting that IL-22 inhibits HSC activation. Moreover, our results suggest that IL-22 promotes HSC senescence. IL-22 treatment increased the number of SA- β -Gal⁺ HSCs but decreased the telomerase activity in HSCs (Fig. 5C and supporting Figs. S5a-b). Additionally, RT-PCR analysis of senescence-associated inflammatory genes showed that IL-22 treatment upregulated the expression of MMP9, IL-6, and MIP2 but downregulated TIMP1 expression (Fig. 5D). Finally, IL-22 exposure upregulated the expression of several cellular senescence-associated proteins including phosphorylated p53 at serine 15 (p-p53^{ser15}), p53, p21, and SOCS3 (Fig. 5E). In contrast, IL-22 challenge failed to promote mouse hepatocyte senescence (supporting Fig. S5c).

IL-22 induces HSC senescence via a STAT3-dependent mechanism

To test the role of STAT3, a key downstream transcription factor of IL-22, in IL-22 induction of HSC senescence, we generated STAT3^{-/-} HSCs. STAT3 protein deletion was confirmed by western blotting (Fig. 6A). As illustrated in Figs. 6B-C and supporting Fig. S6a, IL-22 treatment upregulated SA- β -gal activity and the expression of p-p53^{ser15}, p53, and p21 in WT HSCs but not in STAT3^{-/-} HSCs.

Additionally, we generated caSTAT3⁺ cells that overexpress constitutively activated STAT3 (caSTAT3) to further determine the function of STAT3 in HSC senescence. The expression of Flag-ca-STAT3 was confirmed by western blotting (Fig. 6D). Infection with Ad-Flag-ca-STAT3 markedly decreased α -SMA protein expression (Fig. 6D) but increased SA- β -Gal staining in HSCs (Fig. 6E and sFig. 6b). Moreover, the introduction of Flag-ca-STAT3 increased the expression of p-p53^{ser15}, p53, and p21 in HSCs (Fig. 6F).

SOCS3 is required for IL-22-induced senescence and p53 activation in HSCs

SOCS1 has been shown to induce cellular senescence by binding to p53.¹⁸ Furthermore, the structure of SOCS3 is similar to SOCS1, both of which have a kinase inhibitory region (KIR) capable of binding to p53.¹⁸ This led us to hypothesize that SOCS3, a STAT3-regulated gene, may contribute to the IL-22-mediated induction of HSC senescence. As illustrated in Figs. 7A-B, IL-22 exposure upregulated the expression of SOCS3 mRNA and protein in HSCs. To investigate the importance of SOCS3 in IL-22-induced senescence, we generated SOCS3^{-/-} HSCs and confirmed SOCS3 deletion by real-time PCR (Fig. 7C). IL-22 treatment induced accumulation of a higher number of SA- β -gal⁺ HSCs in WT HSCs when compared with SOCS3^{-/-} HSCs, indicating that SOCS3^{-/-} HSCs are refractory to IL-22-induced senescence (Fig. 7D and supporting Fig. S7). Furthermore, the deletion of SOCS3 abrogated the IL-22-mediated induction of p53 and its target genes (Fig. 7E).

To investigate how SOCS3 modulates the activity of p53, we performed an immunoprecipitation assay with anti-p53 or anti-SOCS3 antibodies in HSCs. As shown in Fig. 7F, IL-22 treatment or caSTAT3 overexpression upregulated both p53 and SOCS3 proteins. Immunoprecipitation assays showed that p53 and SOCS3 bound each other in HSCs and that IL-22 treatment or caSTAT3 overexpression promoted the interaction between p53 and SOCS3.

Discussion

In this study, we have demonstrated for the first time that HSCs, cells of non-epithelial origin, express IL-22R1 and IL-10R2. Through the ligation of both receptors, IL-22 induces HSC senescence, resulting in the inhibition of liver fibrosis. Furthermore, results from our mechanistic studies suggest that IL-22 induction of HSC senescence is mediated via the activation of a STAT3-SOCS3-p53 signaling axis as summarized in Fig. 8.

IL-22R1 has been classically thought to be expressed exclusively in epithelial cells.¹⁻³ Interestingly, our study demonstrates the detection of high levels of IL-22R1 mRNA and protein expression in quiescent and activated primary mouse HSCs, primary human HSCs, and the human HSC cell line LX2. HSCs are thought to originate from mesodermal mesothelial cells/submesothelial cells,¹⁹ and differ from hepatocytes and biliary epithelial cells, which are derived from the embryonic endoderm. Additionally, the expression of IL-22R1 was reported on colonic subepithelial myofibroblasts.²⁰ Therefore, there is evidence that, in addition to epithelial cells, some non-epithelial cells such as quiescent HSCs, activated HSCs/myofibroblasts, subepithelial myofibroblasts, and skin fibroblasts also express IL-22R1.

Upon binding to IL-22R1 and IL-10R2, IL-22 promotes epithelial cell (e.g., hepatocyte) proliferation and survival.⁴ In the present paper, we have demonstrated that IL-22 also promotes HSC survival but induces HSC senescence rather than stimulating HSC proliferation. Our study shows that the overexpression of IL-22 via either gene targeting (transgenic) or the exogenous administration of Ad-IL-22 increased the number of senescent HSCs within the fibrotic scars of the livers of CCl₄-treated mice. Furthermore, we show that IL-22 challenge modulates the expression of “senescence-associated secretory phenotype” genes¹⁰ by upregulating pro-inflammatory genes and MMP9, and by downregulating TIMP1/2 genes in the liver *in vivo* and in cultured HSCs *in vitro*. Finally, *in vitro* IL-22 treatment increased SA- β -gal activity and the expression of the cellular senescence-associated genes p53 and p21. The upregulation of these genes likely contributes to IL-22-mediated HSC senescence, as the p53-p21 axis is known to inhibit the cell cycle.²¹⁻²³

Our study also provided evidence suggesting that the IL-22-dependent upregulation of p53 and p21 is mediated through STAT3 and SOCS3, resulting in HSC senescence. Although there is no evidence suggesting that STAT3 directly promotes cellular senescence, several STAT3 downstream target genes have been shown to induce cellular senescence, including p53, p21, and the suppressor of cytokine signaling (SOCS) family.^{18, 21-24} Our data in this paper showed that the deletion of STAT3 abolished IL-22-mediated induction of p53, p21, and HSC senescence, whereas the overexpression of caSTAT3 promoted HSC senescence (Fig. 6). This suggests that STAT3 plays an important role in the IL-22-mediated HSC senescence via the induction of p53 and p21.

SOCS3 is an important feedback suppressor for STAT3 activation during normal cytokine signaling. Our results support another aspect of SOCS3 function in that SOCS3 directly binds to p53, thus enhancing expression of p53 protein and p53 target genes. As shown in Fig. 7, the deletion of SOCS3 abolished the IL-22-mediated induction of p53 and p53-

regulated genes. Immunoprecipitation experiments between SOCS3 and p53 suggest that these proteins directly interact with each other. However, how SOCS3 activates p53 remains unknown. SOCS1 has been reported to form a ternary complex with p53 and Ataxia telangiectasia mutated (ATM) kinase through the KIR domain of SOCS1, followed by activating p53, increasing p53 protein expression, and subsequently promoting oncogene-induced senescence.¹⁸ SOCS3 also contains a KIR, which suggests that the KIR in SOCS3 might be responsible for SOCS3 binding to and activating p53, and resulting in HSC senescence.

Although many studies have suggested that senescent cells eventually succumb to p53-mediated cell death,²⁵ our findings imply that IL-22 promotes HSC senescence and does not induce but rather prevents HSC apoptosis. We suspect that this may be because IL-22 activates STAT3, which not only promotes cell senescence via the induction of p53 but also acts as a key transcriptional factor for cell survival via the induction of the anti-apoptotic genes Bcl-2 and Bcl-xL. Interestingly, other groups have also reported the simultaneous observation of resistance to apoptosis and senescence in human fibroblasts.²⁶

The induction of activated HSC senescence plays an important role in limiting the fibrogenic response, which is likely mediated by enhancing the *in vivo* clearance of senescent HSCs presumably by NK cells, the downregulation of collagen and TIMP expression, and the upregulation of MMP expression.^{9, 10} Interestingly, the *in vitro* treatment of HSCs with IL-22 not only induces HSC senescence but also downregulates α -SMA expression in these cells (Fig. 5). Because senescent HSCs are not associated with the downregulation of α -SMA,¹⁰ the decreased α -SMA expression in response to IL-22 may not be directly due to senescence, and a distinct mechanism may be involved, which will require further studies to elucidate.

In addition to IL-22/STAT3 induction of HSC senescence, the well documented hepatoprotective and mitogenic effects of IL-22/STAT3 in hepatocytes^{4, 5} may also contribute to the observed amelioration of liver fibrosis by IL-22. Indeed, the administration of Ad-IL-22 markedly inhibited liver fibrosis in STAT3^{Hep-/-} mice, but the degree of inhibition was lower when compared with that in WT mice, indicating that IL-22 inhibits liver fibrogenesis via hepatocyte STAT3-dependent and independent mechanisms. Moreover, the administration of Ad-IL-22 increased liver regeneration (supporting Fig. S4e), which is shown to ameliorate liver fibrosis and may contribute to the anti-fibrotic effect of IL-22.¹⁷ Although the hepatoprotective effects of IL-22 have been well-documented,^{4, 5} IL-22TG mice displayed a similar extent of liver injury after chronic CCl₄-treatment when compared with WT mice (supporting Fig. S3d). Thus, in our model of CCl₄-induced liver injury, the IL-22-mediated inhibition of liver fibrosis is not completely mediated via its hepatoprotective effects. However, IL-22 has been shown to protect against hepatocellular damage in various models of liver injury.^{4, 5} Thus, the hepatoprotective function of IL-22 likely plays an important role in inhibiting liver fibrosis in these models.

It has been reported that hepatic IL-22 levels are elevated in viral hepatitis patients; however, the effect of IL-22 on liver injury and fibrosis in these patients remains obscure. We have previously shown that the number of IL-22⁺ lymphocytes positively correlates with the grade of inflammation and the serum ALT or AST levels in viral hepatitis patients.¹³ However, a recent study has shown that hepatic IL-22 expression inversely correlates with the histological activity index and the fibrosis stage in HBV patients.¹⁴ These findings suggest that elevated hepatic IL-22 levels may play a compensatory role in preventing liver injury and fibrosis in viral hepatitis patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

αSMA	α -smooth muscle actin
caSTAT3	constitutively activated STAT3
CCl₄	carbon tetrachloride
CHX	cycloheximide
FACS	Fluorescence-activated cell sorting
HSCs	hepatic stellate cells
IL-22	interleukin-22
IL-22TG mice	IL-22 liver-specific transgenic mice
KIR	a kinase inhibitory region
MMP9	matrix metalloproteinase-9
p-p53^{ser15}	phosphorylated p53 at serine 15
SA-β-Gal	senescence-associated β -galactosidase
SOCS3	suppressor of cytokine signaling 3
STAT3	signal transducer and activator of transcription 3
TIMP	Tissue inhibitor of metalloproteinase
WT	wild-type

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Fig. 1. IL-22 activates STAT3 in HSCs that express high levels of IL-22 receptors

(A) Primary mouse HSCs (mHSCs) were cultured for various time periods, followed by RT-PCR analyses. Hepatocytes and Kupffer cells served as the positive and negative control for IL-22R1 expression, respectively. (B) 1d- or 7d-cultured HSCs were treated with IL-22 (10ng/ml) for 24h, followed by RT-PCR analyses. (C) 7d-cultured HSCs were treated with IL-22 for 24h or 48h, followed by western blotting. (D) RT-PCR analyses of IL-22R genes from primary human HSCs (hHSCs) and LX2 cells. (E) HSCs and LX2 cells were treated with IL-22 for various time periods, followed by western blotting.

Fig. 2. IL-22 inhibits HSC apoptosis

(A) Primary HSCs were treated with IL-22 for 24h and then CHX for 4h, followed by staining with acridine orange and counting the number of apoptotic HSCs, or by measuring caspase 3/7 activity. RFU: relative fluorescence units. (B) Western blotting of cleaved caspase-3 from IL-22+CHX-treated HSCs that were described in panel A. (C) Primary HSCs were cultured without serum for 24h, followed by adding IL-22, serum, or PDGF for additional 24h. Cell proliferation was measured by BrdU incorporation. (D) HSCs were cultured for 4d and then treated with IL-22 for 24h or 48h and subjected to western blotting. ** $P < 0.01$, * $P < 0.05$.

Fig. 3. IL-22TG mice are resistant to HSC apoptosis and fibrosis induced by chronic CCl₄ treatment

WT and IL-22TG mice were injected with CCl₄ for 8 weeks and sacrificed at 1d and 5d after the final injection. (A) Representative pictures of liver tissue sections stained with TUNEL plus anti-α-SMA antibody (x400), or with anti-α-SMA antibody (x40), or with Sirius red (x200) are shown. Arrows indicate α-SMA⁺TUNEL⁺ cells. (B) The number of α-SMA⁺TUNEL⁺ cells (x400) was quantified from 20 fields of each sample; the α-SMA⁺ areas (x40) and Sirius red areas (x200) were quantified from 10 fields of each sample. Six to ten mice were used in each group. (C) Western blotting and densitometry analyses of the immunoblots are shown. (D) Liver tissues were harvested from the 5d groups in panel A, stained for SA-β-gal activity, and counterstained with eosin. The SA-β-gal⁺ areas are quantified and shown in the right panel. ** $P < 0.01$, * $P < 0.05$.

Fig. 4. Administration of Ad-IL-22 promotes the resolution of liver fibrosis and induces HSC senescence *in vivo*

WT mice were injected with CCl₄ for 8 weeks. Ad-GFP or Ad-IL-22 was administered immediately following the final injection of CCl₄. Mice were sacrificed at 1d and 5d after the final injection. (A) Representative pictures of liver tissues stained with TUNEL plus anti-α-SMA antibody (x400), or with anti-α-SMA antibody (x40), or with Sirius red (x200) are shown. Arrows indicate α-SMA⁺TUNEL⁺ cells. (B) The number of α-SMA⁺TUNEL⁺ cells (x400) was quantified from 20 fields of each sample; the α-SMA⁺ areas (x40) and Sirius red areas (x200) were quantified from 10 fields of each sample. Six to ten mice were used in each group. (C) Western blotting and the associated densitometry analyses are shown. (D) Liver tissues were harvested from the 5d groups in panel A, stained for SA-β-gal activity, and counterstained with eosin. The SA-β-gal⁺ areas are quantified and shown in the right panel. ** $P < 0.01$, * $P < 0.05$.

Fig. 5. *In vitro* IL-22 treatment inhibits HSC activation and promotes HSC senescence
 (A) Primary HSCs were cultured for 1d or 4d followed by treatment with IL-22 for 24h or 48h and were subjected to western blotting. (B) Cultured HSCs were treated with IL-22 for 24h and were subjected to real-time PCR analysis. (C) After a 24-h incubation in serum-free medium, HSCs were incubated with IL-22 for another 2d followed by SA-β-gal staining. The number of SA-β-gal⁺ HSCs was counted. (D) Cultured HSCs were treated with IL-22 for 24h and then were subjected to real-time PCR analyses. (E) Western blotting of senescence-associated proteins from HSCs treated with IL-22. ** $P < 0.01$, * $P < 0.05$.

Fig. 6. IL-22 induces HSC senescence via a STAT3-dependent mechanism

(A) WT and STAT3^{-/-} HSCs were generated by infection of STAT3^{fllox/fllox} HSCs with Ad-GFP or Ad-Cre for 24h, respectively. STAT3 deletion was confirmed by western blotting. (B, C) WT and STAT3^{-/-} HSCs were treated with IL-22 for 2d and were stained for SA-β-gal activity, and the number of SA-β-gal⁺ HSCs was quantified (B), or the cells were subjected to western blot analysis for senescence-associated proteins (C). (D) WT and caSTAT3⁺ HSCs were generated by infection of primary HSCs with Ad-GFP or Ad-caSTAT3 for 48h, respectively. Flag-STAT3 expression was confirmed by western blotting. (E, F) WT and caSTAT3⁺ HSCs were stained for SA-β-gal activity, and the number of SA-β-gal⁺ HSCs was quantified (E), or the cells were subjected to western blot analysis for senescence-associated proteins (F). ** $P < 0.01$.

Fig. 7. SOCS3 contributes to IL-22 induction of HSC senescence via the interaction with p53 protein

(A, B) HSCs were treated with IL-22 followed by real-time PCR and western blotting for SOCS3 mRNA and protein, respectively. (C) WT and SOCS3^{-/-} HSCs were generated by a 24-h infection of SOCS3^{fllox/fllox} HSCs with Ad-GFP and Ad-Cre, respectively. SOCS3 deletion was confirmed by real-time PCR. (D) WT and SOCS3^{-/-} HSCs were incubated with IL-22 for 2d and were stained for SA-β-gal activity. The number of SA-β-gal⁺ HSCs was counted. (E) WT and SOCS3^{-/-} HSCs were treated with IL-22 for 4h and assessed by real-time PCR analysis for p53 and p53-regulated genes. (F) Primary HSCs were treated with IL-22 or were infected with Ad-ca-STAT3 for 2d. Cell lysates were immunoprecipitated with anti-p53 or anti-SOCS3 antibodies followed by immunoblotting for p53 and SOCS3. ***P*<0.01, **P*<0.05.

Fig. 8.

An integrated model depicting the mechanism underlying the IL-22-mediated induction of HSC senescence in a STAT3-SOCS3-dependent fashion.