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IL-10 attenuates IFN-α-activated STAT1 in the liver: involvement of SOCS2 and SOCS3

Xuening Shen, Feng Hong, Van-Anh Nguyen, Bin Gao*

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, P.O. Box 980613, Richmond, VA 23298, USA

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Abstract Interleukin-10 (IL-10) has been used in the treatment of viral hepatitis in interferon-α (IFN-α) non-responders while patients who have high levels of IL-10 are poorly responsive to IFN-α. The mechanism underlying such controversial functions of IL-10 remains unknown. Here we demonstrated that injection of IL-10 into mice attenuated IFN-α-induced signal transducer and activator transcription factor (STAT)1 tyrosine phosphorylation in the liver. Reverse transcriptase-polymerase chain reaction assay demonstrated that mouse liver expressed high levels of IL-10 receptor 2 (IL-10R2) but low levels of IL-10R1. Injection of IL-10 into mice activated STAT3 but not STAT1 tyrosine phosphorylation and induced suppressor of cytokine signal 2 (SOCS2), SOCS3, and cytokine-inducible SH2 protein (CIS) mRNA expression in the liver. Furthermore, overexpression of SOCS2 or SOCS3 inhibited IFN-α-induced reporter activity in hepatic cells. These findings suggest that IL-10 inhibits IFN-α-activated STAT1 in the liver, at least in part, by inducing SOCS2, SOCS3, and CIS expression, which may be responsible for the resistance of IFN-α therapy in patients who have high levels of IL-10 and recommends that IL-10 treatment for viral hepatitis should be cautious. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Interleukin 10; Interferon; Liver; Signal transducer and activator transcription factor; Suppressor of cytokine signal; Viral hepatitis

1. Introduction

Interleukin 10 (IL-10) is a pleiotropic cytokine produced by many cell types including Th0, Th2 $CD4^+$ T cells, $CD5^+$ B cells, thymocytes, keratinocytes, and macrophages. IL-10 exerts the anti-inflammatory effect by suppressing expression of macrophage inflammatory proteins (reviewed in [1–3]). The anti-inflammatory and immunosuppressive activities of IL-10 in vivo are clearly demonstrated in IL-10-deficient [4] and IL-10 receptor 2 (IL-10R2)-deficient (CRFB4−/−) [5] mice. In both mice, chronic colitis and splenomegaly reminiscent were developed. IL-10 treatment has been shown to decrease the severity of inflammatory processes in a variety of experimental models and clinical patients [6–11]. It is believed that IL-10 exerts its function by acting on IL-10 receptors, which consist of an α chain (IL-10Rα or IL-10R1) and a β chain (IL-10Rβ or IL-10R2). Upon IL-10 binding, IL-10R-associated tyrosine kinases (JAK1 and Tyk2) are activated and followed by phosphorylating Y446 and Y496 of IL10R1, which in turn phosphorylate signal transducer and activator transcription factor (STAT)1 and STAT3. Phosphorylated STAT1 and STAT3 form a heterodimer or homodimer, which then translocates to the nucleus to activate the transcription of many target genes, including suppressor of cytokine signal 3 (SOCS-3) (reviewed in [1–3]).

Recently, it has been shown that IL-10 has antifibrogenic and anti-inflammatory effects in the liver [12–14]. IL-10-deficient mice are more susceptible to severe CCl4-induced liver fibrosis [13,14]. The people with possession of the A allele at position −627 in the IL-10 promoter region are more susceptible to alcoholic liver disease [15]. IL-10 treatment has a protective role for liver transplantation, hepatic ischemia/reperfusion injury, and toxic liver injury [16,17]. The molecular mechanism underlying the protective effects of IL-10 in the liver is poorly understood. It is believed that IL-10 protects against hepatic liver injury by suppressing NFkB activation and subsequent expression of proinflammatory mediators (reviewed in [1–3]). In addition to T cells, B cells, and macrophages, many cell types within the liver can synthesize IL-10. These include Kupffer cells [18], hepatocytes [19], and stellate cells [20]. IL-10 expression in the liver or the levels of IL-10 in the plasma are significantly elevated in many chronic liver diseases including viral hepatitis [21]. High expression of IL-10 has also been associated with the IL-10 polymorphisms −627*C allele [22] and GCC haplotype [23,24]. Although IL-10 has been shown to play an important role in anti-inflammatory and anti-fibrosis in the liver [12–14], patients who are genetically predisposed to high IL-10 production have a poor response to IFN-α [24]. In attempting to define the mechanism by which IL-10 may antagonize IFN-α-action, we also reviewed the IFN-α signaling pathway. The binding of IFN-α to its receptor activates the receptor-associated JAK tyrosine kinases. This receptor–kinase complex interacts with and activates the SH2-containing cytoplasmic STAT transcription factors (such as STAT1, 2, or 3). Activated STAT proteins then form homo- or hetero-complexes that translocate to the nucleus to activate the transcription of many target genes, including antiviral proteins Mx, PKR, and 5-3 OAS (reviewed in [25–27]). The essential role of IFN-activated STAT1 in antiviral and anti-tumor activities is clearly demonstrated in STAT1 knockout mice [28,29]. In these mice, IFN signaling is

*Corresponding author. Fax: (1)-8048282117.
E-mail: bgao@hsc.vcu.edu

Abbreviations: IL-10, interleukin-10; IFN, interferon; STAT, signal transducer and activator transcription factor; SOCS, suppressor of cytokine signal; RT-PCR, Reverse transcriptase-polymerase chain reaction

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defective and the innate response to viral or bacterial infection is absent.

In this study, we demonstrated that injection of IL-10 attenuated IFN-α-induced STAT1 tyrosine phosphorylation in the liver, and induction of suppressor of cytokine signal 2 (SOCS2) and SOCS3 was, at least in part, responsible for such inhibition. This may explain a poor response to IFN-α in patients with high levels of IL-10 in the serum [24].

2. Materials and methods

2.1. Materials

Female ICR mice (15–20 g) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, USA). STAT1 and STAT3 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phosphotyrosine-STAT1 (Tyr^{701}) and anti-phosphotyrosine-STAT3 (Tyr^{703}) antibodies were obtained from Bio-lab (Beverly, MA, USA). IL-10 and IFN-α were purchased from Bioscience International (Camarillo, CA, USA). The SOSC2 and SOCS3 expression vectors were kindly provided by Dr. Douglas Hilton (The Walter and Eliza Hall Institute of Medical Research, Vic., Australia). The IFN-responsive reporter gene (pISG54-LUC), which was constructed by incorporating a fragment of the hamster ISG54 promoter from −429 to +31 [30] fused to the luciferase gene, was kindly provided by Dr. Levy (New York University School of Medicine, New York, USA).

2.2. Western blot analysis

Tissues were homogenized in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1% Nonidet P-40, 10% glycerol) and then centrifuged for 10 min at 4°C. The protein concentration of the supernatant (protein fraction) was calculated using the Bio-Rad protein assay. An aliquot of 40 μg of protein was mixed with an equivalent volume of 2× protein loading buffer containing β-mercaptoethanol and boiled for 5 min before loading onto an SDS/8%polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary antibodies. Membranes were washed with TPBS (0.05%, vol./vol.) Tween 20 in phosphate-buffered saline (pH 7.4) and incubated with a 1:4000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were visualized by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.3. Transient transfection and luciferase assay

Transient transfections were performed by using the lipofectin reagent (Gibco BRL) as recommended by the manufacturer. Briefly, the cells were washed twice with Opti-MEN I reduced-serum medium (Gibco BRL). For luciferase assays, 2 μg of plasmid DNA per 35 mm plate was cotransfected with 1 μg of a β-galactosidase vector (Promega, Madison, WI, USA) to allow for adjustments of transfection efficiency. After transfection, the cells were continuously incubated in reduced-serum medium for 8 h, then changed to normal growth medium for 16 h. Cells were stimulated with IFN-α and harvested after an additional 8 h period, lysed by freeze-thawing, and assayed for β-galactosidase and luciferase activities. β-Galactosidase activity was assayed as described by Sambrook et al. (1989) [31] and was expressed as $A_{420} \times \mu g\ protein^{-1} \times h^{-1} \times 100$. Protein concentrations were determined using the Bio-Rad protein assay solution as described by the manufacturer, and bovine serum albumin as standard. Luciferase activity was assayed using the luciferase enzyme assay system (Promega) on a luminometer (Bio-Rad). All values of luciferase in cell extracts were normalized to the β-galactosidase activity in the same extracts. All transfections were replicated at least three times with similar results.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The RT-PCR was carried out as described previously [32]. The following mouse primer pairs were used: IL-10R1 (508 bp): forward (5′ AGG CAG AGG CAG CAG GCC CAG CAG AA 3′) and reverse (5′ TGG AGC CTG GCT AGC TGG TCA CAG 3′); IL-10R2 (400 bp): forward (5′ GCC AGC TCT AGG AAT GAT TC 3′) and reverse (5′ AAT GTT CTT CAA GGT CCA C 3′); SOCS1 (350 bp): forward (5′ CAC GCC GAT TAC CGG CGC ATC 3′) and reverse (5′ GCT CCT GCA GCG GCC GCA CG 3′); SOCS2 (300 bp): forward (5′ AAG ACA TCA GCC GGG CCG ACT A 3′) and reverse (5′ GTC TTG TTG GTA AAG GTA GTC 3′); SOCS3 (450 bp): forward (5′ GGA CCA GCG CCA CTT CTT CAC 3′) and reverse (5′ TAC TGG TCC AGG AAC TCC CGA 3′); cytokine-inducible SH2 protein (CIS) (213 bp): forward (5′ TAG TGA CTC GGT GCT GCC TAT C 3′) and reverse (5′ GTG CCT GGC TCA GTC AGA GTT G 3′).

3. Results

3.1. IL-10 attenuates IFN-α-induced STAT1 tyrosine phosphorylation without affecting the levels of STAT1 protein expression in the liver

To test whether IL-10 regulates IFN-α signaling pathway in the liver, ICR mice were intravenously injected with IL-10 for various time periods and followed by administration of IFN-α for 30 min. Liver extracts were prepared and IFN-α-activated STAT1 was analyzed. As phosphorylation of STAT1 at Tyr^{701} is essential for dimerization and DNA binding of STAT induced by IFN-α, phosphorylation at this site is an excellent marker of the IFN-α signaling pathway [25–27]. As shown in Fig. 1, injection of IL-10 for 2 h markedly attenuated IFN-α-induced STAT1 tyrosine phosphorylation in the liver. Injection of IL-10 for 4 or 8 h almost completely abolished IFN-α-induced STAT1 tyrosine phosphorylation. The same density of unphosphorylated STAT1 in the bottom panel indicated that IL-10 and/or IFN-α treatment did not affect the levels of STAT1 protein expression in the liver. These findings indicate that IL-10 is able to suppress the IFN-α signaling pathway in the liver in vivo.

3.2. Mouse liver expresses high levels of IL-10R2 but low levels of IL-10R1

The above data suggested that IL-10 might directly target the liver. To further confirm this notion, we examined whether mouse liver expresses IL-10R. As shown in Fig. 2, mouse spleen and thymus expressed high levels of both IL-10R1 and IL-10R2, while the liver expressed high levels of IL-10R2 but low levels of IL-10R1. These findings suggest that mouse liver expresses both IL-10R1 and IL-10R2.

3.3. IL-10 activates STAT3 but not STAT1 tyrosine phosphorylation in the liver and spleen

It has been shown that IL-10 activated STAT1 and STAT3

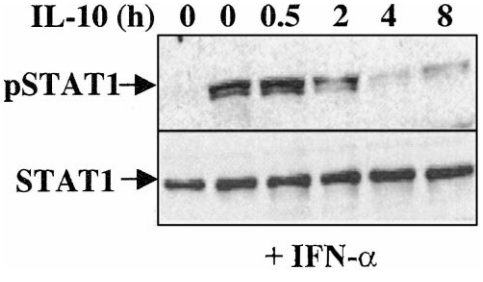

Fig. 1. IL-10 attenuates IFN-α-induced STAT1 tyrosine phosphorylation in the liver in vivo. Mice were injected intravenously with IL-10 (40 μg/kg body weight (bwt)) for various time periods as indicated and followed by injection of IFN-α (40 μg/kg bwt) for 30 min. Liver extracts were prepared and then subjected to Western blot analysis by using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 antibodies. Blots shown are representative of three independent experiments.

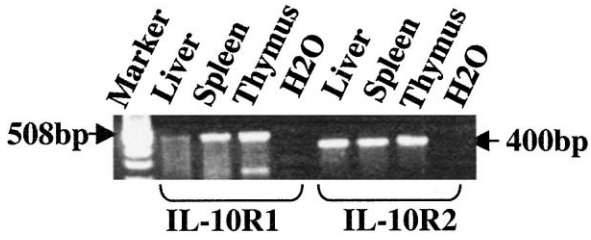

Fig. 2. Mouse liver expresses high levels of IL-10R2 but low levels of IL-10R1. Total RNA was isolated from mouse liver, spleen, or thymus, and then subjected to RT-PCR by using mouse IL-10R1 or IL-10R2 primers, as described in Section 2. The PCR products were run on a 1.5% agarose gel and visualized by staining with ethidium bromide.

in a variety of cells [1–3], but the IL-10 signaling pathway in the liver remains unknown. To determine IL-10 signal transduction in the liver, mice were intravenously injected with IL-10 and phosphorylation of STATs was detected. As shown in Fig. 3, injection of IL-10 markedly induced STAT3 but not STAT1 tyrosine phosphorylation in the liver and spleen, while the positive control IFN-α markedly activated STAT1 in both the liver and spleen. Interestingly, IFN-α activated STAT3 in the spleen but not in the liver. The unchanged levels of STAT1 and STAT3 proteins in the liver and spleen indicated that IL-10 treatment did not affect the levels of STAT1 and STAT3 protein expression. These findings demonstrate that IL-10 is able to activate STAT3 tyrosine phosphorylation in the liver and spleen.

3.4. *IL-10 induces SOCS2, SOCS3, and CIS mRNA expression in the liver*

It has been shown that IL-10 was able to induce SOCS mRNA expression in a variety of cells [33,34], we asked whether IL-10 could also induce SOCS mRNA expression in the liver. As shown in Fig. 4, injection of IL-10 markedly induced SOCS2, SOCS3, and CIS mRNA expression in the liver, whereas the SOCS1 mRNA was not induced. The peak of induction of SOCS2 and SOCS3 mRNA expression occurred after 8 h stimulation while the peak for CIS mRNA induction was between 2 and 8 h stimulation. After 24 h stimulation, the levels of SOCS2, SOCS3, and CIS mRNA returned to the control levels. The unchanged intensity of the β-actin in each lane indicated that the observed increase in SOCS2, SOCS3, and CIS mRNA was real and not the result of uneven loading. These findings indicate that IL-10 was able to induce expression of SOCS2, SOCS3, and CIS mRNAs in the liver in vivo.

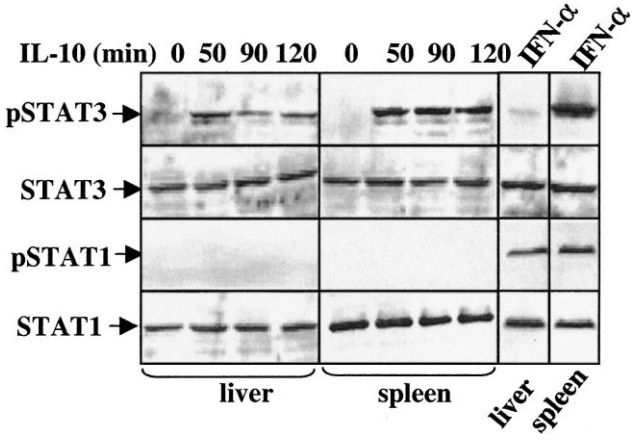

Fig. 3. IL-10 activates STAT3 but not STAT1 tyrosine phosphorylation in the liver and spleen in vivo. Mice were injected intravenously with IL-10 (40 μg/kg bwt) for various time periods as indicated, or injected with IFN-α (40 μg/kg bwt) for 30 min. Liver or spleen extracts were prepared and then subjected to Western blot analysis by using anti-phospho-STAT1 (Tyr^{701}), anti-phospho-STAT3 (Tyr^{727}), anti-STAT1 or anti-STAT3 antibodies. Blots shown are representative of three independent experiments.

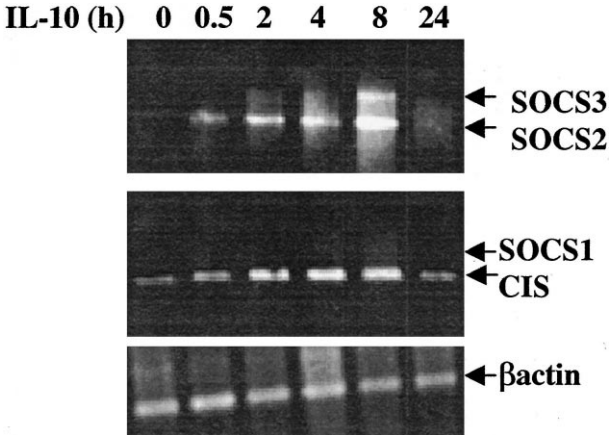

Fig. 4. IL-10 induces expression of SOCS2, SOCS3, and CIS mRNAs in the liver in vivo. Mice were injected intravenously with IL-10 (40 μg/kg, bwt) for various time periods as indicated. Total RNA was prepared from the liver and subjected to RT-PCR analysis by using various primer pairs as described in Section 2. The photograph shown is representative of two independent experiments.

3.5. *Overexpression of SOCS2 or SOCS3 attenuates IFN-α-activated reporter activity*

Induction of SOCS3 has been implicated in IL-10 suppression of IFN-α signaling pathway [33,34]. Here we examined

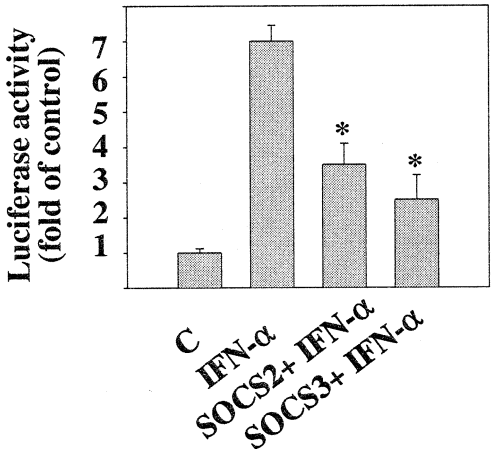

Fig. 5. Overexpression of SOCS2 or SOCS3 attenuates IFN-α-activated reporter activity in HepG2 cells. HepG2 cells were transfected with pISG54-Luc alone, or cotransfected with SOCS2 or SOCS3 expression vectors. After transfection for 16 h, cells were treated with IFN-α (250 units/ml) for 8 h. Cell extracts were then harvested and assayed for luciferase activity. C (control) represents transfection with pISG54-Luc alone without IFN-α stimulation. Values shown are means ± S.E.M. from three independent experiments, expressed as fold changes over controls. Significant differences from the IFN-α stimulation group are indicated by asterisks; i.e., * denotes $P < 0.01$.

whether overexpression of SOCS2 or SOCS3 also attenuated IFN-α-activated reporter activity in HepG2 cells. As shown in Fig. 5, IFN-α treatment markedly induced ISG54-luciferase activity (about 7 fold control). Cotransfection of SOCS2 or SOCS3 significantly inhibited IFN-α-induced ISG54-luciferase activity. These findings suggest that induction of SOCS2 or SOCS3 in the liver by IL-10 may be, at least in part, responsible for IL-10 suppression of IFN-α-activated STAT1.

4. Discussion

Although it has been shown that IL-10 has anti-fibrogenic and anti-inflammatory effects in the liver, IL-10 signaling pathway in the liver has not been explored. Here we demonstrate that mouse liver expresses both IL-10R1 and IL-10R2, and injection of IL-10 markedly activated STAT3 but not STAT1 tyrosine phosphorylation in the liver. These findings suggest that IL-10 can directly target the liver. Since the STAT3 activation was measured in the whole liver extracts, it remains to be determined whether IL-10 activated STAT3 in hepatocytes or other hepatic cells. However, the whole liver extracts are largely representative of hepatocytes, it is very likely that IL-10 can activate STAT3 in hepatocytes in vivo. Further studies will be required to confirm this notion. STAT3 activation by IL-6 has been shown to initiate liver regeneration and protect liver injury [35,36]. Therefore, in addition to its anti-inflammatory effect, IL-10 activation of STAT3 in the liver may also be involved in the protective role of IL-10 in liver transplantation, hepatic ischemia/reperfusion injury, and toxic liver injury [16,17].

The molecular mechanism by which IL-10 attenuates IFN-α signaling in the liver in vivo was also explored in this paper. Several mechanisms responsible for inhibition of the JAK-STAT signaling pathway have been proposed. For example, activated JAK-STAT can be attenuated by (1) dephosphorylation [37–39], (2) proteolytic degradation [40,41], (3) inhibitory molecules such as SOCS/JAB/SSI/CIS [42–44], which are relatively small proteins that contain a central SH2 domain and a conserved C-terminal SOCS-box. It is believed that SOCS and CIS attenuated the JAK-STAT signaling pathway by binding to the phosphorylated tyrosine residues on JAK proteins [42–44]. Induction of inhibitory protein SOCS3 has been implicated in IL-10 suppression of IFN-α-activated STAT1 in human monocytes [33] and neutrophils [34] in vitro. Two lines of evidence from this report suggest that SOCS2 and SOCS3 may also be involved in IL-10 suppression of IFN-α-activated STAT1 in the liver in vivo. First, injection of IL-10 induced expression of SOCS2, SOCS3, and CIS mRNAs. Second, overexpression of SOCS2 or SOCS3 attenuated IFN-α-activated reporter activity in hepatic cells. However, how IL-10 induces SOCS2, SOCS3, and CIS mRNAs in the liver in vivo remains unknown. It has been shown that IL-10 only selectively induces SOCS3 in human monocytes [33] and neutrophils [34] in vitro, and this induction is rapid and independent of STAT1/STAT3 phosphorylation. Here we have shown that IL-10 was able to induce not only SOCS3 but also SOCS2 and CIS in the liver in vivo, the peak of induction observed at 8 h after injection. This suggests that IL-10 induction of SOCS in the liver in vivo may be indirect. Further studies will be required to clarify this mechanism.

In summary, in the present paper, we demonstrated for the first time that IL-10 inhibits IFN-α-activated STAT1 in the liver in vivo. These findings may have two clinical implications. First, IL-10 inhibition of IFN-α signaling pathway in the liver may explain why patients who are genetically predisposed to high IL-10 production have a poor response to IFN-α [24]. Second, current forms of IL-10 treatment for viral hepatitis [45,46] must be carefully designed. A combination of IL-10 and IFN-α treatment for viral hepatitis should be avoided. It has been reported that IL-10 also attenuated IFN-α-activated STAT1 in human monocytes [33] and neutrophils [34], which may further decrease the antiviral activity of IFN-α in vivo.

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