ROLE OF IL-6 IN THE INDUCTION OF THE LIVER MONOOXYGENASE SYSTEM

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Summary

IL-6 is a soluble mediator with pleiotropic effects on inflammation, immune response, and hematopoiesis. The various functions of IL-6 were first studied and given different names based on their biological activities. For example, the name B cell stimulating factor 2 (BSF-2) was based on the ability to induce the differentiation of activated B cells into antibody (Ab)-producing cells (Kishimoto 1985), on the effect of acute phase protein synthesis on hepatocytes, the name hybridoma growth factor (HGF) to enhance the growth of fusion cells between plasma cells and myeloma cells or the name interferon (IFN)-β2 due to its antiviral IFN activity. Aim of the study: to evaluate the role of IL-6 in the induction of the liver monooxygenase system.

Key words: IL-6, immunity system, liver monooxygenase system

References to the article

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IL-6 is a soluble mediator with pleiotropic effects on inflammation, immune response, and hematopoiesis. The various functions of IL-6 were first studied and given different names based on their biological activities. For example, the name B cell stimulating factor 2 (BSF-2) was based on the ability to induce the differentiation of activated B cells into antibody (Ab)-producing cells (Kishimoto 1985), on the effect of acute phase protein synthesis on hepatocytes, the name hybridoma growth factor (HGF) to enhance the growth of fusion cells between plasma cells and myeloma cells or the name interferon (IFN)-β2 due to its antiviral IFN activity. When BSF-2 DNA was successfully cloned in 1986 (Hirano et al. 1986), it was found that molecules with different names studied by different groups were virtually identical, leading to the common name IL-6 (Kishimoto 1989). Human IL-6 consists of 212 amino acids, including a 28 amino acid signal peptide, and its gene is mapped to chromosome 7p21. Although the core protein is about 20 kDa, glycosylation accounts for 21–26 kDa of native IL-6. After the synthesis of IL-6 in the local focus at the initial stage of inflammation, it moves through the bloodstream to the liver, after which there is a rapid induction of a wide range of acute-phase proteins, such as C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, haptoglobin and α1-antichymotrypsin (Fig. 1) (Heinrich et al., 1990). On the other hand, IL-6 reduces the production of fibronectin, albumin and transferrin. These biological effects on hepatocytes were first studied as being related to HSF. When high concentrations of SAA are maintained for long periods of time, it leads to serious complications in a number of chronic inflammatory diseases through the formation of amyloid A amyloidosis (Gillmore et al., 2001). This leads to the deposition of amyloid fibrils, which causes progressive deterioration of various organs. IL-6 is also involved in the regulation of serum iron and zinc levels through the control of their transporters. Regarding serum iron, IL-6 induces the production of hepcidin, which blocks the action of the iron transporter ferroportin 1 on the intestine and thus reduces serum iron levels (Nemeth et al. 2004). This means that the IL-6-hepcidin axis is responsible for the hypoferremia and anemia associated with chronic inflammation. IL-6 also upregulates the expression of the zinc importer ZIP14 on hepatocytes and thus causes the hypozincemia observed in inflammation (Liuzzi et al. 2005). When IL-6 reaches the bone marrow, it promotes the maturation of megakaryocytes, which leads to the release of platelets (Ishibashi et al., 1989). These changes in acute phase protein levels and red blood cell and platelet counts are used to assess the severity of inflammation in routine clinical laboratory tests.

Aim of the study. to evaluate the role of IL-6 in the induction of the liver monooxygenase system.

Materials and methods of research. 50 Wistar rats of both sexes weighing 20-25 g were studied. Aseptic inflammation was created in 30 rats. To determine the content of IL-6 in blood serum, a set of reagents was used for its quantitative determination in human biological fluids and cultural

media produced by Vector-Best CJSC, Novosibirsk, Russian Federation. Before testing, a set of reagents and serum samples were thawed and heated to 18-25 degrees with ambient air for 30 minutes. The sensitivity of the method is 1 pg/ml. Measuring range is 0-500 pg/ml.

Control samples and test sera (100 µl each), pre-diluted with a buffer solution in a ratio of 1:1, were added to the wells and incubated for 120 minutes at room temperature in an automatic shaker-incubator (Elmi, Latvia) at 700 RPM. The strips were then washed five times using a BioRad automatic washer with 300 µL of 1:25 Tween phosphate-buffered saline wash solution as recommended by the reagent kit manufacturer.

At the next stage, a solution of conjugate No. 1 - biotinylated antibodies to IL-6 - was added to the wells in an amount of 100 µl. The plates were incubated under film for 60 minutes at room temperature in a shaker at 700 RPM, then washed five times with 300 µl of Tween-supplemented phosphate-buffered saline wash solution.

Then, 100 µl of a solution of conjugate No. 2, streptavidin-horseradish peroxidase, was added to each well. Incubation was carried out in a shaker at 700 RPM at room temperature for 30 minutes, then the strips were washed five times with 300 µl of a washing solution of phosphate-buffered saline with the addition of Tween.

Next, 100 µl of tetramethyl benzoate solution was added to each well with the addition of a substrate buffer solution at the concentration specified by the manufacturer. The plates were incubated protected from light at room temperature for 30 minutes.

The reaction was stopped by adding 100 µl of a stop reagent solution and the optical density of the solutions in the wells was measured using a Uniplan spectrophotometer with a vertical beam with a wavelength of 450 nm within 15 minutes after stopping the reaction relative to air.

The concentration of IL-6 in the studied blood serum samples was determined by measuring the optical density of the solution using a calibration curve. The calibration curve was constructed using the optical density values of the solution in the control wells (ordinate axis). Solutions with a given concentration of IL-6 (abscissa axis) were used as controls. IL-6 in physiological solution was administered intravenously to Wistar rats into the tail vein at a rate of 0.25 mg per kg of body weight and decapitated after 1, 2, 4, 8, 16 and 24 hours under ether anesthesia.

Statistical processing was carried out using parametric and non-parametric research methods.

Results. Acute liver injury was induced by a single injection of 3 ml/kg body weight of 20% (v/v) CCl4 solution. Serum sIL-6R levels measured by ELISA were significantly increased after CCl4 treatment. As shown in Fig. 1, immediately after induction of damage there was a more than twofold increase in the level of endogenous sIL-6R. Increased levels of sIL-6R persisted for 48 hours, indicating that IL-6 signaling was induced upon liver injury by CC l4 treatment.

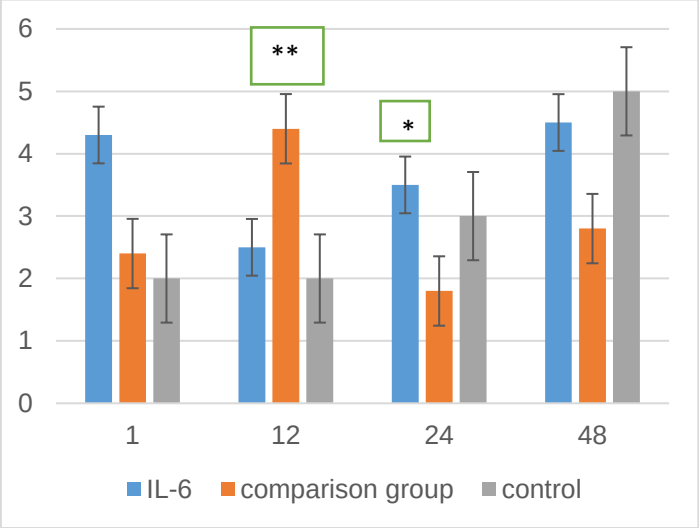


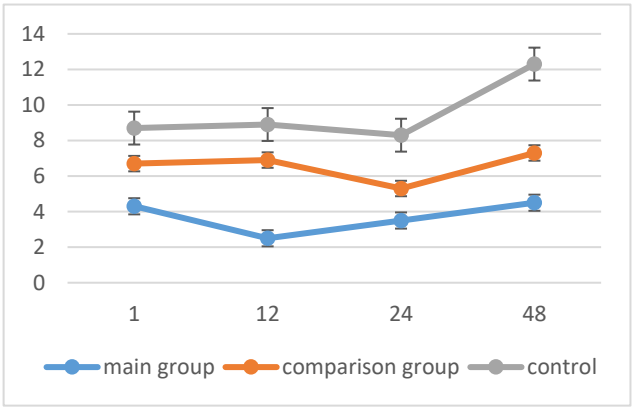
Figure 1. Serum levels of soluble IL-6 receptor (sIL-6R) after CC l4-induced liver injury. C57Bl/6 N mice were administered i.p. with a single dose of 3 ml/kg body weight of 20% (v/v) CCl4 solution or sham treatment. Serum sIL-6R levels were measured by ELISA at the indicated time points. sIL-6R levels were significantly increased immediately ($P < 0.0001$), 24 hours ($P < 0.005$), and 48 hours ($P < 0.0001$) after CCl4 treatment.

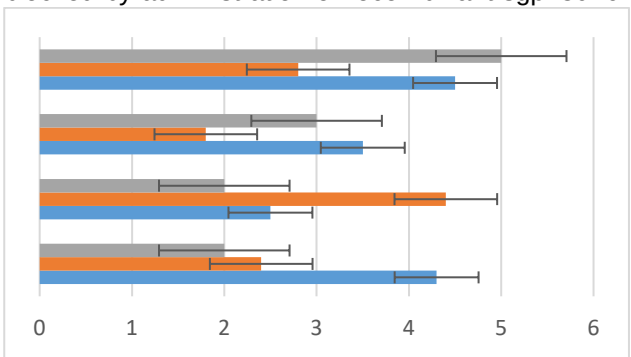
Liver damage after CCl4 treatment was quantified by measuring serum levels of ALT and

AST (Fig. 2A, B). To specifically block IL-6 signaling, mice were administered 250 μg of sgp130Fc intraperitoneally per mouse 18 h before CCl4 treatment. Serum sgp130Fc levels ranged between 35-55 μg/ml, which was confirmed by ELISA measurements and was therefore shown to be high enough to inhibit the activity of the IL-6/sIL-6R complexes [20,22] throughout the course of the experiment (adj. Fig. 1). Treatment of mice with the cytokine constructor Hyper-IL-6 did not result in significant changes in serum ALT levels compared to treatment with CCl4 alone. It should be noted, however, that Hyper-IL-6 was administered only once, 18 hours before CCl4 treatment.

Since the half-life of Hyper-IL-6 is approximately 24 hours, the cytokine is expected to remain in the bloodstream after 48 hours. We therefore believe that in order to thoroughly test the beneficial effects of Hyper-IL-6 on liver injury, experiments with daily injections of the Hyper-IL-6 protein or with genetic delivery via adenovirus should be performed.

CCl4 treatment increased ALT and AST levels at 24 and 48 hours. When IL-6 signaling was blocked by administration of recombinant sgp130Fc. protein, ALT and AST levels increased sharply after 24 hours and the increased levels were maintained after 48 hours. Hyper-IL-6 injection resulted in a slight reduction in CCl4 treatment-induced liver damage after 48 hours. Histological examination of overall liver damage 48 h after CCl4 administration showed smaller areas of necrosis in CCl4-treated mice compared to sgp130Fc pretreated mice (Fig. 2B, a, b, c, d). Quantification of liver damage 48 hours after CCl4-induced liver injury showed that only 33.2% of the liver was damaged. necrotic in CCl4-treated mice compared with 58.6% in sgp130Fc-pretreated mice. mice (Fig. 2E). There was no significant difference in quantity. Apoptotic nuclei measured by TUNEL staining (Fig. 2B, e, f). Using DAPI staining, we noted less intact nuclei and expanded areas of necrosis in animals pretreated with sgp130Fc (Fig. 2B, g, h). The decrease in hepatocyte DAPI positivity is shown in Fig. 2D. Interestingly, at 6 and 24 h, IL-6 levels were significantly higher in mice pretreated with sgp130Fc compared to control animals (Suppl. Fig. 2). These results indicated that endogenous IL6 trans-signaling was protective after CCl4 treatment





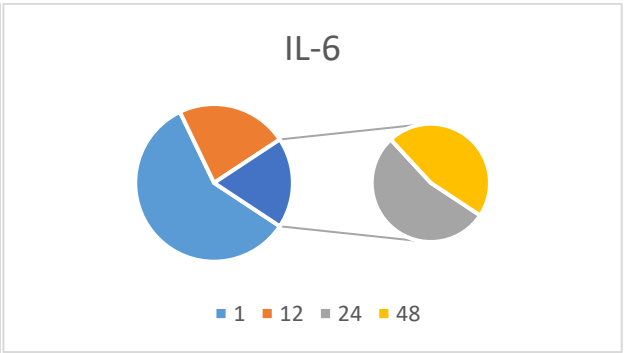


Figure 2. IL-6 indicators after exposure to CC4 at certain time intervals

Thiobarbituric acid reactive substances (TBARS) are indicators of lipid peroxidation. TBARS were elevated in sgp130Fc liver tissues. treated animals 4 and 6 h after CCl4 treatment (Fig. 3A). This increase in lipid peroxidation could be reversed when mice were treated with Hyper-IL-6 to CCl4 (data not shown). Moreover, following CCl4 treatment, blood uric acid and potassium levels were increased by sgp130Fc

treatment and decreased by Hyper-IL-6 treatment (Suppl. Fig. 3). Because uric acid and potassium are markers for oxidative and general cell damage, these data suggest that oxidative cell damage is greater when IL-6 trans-signaling is blocked.

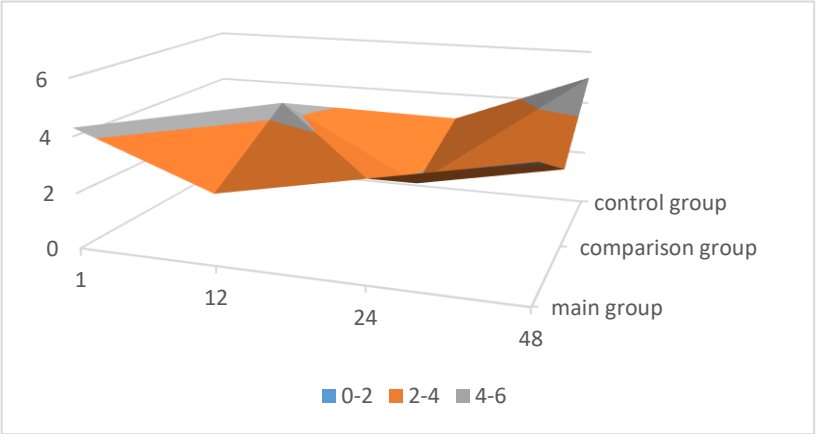


Figure 3. Statistical significance of IL-6 trans-signaling.

CCl4 liver injury depends on biotransformation catalyzed by cytochrome P450 2E1 (Cyp2E1). To measure Cyp2E1 protein expression levels, microsomes were prepared and Western blots were performed. In Fig. Figure 3B shows that Cyp2E1 protein expression is highly induced at 24 and 48 h after CCl4 treatment compared with 6 h after CCl4 treatment. This is interesting because in a recent publication, Horiguchi et al showed that when mice were treated with CCl4, CYP2E1 expression was markedly decreased 6 and 12 h after CCl4 treatment [24]. Notably, treatment of animals with sgp130Fc had no effect on Cyp2E1 protein levels. Thus, the greater liver damage in sgp130Fc-treated mice was not a result of different levels of Cyp2E1 in the liver.

Treatment of mice with CCl4 resulted in activation of the STAT3 pathway at 2 h with a peak at 4 h as measured by STAT3 phosphorylation (Fig. 4A). Treatment of mice with the sgp130Fc protein resulted in a strong decrease in phosphorylated STAT3 protein in CCl4-treated mice. Interestingly, only partial blockade of STAT3 activation was achieved by sgp130Fc treatment, indicating that gp130 on liver cells was also stimulated through membrane-bound IL-6R after CCl4 treatment. Of note, treatment of mice with sgp130Fc alone had no effect on STAT3 protein phosphorylation. Liver cell proliferation was quantified by BrdU staining. Mice were injected with BrdU 2 hours before sacrifice, and liver sections were analyzed by immunohistochemistry. Quantification of positive cells showed that liver cell proliferation is, at least in part, dependent on signaling through IL-6/sIL-6R, as blockade of this pathway significantly reduced the number of proliferating cells.

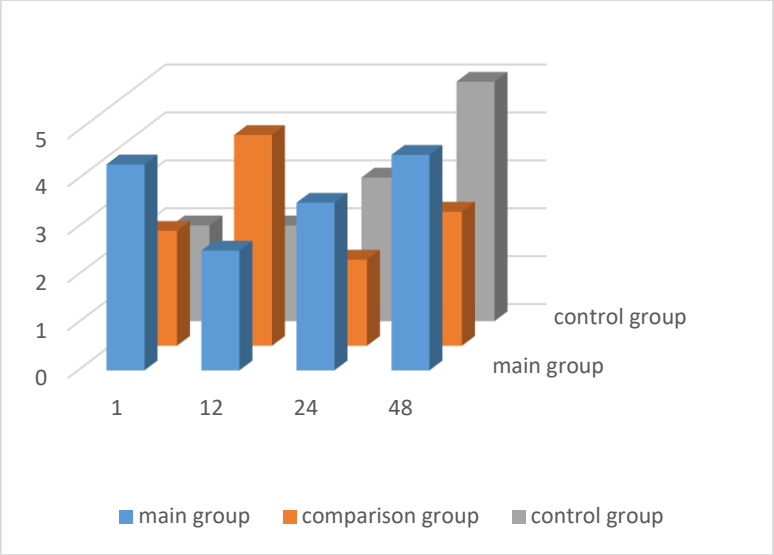


Figure 4. Some indicators of statistically significant values in the main group, comparison group and control group.

Conclusion. In the present study, we show that the liver response to acute CCl4 insult is strongly influenced by IL-6 trans-signaling through sIL-6R. Shortly after CCl4 treatment, sIL-6R levels were significantly increased. The sgp130 protein is a natural specific inhibitor of IL-6 trans-signaling [9], and we have developed a fusion protein sgp130Fc that blocks IL-6 trans-signaling without affecting classical IL-6 signaling through the membrane-bound IL-6R. [28]. In this study, we show that blockade of IL-6 trans signaling by sgp130Fc results in increased IL-6 status levels at early time points after injury. This increase in IL-6 may be explained by the increased half-life of IL-6 extended by the formation of sgp130F/sIL-6R/IL-6 immune complexes. A similar effect was found after administration of neutralizing antibodies to IL-6R to patients with rheumatoid arthritis and Castleman disease [29].

In gene-deficient mice, CCl4-mediated damage was shown to be more severe in the absence of IL-6 [19], [30]. We now demonstrate that liver damage is increased when only IL-6 signaling is blocked. Various parameters of liver injury were quantified. Serum ALT and AST levels were found to be significantly higher in the presence of sgp130Fc protein. Increased liver damage was also seen morphologically with HE staining showing massive necrotic areas. DAPI staining showed fewer intact nuclei than in the livers of control mice, indicating necrotic tissue damage.

An additional aspect of CCl4-induced liver injury is the induction of inflammation and oxidative damage. Oxidative stress is usually quantified by the amount of lipid peroxidation in liver tissue. In the present study, we show increased lipid peroxidation when IL-6 signaling was blocked. Similar results were obtained in IL-6-deficient mice [19]. Other parameters of oxidative stress or general cell damage are uric acid and potassium, which play a critical role in antioxidant defense and lipid peroxidation in human plasma [31]. These factors were activated when IL-6 trans signaling was blocked, indicating that this signaling pathway is important for protecting the body from oxidative stress.

In exploring possible explanations for these observations, we focused on the influence of infiltrated neutrophils in the liver. Recently, it was found that in a kidney injury model, the number of infiltrating neutrophils and tissue damage were lower in IL-6-deficient mice [32]. For CCl4-induced liver injury, neutrophils were found to be involved in CCl4-induced liver injury, which was reduced by neutrophil depletion [25]. We have previously demonstrated that fewer infiltrating neutrophils are detected during acute inflammation when IL-6 trans-signaling is blocked [20], [22]. In CCl4-induced liver injury, we confirmed that neutrophil depletion before CCl4 treatment reduced liver injury. However, sgp130Fc-pretreated mice had higher levels of ALT and other markers of liver damage than CCl4-treated animals, while neutrophil levels remained unchanged. Thus, we hypothesize that the effect of IL-6 trans-signaling on the extent of induced liver injury is independent of neutrophils.

It should be noted that pretreatment with sgp130Fc could very likely reduce STAT3 activation not only in the liver, but also in immune cells such as macrophages and neutrophils. Thus, inhibition of STAT3 in macrophages and neutrophils may explain the increased levels of IL-6, as in a recent study,

myeloid-specific conditional knockout of STAT3 resulted in increased IL-6 levels after CCl4 treatment compared to wild-type mice [24].

Liver glycogen is consumed 24 hours after CCl4 treatment, but is replenished 48 hours after CCl4 treatment. However, when IL-6 signaling was blocked, glycogen replenishment was significantly delayed. Interestingly, we recently noted that glycogen consumption was blocked 24 hours after d-galactosamine injury in sgp130 Fc transgenic mice, highlighting a different mechanism of CCl4 liver injury [13].

In earlier reports, we demonstrated the ability of the IL-6/sIL-6R complex to induce liver regeneration [6], [7], [8]. In the present study, we show that the concentration of sIL-6R increased after CCl4 administration. Moreover, by blocking endogenous IL-6 trans-signaling, we demonstrate that this pathway is important for the liver response to chemical injury. It is important to note that very recently the role of STAT3 was addressed in two elegant studies using conditional gene ablation [24], [33]. The authors showed that hepatocyte-specific knockout of STAT3 in mice resulted in increased liver injury when mice were treated with CCl4, but decreased liver regeneration in response to partial liver ablation [24], [33]. These studies clearly demonstrate that the role of STAT3 in the liver response to injury and injury is complex. Moreover, it should be noted that other signaling pathways induced by gp130, such as the AKT pathway, may also be important for liver integrity during injury and damage. As strategies to neutralize IL-6 have been approved in clinics in Europe and the United States for the treatment of inflammatory diseases, it will be important to monitor which IL-6 activities, when blocked, may be harmful to the body.

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