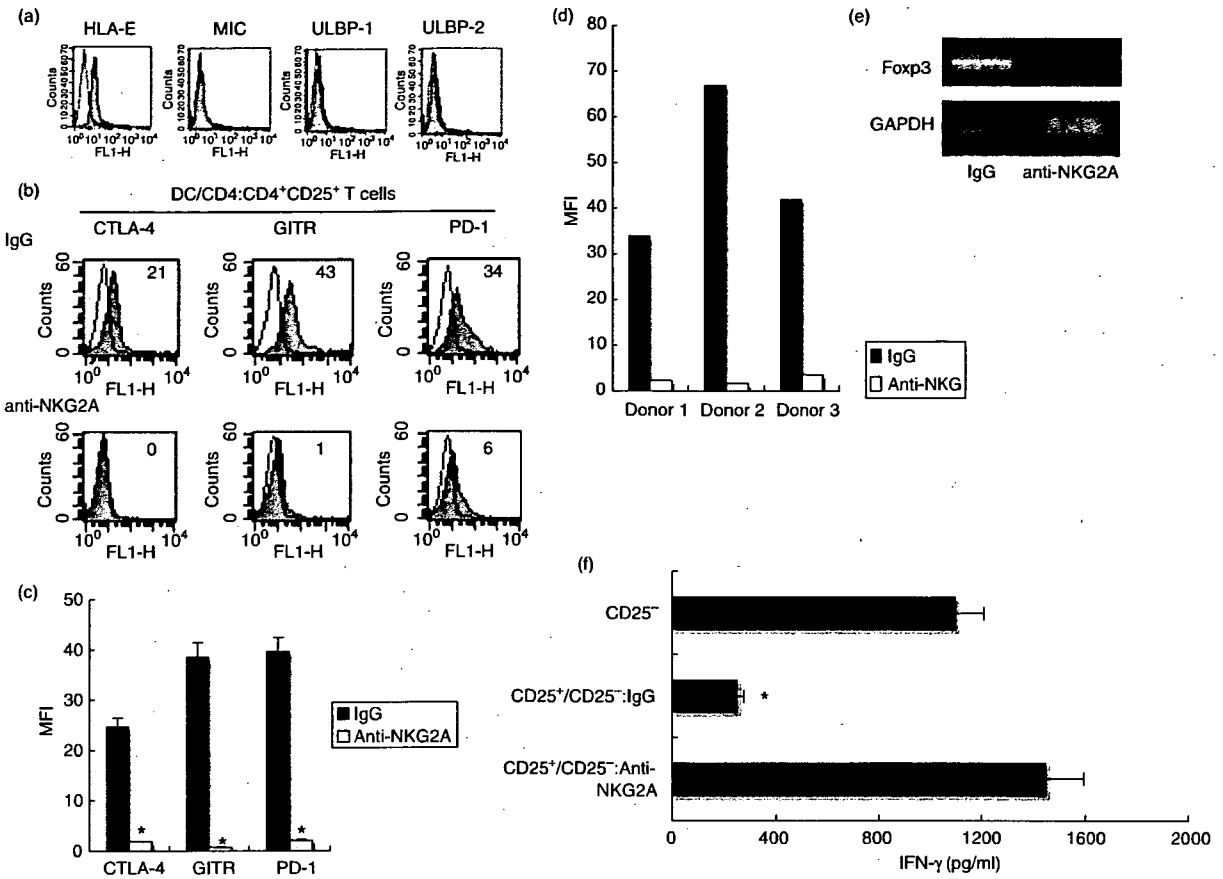

Figure 2. NKG2A signals of natural killer (NK) cells are required for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ T cells with the regulatory phenotype. (a) Surface expression of the ligands of NKG2A (HLA-E) as well as NKG2D (MIC, ULBP1 and ULBP2) in human non-transformed hepatocytes (NHs) were assessed by flow cytometry (closed histograms). Open histograms show isotype control staining. (b, c) Interleukin (IL)-2-preactivated NK cells were co-cultured with NHs in the presence of 30 μg/ml of anti-NKG2A neutralizing antibody (Ab) (anti-NKG2A) or control IgG. DCs (1×10^5) were then stimulated with the supernatant obtained from the co-cultured medium for 24 hr. After washing three times, DCs were cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ cells isolated from the co-culture were subjected to FCM for their surface expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and programmed death-1 (PD-1) (closed histograms). Open histograms show isotype control staining. Numbers on the upper right indicate the mean fluorescence intensity (MFI) of each type of stained cell. All experiments were performed three times. Representative data (b) and composite results with statistical analysis (c) are shown as the MFI of the staining cells. $^*P < 0{\cdot}05$ vs. responses of IgG group. The experiment was performed in different set of donors and similar results were obtained. (d) The inhibitory effect of anti-NKG2A Ab on PD-1 expression of $CD4^+$ $CD25^+$ T cells stimulated with allogeneic DCs from three different donors. Data are shown as MFI. (e) $CD4^+$ $CD25^+$ T cells were stimulated and purified as described above. The mRNA expression of Foxp3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was examined by reverse transcription-polymerase chain reaction (RT-PCR. (f) $CD4^+$ $CD25^+$ T cells (1×10^5/well) isolated from DC and $CD4^+$ T cell co-cultures were cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab ($CD25^+/CD25^-$). The anti-CD3 Ab-activated $CD4^+$ $CD25^-$ T cells alone were used as a positive control ($CD25^-$). Interferon (IFN)-γ was measured for each supernatant obtained after 48 hr of co-culture by enzyme-linked immunosorbent assay (ELISA). $^*P < 0{\cdot}05$. All experiments were performed three times; representative results are shown.

Suppressive actions of $CD4^+$ $CD25^+$ Treg cells, induced by NH/IL-2 NK-primed DCs, depends on PD-1-mediated negative costimulatory signals

The suppressive activities of $CD4^+$ $CD25^+$ Treg cells reportedly depend on various kinds of mediators, such as CTLA-4, IL-10 and/or TGF-β, but the exact mechanisms of the actions have not been fully elucidated.[1,6,12–14] PD-1, recently identified as a negative costimulatory receptor of the B-7 family, is expressed in $CD4^+$ $CD25^+$ Treg cells, indicating that PD-1-mediated negative signals may be involved in the regulatory functions of $CD4^+$ $CD25^+$ Treg cells.[9] Thus, we evaluated the involvement of these molecules in the suppressive activities of $CD4^+$ $CD25^+$ Treg cells. For this purpose, the blocking Ab of CTLA-4, GITR, PD-1, TGF-β or IL-10 was added

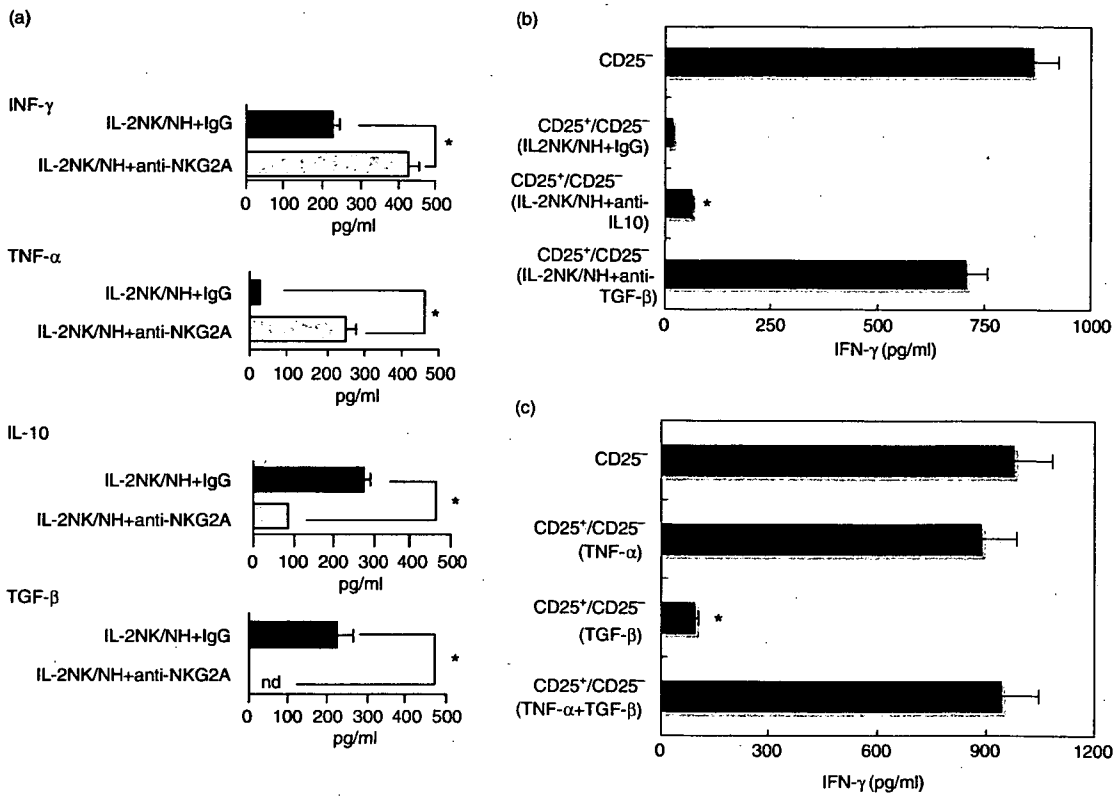

Figure 3. Change of cytokine production pattern of natural killer (NK) cells through NKG2A signals is responsible for the dendritic cell (DC) induction of $CD4^+$ $CD25^+$ Treg cells. (a) NK cells prestimulated with interleukin (IL)-2 were cultured with human non-transformed hepatocytes (NHs) in the presence of masking antibodies (Abs) of NKG2A (IL-2 NK/NH + anti-NKG2A) or isotype control IgG (IL-2 NK/NH + IgG) for 24 hr. *$P < 0{\cdot}05$. (b) IL-2 activated NK cells were co-cultured with NHs (IL-2 NK/NH). DCs (1×10^5) were stimulated with the culture supernatant in the presence of anti-IL-10, anti-transforming growth factor (TGF)-β neutralizing Ab or control IgG for 24 hr. DCs were washed thoroughly and co-cultured with allogeneic $CD4^+$ T cells for 48 hr. Next, the isolated $CD4^+$ $CD25^+$ T cells (1×10^5/well) were co-cultured with autologous $CD4^+$ $CD25^-$ T cells in the presence of plate-bound anti-CD3 Ab at a ratio of 1 : 1. Interferon (IFN)-γ production from the culture supernatant was examined by enzyme-linked immunosorbent assay. *$P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells. (c) DCs (1×10^5) were stimulated with 50 ng/ml TNF-α, 100 ng/ml TGF-β or both for 24 hr. After thorough washing, they were co-cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ T cells (1×10^5/well) were isolated from the DC and $CD4^+$ co-cultures and cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab. IFN-γ production was examined as described above. *$P < 0{\cdot}05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells.

during co-cultures of $CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$ T cells in the presence of anti-CD3 Ab. In case of natural $CD4^+$ $CD25^+$ T cells, their suppressive action was partially reversed on addition of anti-CTLA-4 Ab. By contrast, they preserved their suppressive capacity even in the presence of the blocking Ab of GITR, PD-1, TGF-β or IL-10 (Fig. 4a). When $CD4^+$ $CD25^+$ Treg cells induced by NH/IL-2 NK-primed DCs were used instead of natural $CD4^+$ $CD25^+$ T cells, their suppressive activity was markedly reduced on addition of the blocking Ab of PD-1 but not CTLA-4, IL-10, TGF-β or GITR (Fig. 4a). The regulatory functions of these Treg cells were required for direct cell-to-cell contact because separation of $CD4^+$ $CD25^+$ Treg cells and $CD4^+$ $CD25^-$ T cells in transwell chambers virtually abolished their suppressive effects (data not shown). We also confirmed the presence of PDL-1 expression on $CD4^+$ $CD25^-$ T cells when they were activated with anti-CD3 Ab (Fig. 4b), suggesting that effector cells themselves induce suppressive activities of $CD4^+$ $CD25^+$ Treg cells. Taken together, these results further reinforced the hypothesis that $CD4^+$ $CD25^+$ Treg cells induced by NH/IL-2 NK-primed DCs were different from natural $CD4^+$ $CD25^+$ Treg cells in their PD-1-dependent suppressive functions.

Discussion

Recent studies have revealed that activated NK cells positively regulate DC activation and maturation either through direct contact via NK cell receptors (NKp30, NKG2D, etc.) or in co-ordination with various kinds of cytokines (IFN-γ, TNF-α, etc.).[15–18] However, the issue of

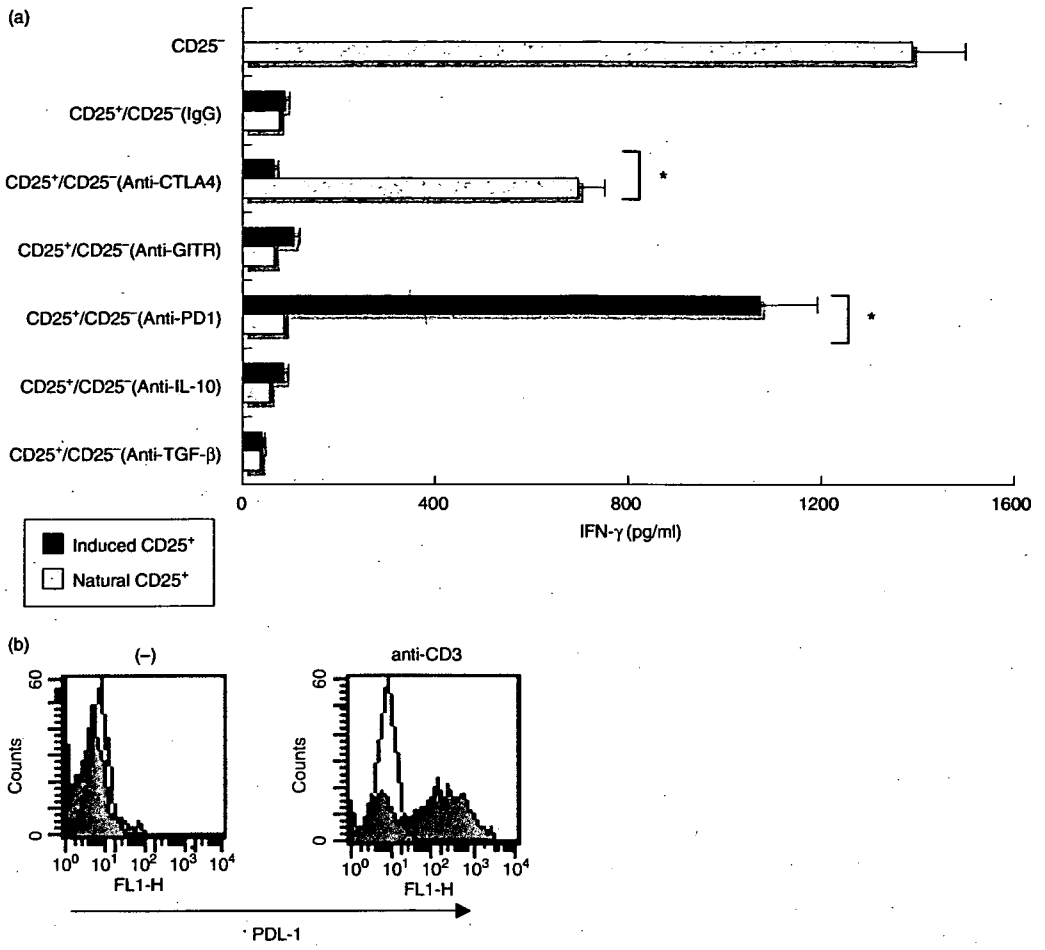

Figure 4. $CD4^+$ $CD25^+$ Treg cells induced by interleukin (IL)-2 natural killer (NK)/human non-transformed hepatocytes (NH)-treated dendritic cell (DC) suppressed T cell activation through programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) interactions. (a) DCs (1×10^5) were stimulated with the IL-2 NK/NH supernatant for 24 hr, and then cultured with allogeneic $CD4^+$ T cells for 48 hr. $CD4^+$ $CD25^+$ fractions were isolated from the DC/$CD4^+$ T cell mixtures. Freshly isolated $CD4^+$ $CD25^+$ T cells (natural $CD25^+$) or $CD4^+$ $CD25^+$ T cells induced by NK/NH-primed DCs (induced $CD25^+$) were co-cultured with freshly isolated autologous $CD4^+$ $CD25^-$ T cells at a ratio of 1 : 1 upon stimulation of plate-bound anti-CD3 antibody (Ab). Anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) Ab, anti-GITR (glucocorticoid-induced TNF receptor) Ab, anti-PD-1 Ab, anti-IL-10 Ab, anti-TGF-β Ab or isotype control IgG (20 μg/ml for each) were incubated during $CD4^+$ $CD25^+$/$CD4^+$ $CD25^-$ T cell co-cultures. Interferon (IFN)-γ was measured for each supernatant obtained after 72 hr of co-culture by enzyme-linked immunosorbent assay. $^*P < 0.05$ vs. responses of anti-CD3 Ab-stimulated $CD4^+$ $CD25^-$ T cells. (b) Freshly isolated $CD4^+$ $CD25^-$ T cells were incubated with (anti-CD3) or without (–) plate-bound anti-CD3 Ab for 24 hr. PDL-1 expression was assessed by flow cytometry (closed histograms). Open histograms show isotype control staining.

whether NK cells are involved in DC-mediated Treg cell induction has not been resolved. In the present study, we report that the expression of regulatory markers and functions was markedly decreased on $CD4^+$ $CD25^+$ T cells upon exposure to IL-2 NK-primed DCs. By contrast, the interaction of activated NK cells and NH through the NKG2A inhibitory receptor led to DC induction of $CD4^+$ $CD25^+$ T cells with regulatory properties. Furthermore, NKG2A-mediated increase in TGF-β as well as decrease in TNF-α in an NH and NK cell mixture contributed to DC induction of $CD4^+$ $CD25^+$ Treg cells. This is consistent with previous reports showing that TGF-β plays a role in generating the specific DC that activates $CD4^+$ $CD25^+$ Treg cells.[10,11] The findings that TNF-α suppressed TGF-β-mediated priming of DCs to induce Treg cells also extended the previously identified role of TNF-α as a positive regulator of DC activation. In line with our findings, previous reports showed that impairment of $CD4^+$ $CD25^+$ Treg cell activities restored their suppressive functions after blocking TNF-α signals in non-obese diabetic (NOD) mice or in patients with Crohn's disease.[28,29] To our knowledge, the present study is the first description of modulation of NK cells and human hepatocytes through NKG2A-mediated inhibitory

signals that profoundly affect DC functions towards $CD4^+$ $CD25^+$ Treg cells. Because NK cell functions are regulated by the balance between inhibitory and activating signals, any future clarification of the role of other NK inhibitory and activating receptors in DC modulation and Treg cell activation will be of great interest.

The cross-presentation of self-antigens by major histocompatibility complex (MHC) class II pathways constitutes an important step towards generating and/or expanding peripheral Treg cells.[30] However, we initially settled our experimental design by using DCs and Treg cells from different donors, and DCs encountered $CD4^+$ T cells in an 'antigen-free' condition. Therefore, Treg cells induced by NK/NH-primed DCs are generated independently of MHC class II-mediated self-antigen recognition. These results give rise to the possibility that the cross-talk of NK cells, DCs and hepatocytes represents an alternative pathway in the generation and expansion of peripheral Treg cells. However, it should be noted that these results may not apply to all donors because of the complexity of the allogeneic system and the relatively few donors tested.

PD-1-mediated suppressive activities were characteristic for $CD4^+$ $CD25^+$ Treg cells generated by NH/IL-2 NK-primed DCs. By contrast, natural $CD4^+$ $CD25^+$ Treg cells exerted their suppressive function, at least in part, in a CTLA-4-dependent fashion. Recent reports have clarified the existence of two subtypes of Treg cells: natural and inducible $CD4^+$ $CD25^+$ Treg cells. Inducible Treg cells exert suppressive activities by using molecular mechanisms distinct from those of natural regulatory cells.[31] Our findings further identify the novel pathways by which inducible $CD4^+$ $CD25^+$ Treg cell activities triggered by NKG2A inhibitory signals are dependent on PD-1-mediated negative costimulation. A recent report identified the interaction of B7 on effector T cells with costimulatory molecules CD28/CTLA-4 on $CD4^+$ $CD25^+$ Treg cells as molecular mechanisms of their suppressor activity.[32] Thus, it is possible that reverse signalling of PDL-1 on effector cells may also be crucial for the negative costimulator-mediated suppressive action of $CD4^+$ $CD25^+$ Treg cells. In the present study, we did not address the mechanisms by which NH/IL-2 NK-primed DCs induce $CD4^+$ $CD25^+$ Treg cells with PD-1-dependent suppressive functions. Further study will be needed to clarify this issue.

We previously showed that NKG2A is expressed at higher levels from NK cells isolated from peripheral blood in patients with chronic hepatitis C virus (HCV) infection than from those in healthy donors.[20] HCV frequently persists in humans, at least in part, due to inefficient induction of NK activity as well as specific T cell responses.[33–35] The small percentage of patients who spontaneously clear the virus and recover from chronic hepatitis C mount vigorous HCV-specific $CD4^+$ and $CD8^+$ T cell responses.[36,37] Research has described an increased frequency of $CD4^+$ $CD25^+$ T cells in the blood of patients with persistent HCV infection compared with those who have spontaneously cleared HCV.[38,39] Our current findings raise the interesting possibility that increased NKG2A expression on NK cells may lead to DC-mediated induction of Treg cells, leading to the inhibition of adaptive responses to HCV and failure to eliminate this virus. Indeed, $CD4^+$ $CD25^+$ T cells induced by HCV-NK/Hep3B hepatoma cell-primed DCs expressed and suppressed effector T cell functions at greater levels than those induced by N-NK/Hep3B-primed DCs (our unpublished data). Interestingly, a recent study identified PD-1-mediated signals as a critical pathway to induce anergic $CD8^+$ T cells and impair antiviral CTL responses in chronic viral infection.[40] In this regard, the therapeutic modification of the PD-1 pathway may synergistically augment antiviral immunity by suppressing Treg activity and recovering CTL responses. It is important to establish whether the PD-1 pathway in liver lymphocytes may be operable *in vivo* and play a critical role in suppression of virus-specific immunity in HCV infection.

In conclusion, we have demonstrated that interaction of NK cells and hepatic cells via NKG2A leads to DC induction of $CD4^+$ $CD25^+$ T cells with PD-1-dependent regulatory activities. These findings also imply that NK receptor signals of NK cells may dictate DC-mediated adaptive immune responses towards tolerogenic or immunogenic status via induction of Treg cells.

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Signal Transducer and Activator of Transcription 3 Signaling Within Hepatocytes Attenuates Systemic Inflammatory Response and Lethality in Septic Mice

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Sepsis is an infection-induced syndrome with systemic inflammatory response leading to multiorgan failure and occasionally death. During this process, signal transducer and activator of transcription 3 (STAT3) is activated in the liver, but the significance of this molecule has not been established. We generated hepatocyte-specific STAT3-deficient mice (L-STAT3 KO) and examined the susceptibility of these mice to cecal ligation and puncture–induced peritonitis, a well-established septic model. L-STAT3 KO mice showed significantly higher mortality and produced lesser amounts of various acute phase proteins than control littermates. Although blood bacterial infection did not differ between L-STAT3 KO mice and control mice, the former showed deterioration of the systemic inflammatory response as evidenced by a significant increase in various cytokines such as tumor necrosis factor α, IFN-γ, IL-6, IL-10, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1β. A similar hyperinflammatory response was observed in another septic model caused by lipopolysaccharide (LPS) injection. In vitro analysis revealed that soluble substances derived from hepatocytes and dependent on STAT3 were critical for suppression of cytokine production from LPS-stimulated macrophage and splenocytes. *Conclusion:* STAT3 activation in hepatocytes can attenuate a systemic hyperinflammatory response and lethality in sepsis, in part by suppressing immune cell overactivation, implying a critical role of hepatocyte STAT3 signaling in maintaining host homeostasis. (HEPATOLOGY 2007;46: 1564-1573.)

Signal transducer and activator of transcription 3 (STAT3) mediates a signal from the IL-6 family of cytokines such as IL-6, oncostatin M, leukemia inhibitory factor, and ciliary neutrophic factor, and activates transcription of various target genes.[1] Although a STAT3 is now known to be ubiquitously expressed in variety of cells and has pleiotropic functions, it was formerly termed *acute phase response factor* and was first identified in the liver as an inducible DNA binding protein binding to type 2 IL-6–responsive elements within the promoter of hepatic acute phase protein (APP) genes.[2,3] Because deletion of STAT3 leads to embryonic lethality in mice, the significance of STAT3 in adult organs has been investigated using conditional knockout animals generated by the Cre/loxP recombination system.[4] Research has shown that STAT3 signaling within hepatocytes controls a variety of physiological or pathological processes, including hepatocyte proliferation after partial hepatectomy,[5] apoptosis resistance of hepatocytes during Fas-mediated liver injury,[6] and regulation of hepatic gluconeogenic genes.[7] Although STAT3 is activated in response to a rise of circulating cytokines, the significance of hepatic STAT3 has not been elucidated under systemic inflammatory conditions.

Sepsis is an infection-induced systemic syndrome, the incidence of which is estimated at 750,000 cases annually in North America with overall mortality being approxi-

Abbreviations: APP, acute phase protein; CLP, cecal ligation and puncture; LPS, lipopolysaccharide; L-STAT3 KO, hepatocyte-specific STAT3-deficient mice; STAT3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

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mately 30%, but rising to 40% in the elderly.[8] Sepsis develops when the initial, appropriate host response to an infection becomes amplified and then dysregulated.[9] Among those harmful or damaging responses is the rise of a variety of circulating cytokines such as IL-6, tumor necrosis factor α (TNF-α), IL-10, and IFN-γ. These cytokines lead directly to the development of systemic inflammatory response syndrome. During this process, an increasing proportion of patients will develop adult respiratory distress syndrome, disseminated intravascular coagulation, and/or acute renal failure, leading to the multiple organ dysfunction syndrome.[10] The liver is also one of the target organs of multiple organ dysfunction syndrome, although liver dysfunction may cause patient death less frequently than cardiovascular dysfunction.[11] Conversely, sepsis is a serious complication of severe liver diseases such as fulminant hepatitis[12] and decompensated cirrhosis.[13] Thus, research on the relevance of signal transduction in liver cells in the septic condition would not only satisfy basic scientific interest but would also have clinical implications.

In the present study, we used hepatocyte-specific STAT3-deficient (L-STAT3 KO) mice and examined the significance of STAT3 signaling within hepatocytes in a well-established murine model of sepsis. We found that STAT3 deficiency in hepatocytes causes exacerbation of the hyperinflammatory response by attenuating hepatic production of soluble substances that can suppress immune cell activation and also increases mortality in septic mice. This study identified an anti-inflammatory function of hepatic STAT3 signaling and its protective role against systemic inflammation, providing genetic evidence for a close link between hepatocytes and the immune system.

Materials and Methods

Animals. Mice carrying a STAT3 gene with 2 *loxP* sequences flanking exon 22 and a STAT3 null allele (*STAT3 fl/−*) have been described previously.[14] To generate mice with hepatocyte-specific STAT3 deficiency, we crossed STAT3 fl/− mice and Alb-Cre transgenic mice,[15] which express the Cre recombinase gene under the regulation of the albumin gene promoter. We crossed Alb-Cre STAT3 fl/fl mice and STAT3 fl/− mice. The resulting Alb-Cre STAT3 fl/− mice were used as L-STAT3 KO mice. Sex-matched STAT3 fl/− mice obtained from the same litter were used as control mice. All mice were used at the age of 12-15 weeks. All animals were housed under specific pathogen-free conditions and were treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Cecal Ligation and Puncture and Lipopolysaccharide Injection. Cecal ligation and puncture (CLP) is a well-established murine model of septic shock. The mice underwent CLP surgery as described previously.[16] In brief, the mice were anesthetized via intraperitoneal injection of sodium pentobarbital. Under sterile condition, the cecum was assessed via a 1-cm midline incision of the lower abdomen, ligated with a suture below the ileocecal valve, and punctured once with a 23-gauge needle. The cecum was replaced in the peritoneum, and the abdomen was closed with sutures. The mice were injected with 1 mL of lactate Ringer's solution subcutaneously for fluid resuscitation. As another septic model, lipopolysaccharide (LPS) (form *Escherichia coli* 055: B5; Sigma, St. Louis, MO) was injected intraperitoneally at a dose of 4 mg/kg body weight.

Preparation of Peritoneal Macrophage. To isolate peritoneal macrophages, we injected mice intraperitoneally with 2 mL of 4% thioglycollate. Peritoneal exudates cells were isolated from the peritoneal cavity 4 days after injection. The cells were incubated for 4 hours in 96-well plates and washed 3 times with phosphate-buffered saline. We used the adherent cells as peritoneal macrophages for further experiments.

Determination of the Bacterial Load. Mice were sacrificed 24 hours after CLP surgery. Samples of blood were obtained in sterile condition. Fifty microliters of the blood were then plated on heart-infusion plates. The heart-infusion plates were incubated at 37°C overnight, and the number of bacteria colonies was counted. Results were expressed as $\log_{10}$ of CFU.

Blood Biochemistry. Blood samples were obtained 24 hours after CLP or LPS injection. Acute phase proteins, cytokines, and chemokines in plasma were determined via MultiAnalyte Profile testing (Rules Based Medicine, Austin, TX). Levels of serum ALT and creatinine were measured with a standard UV method using a Hitachi type 7170 automatic analyzer (Tokyo, Japan).

Measurement of Culture Supernatant. Levels of cytokines (TNF-α, IL-6, IL-10, and IFN-γ) in the culture supernatants were measured using commercially available ELISA kits in accordance with the manufacturer's instructions (BD Biosciences-Pharmingen, San Diego, CA). Haptoglobin was determined in cell-free supernatants by using a commercially available ELISA kit (Immunology Constants Laboratory, Newberg, OR).

Western Blot Analysis. The total cellular protein was extracted with the RIPA buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, and 50 mM sodium fluoride in phosphate-buffered saline (pH 7.4). Twenty micrograms of protein were separated

via 7.5% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. After blocking with Tris-buffered saline 0.1% Tween 20 containing 5% skim milk or Blocking One-P (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, the membrane was incubated overnight at 4°C with antibodies to STAT3 or tyrosine^{705}-phosphorylated STAT3 (Cell Signaling Technology, Danvers, MA), respectively. After washing with Tris-buffered saline 0.1% Tween 20, the membrane was incubated with anti–horseradish peroxidase–linked antibody for 1 hour at room temperature. The immune complex was detected by an enhanced chemiluminescent assay. In some experiments, tyrosine^{701}-phosphorylated STAT1 antibody (Cell Signaling Technology) was also used. This antibody recognizes the phosphorylated form of both STAT1α and STAT1β.

Histology and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling. The formalin-fixed livers were paraffin-embedded, and liver sections were analyzed by hematoxylin-eosin staining. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed using an ApopTag kit according to the manufacturer's instructions (Serological Corporation, Norcross, GA).

Primary Culture of Hepatocytes. Livers were digested using a standard in situ 2-step collagenase perfusion procedure (Gibco BRL, Rockville, MD). Hepatocytes were isolated from nonparenchymal cells via subsequent centrifugation at 50*g* for 1 minute. In a selected experiment, nonparenchymal cells in the supernatants were pelleted at 1,500 rpm for 5 minutes and subjected to western blot analysis. Isolated hepatocytes with >90% viability were cultured in Williams' medium E containing 10% fetal bovine serum overnight. On the next day, the cells were stimulated with recombinant IL-6 (PeproTech, London, UK). The cells were harvested after 2 hours for the analysis of STAT3 activation. In another experiment, supernatants were harvested after 48 hours.

Cytokine Production by Macrophage and Splenocytes. The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA). RAW cells were plated at a density of 5×10^5/well in a 96-well plate and were incubated at 37°C in culture supernatants of hepatocyte from L-STAT3 KO mice or control mice. As a control, RAW cells were also cultured in Williams' medium E. After 24 hours, LPS was added to achieve a final concentration of 100 ng/mL. After 24 hours of incubation at 37°C in an atmosphere of 5% CO_2, the supernatant was collected and stored at −80°C for measurement of TNF-α, IL-6, and IL-10. Splenocytes were isolated by way of a standard procedure for wild-type mice[17] and incubated with hepatocyte culture supernatant. Twenty-four hours after incubation, the cells were stimulated with LPS (1,000 ng/mL) for 24 hours. The resultant culture supernatant was subjected to IFN-γ ELISA.

Statistics. Kaplan-Meier curves were used to show survival over time. Data are expressed as interquartile range and median and compared using the Mann-Whitney *U* test. Statistical significance was set at $P < 0.05$.

Results

Mice with hepatocyte-specific STAT3 deficiency were produced by crossing floxed STAT3 mice and Alb-Cre transgenic mice carrying the Cre recombinase gene under the regulation of the albumin gene promoter. L-STAT3 KO mice were born and grew without any gross abnormality. Western blot analysis revealed that STAT3 expression was substantially decreased in the liver but not in other organs (Fig. 1A). Isolation of hepatocytes from nonparenchymal cells by liver perfusion followed by centrifugation confirmed that STAT3 deficiency is specific in hepatocytes (Fig. 1B). In addition, STAT3 expression did not differ in peritoneal macrophages between L-STAT3 KO mice and control littermates (Fig. 1C). Those cells isolated from L-STAT3 KO mice produced similar levels of TNF-α in response to LPS compared with those from control littermates (Fig. 1D).

L-STAT3 KO Mice Are More Vulnerable to Septic Shock. To examine the role of hepatic STAT3 during septic shock, we used a well-examined clinically relevant murine model of sepsis performed by CLP.[16] CLP clearly activated liver STAT3, which was determined via phosphorylation of STAT3 in control mice (Fig. 2A), in agreement with a previous report.[18] Liver STAT3 activation during sepsis is mostly due to the activation of STAT3 in hepatocytes, because liver STAT3 was only marginally activated in L-STAT3 KO mice. CLP activated liver STAT1 both in L-STAT3 KO mice and wild-type mice, suggesting that the absence of STAT3 does not affect the activation of other STATs. Given that STAT3 is a well-known mediator for APP,[19] we measured APPs such as fibrinogen and haptoglobin in plasma after CLP (Fig. 2B). The levels of fibrinogen and haptoglobin clearly increased after CLP in wild-type mice. In contrast, induction of fibrinogen was completely diminished in L-STAT3 KO mice, whereas that of haptoglobin was partially inhibited. This is consistent with the previous notion that fibrinogen is a class 2 gene and haptoglobin is a class 1 gene; the class 2 gene is predominantly regulated by type 2 IL-6 responsive elements binding to STAT[20] and the class 1 gene by both type 1 IL-6 responsive elements

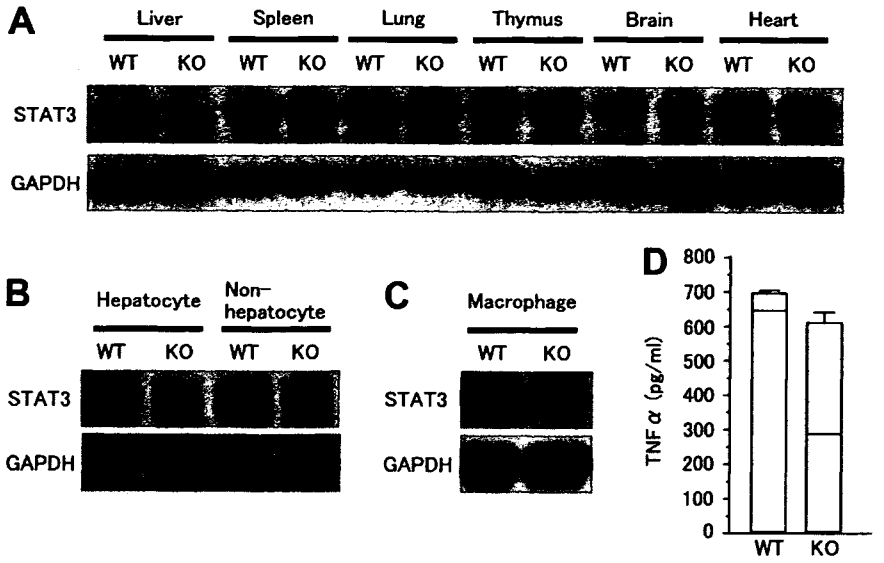

Fig. 1. Hepatocyte-specific STAT3 deficiency in mice. Floxed STAT3 mice were crossed with Alb-Cre transgenic mice. Floxed STAT3 mice having the Alb-Cre transgene were regarded as L-STAT3 KO mice (KO); those not having the Alb-Cre gene were used as a wild-type control (WT). (A) STAT3 expression in a variety of organs from L-STAT3 KO mice and wild-type mice via western blot analysis. Expression of GAPDH was served as a loading control. Representative blots are shown. (B) Expression of STAT3 of isolated hepatocytes and nonhepatocytes. Liver of L-STAT3 KO mice or wild-type mice was collagenase-perfused and separated into hepatocyte and nonhepatocyte fractions. STAT3 expression was determined via western blot analysis. Expression of GAPDH was served as a loading control. Representative blots are shown. (C) Expression of STAT3 in isolated macrophage. Peritoneal macrophage was isolated from L-STAT3 KO mice or wild-type mice and subjected to western blot analysis of STAT3 expression. Representative blots are shown. (D) LPS-stimulated TNF-α production of peritoneal macrophages. Peritoneal macrophages were isolated from L-STAT3 KO mice or wild-type mice ($n = 6$ for each group) and stimulated with LPS (100 ng/mL) for 24 hours. TNF-α production was determined via ELISA in culture supernatants.

binding to CCAAT enhancer-binding protein (C/EBP) and type 2 IL-6 responsive elements.[21]

To address the issue of whether hepatic STAT3 is involved in the outcome of CLP-induced lethality, we performed CLP blinded to the genetic background and checked the survival of the mice every 6 hours. L-STAT3 KO mice were significantly more vulnerable to CLP-induced lethality than wild-type littermates (Fig. 2C). To examine the possible difference in bacterial infection after CLP, we measured colony forming unit of blood bacteria 24 hours after CLP. Because there was no significant difference in bacterial amount between L-STAT3 KO mice and wild-type mice (Fig. 2D), we considered hepatic STAT3 to have had a beneficial effect on the outcome of septic shock without affecting bacterial infection.

Hepatic STAT3-Deficient Mice Show Exacerbated Liver Injury. To examine liver injury and renal dysfunction in CLP-induced sepsis, we measured ALT and creatinine levels. L-STAT3 KO mice showed increased levels of serum ALT and creatinine compared with wild-type littermates, although the difference in creatinine did not reach a significant level (Fig. 3A). TUNEL of the liver revealed that the number of apoptotic hepatocytes was significantly higher in L-STAT3 KO mice than in wild-type littermates (Fig. 3B,C). However, the liver injury itself presumably is not a direct cause of animal death, because histologic abnormality was modest. Furthermore, LPS injection, which is another model of septic shock, induced more hepatocyte apoptosis than CLP but did not kill any mice tested (Fig. 3A-C and data not shown), supporting the idea that increased liver injury could not explain the increased lethality in L-STAT3 KO mice.

Exacerbated Systemic Inflammatory Response in L-STAT3 KO Mice. Hypercytokinemia underlying systemic inflammatory response syndrome may play an important role in the development of multiple organ dysfunction syndrome and lethality.[9] We measured several circulating cytokines and chemokines in septic mice and found that TNF-α, IFN-γ, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1β (MIP-1β) had clearly increased 24 hours after CLP in L-STAT3 KO mice. Of importance is the finding that the plasma levels of these cytokines and chemokines were significantly higher in L-STAT3 KO mice than in wild-type mice, although they did not differ before CLP. This result indicates that the increased lethality found in L-STAT3 KO mice is associated with hypercytokinemia (Fig. 4A). Although plasma insulin levels significantly increased 24 hours after CLP, there was no significant difference between L-STAT3 KO mice and wild-type mice, suggesting that insulin levels do not affect the difference in animal lethality (Supplementary Fig. 1).

Given that bacterial infection did not differ between L-STAT3 KO mice and wild-type mice, we examined the

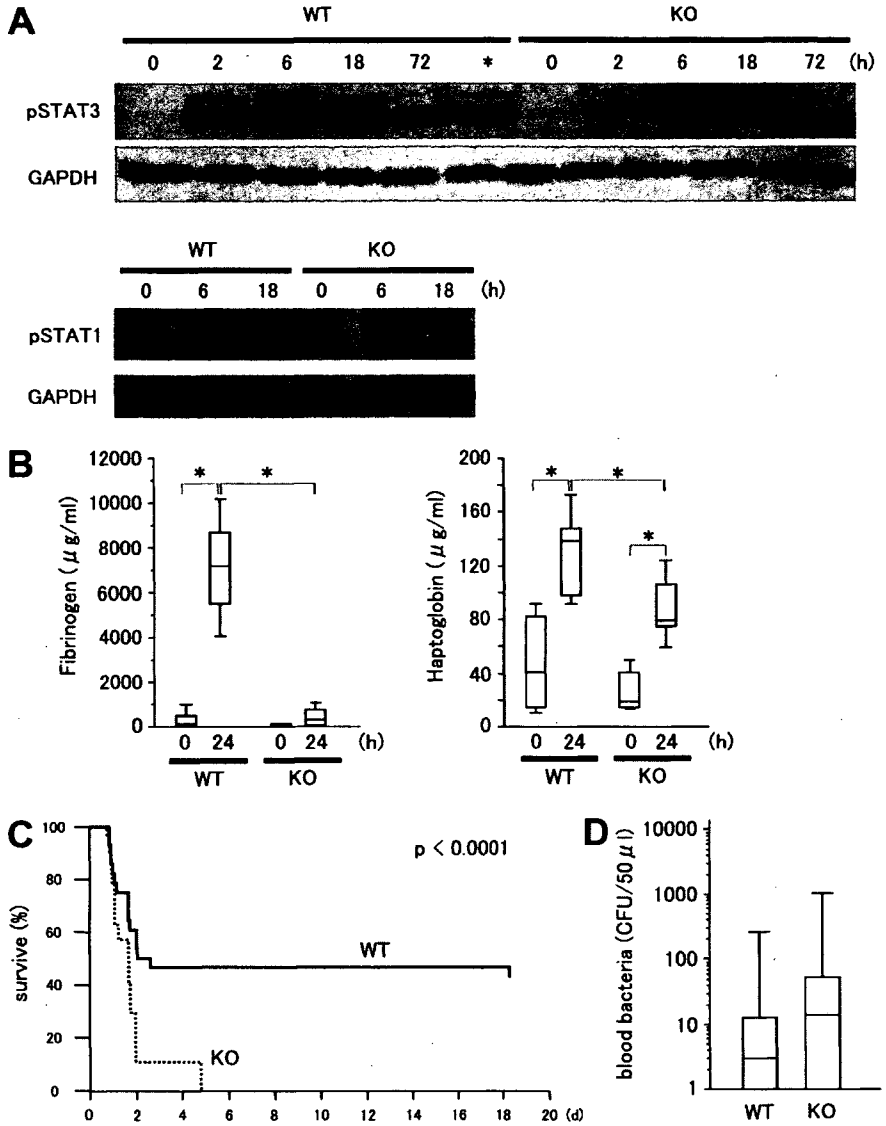

Fig. 2. STAT3 activation, APP production, and survival in CLP mice. (A) Western blot analysis of phosphorylated STAT3 and STAT1 in CLP mice. L-STAT3 KO mice (KO) and wild-type mice (WT) were treated with CLP and sacrificed at the indicated time points. Their liver tissues were subjected to analysis of Tyrosine 705 phosphorylation of STAT3 or Tyrosine 701 phosphorylation of STAT1 via western blot analysis. GAPDH expression served as a control. Representative blots are shown. *7 days. (B) Levels of circulating haptoglobin and fibrinogen before and 24 hours after CLP (n = 8 for each group). *$P < 0.05$. (C) Comparison of survival after CLP between L-STAT3 KO (n = 27) mice and wild-type littermates (n =28). (D) Colony-forming units of blood bacteria after CLP. L-STAT3 KO or wild-type mice were sacrificed 24 hours after CLP. Blood samples were subjected to analysis of bacterial growth (n = 10 for knockout mice and n = 9 for wild-type mice).

response of cytokine production upon endotoxin stimulation. To this end, we injected the same amount of LPS to L-STAT3 KO mice and control mice and measured circulating cytokines. LPS injection into L-STAT3 KO mice upregulated those cytokines to a lesser extent than CLP. In agreement with the finding on the CLP model, the levels of TNF-α, IL-10, MCP-1, and MIP-1β were significantly higher in L-STAT3 KO mice than in wild-type mice after LPS injection (Fig. 4B), indicating that L-STAT3 KO mice were highly sensitive to endotoxin and prone to show hypercytokinemia.

STAT3-Regulated Soluble Factors Produced by Hepatocytes Suppress Cytokine Production From Immune Cells. To examine the underlying mechanisms of the hyperimmune response in L-STAT3 KO mice, we hypothesized that STAT3-mediated soluble factors from hepatocytes repress cytokine production from immune cells. We isolated hepatocytes from L-STAT3 KO mice and control mice and stimulated them with or without IL-6, collecting the conditional medium of hepatocytes. Wild-type hepatocytes displayed STAT3 activation in primary culture without stimulation, but the levels increased upon IL-6 exposure, whereas KO hepatocytes did not show any STAT3 activation (Fig. 5A). Consistent with this was the finding that the wild-type hepatocytes produced more haptoglobin than KO hepatocytes, even in the absence of IL-6 (Fig. 5B).

Next, we cultured RAW cells, a murine macrophage cell line, in the presence or absence of culture supernatant of hepatocytes. RAW cells produced TNF-α, IL-6, and IL-10 but not IFN-γ upon stimulation of LPS, and hepatocyte culture supernatant suppressed the production of these cytokines (Fig. 5C). Importantly, the suppression was significantly weaker in the presence of conditional medium of KO hepatocytes than in the presence of conditional medium of wild-type hepato-

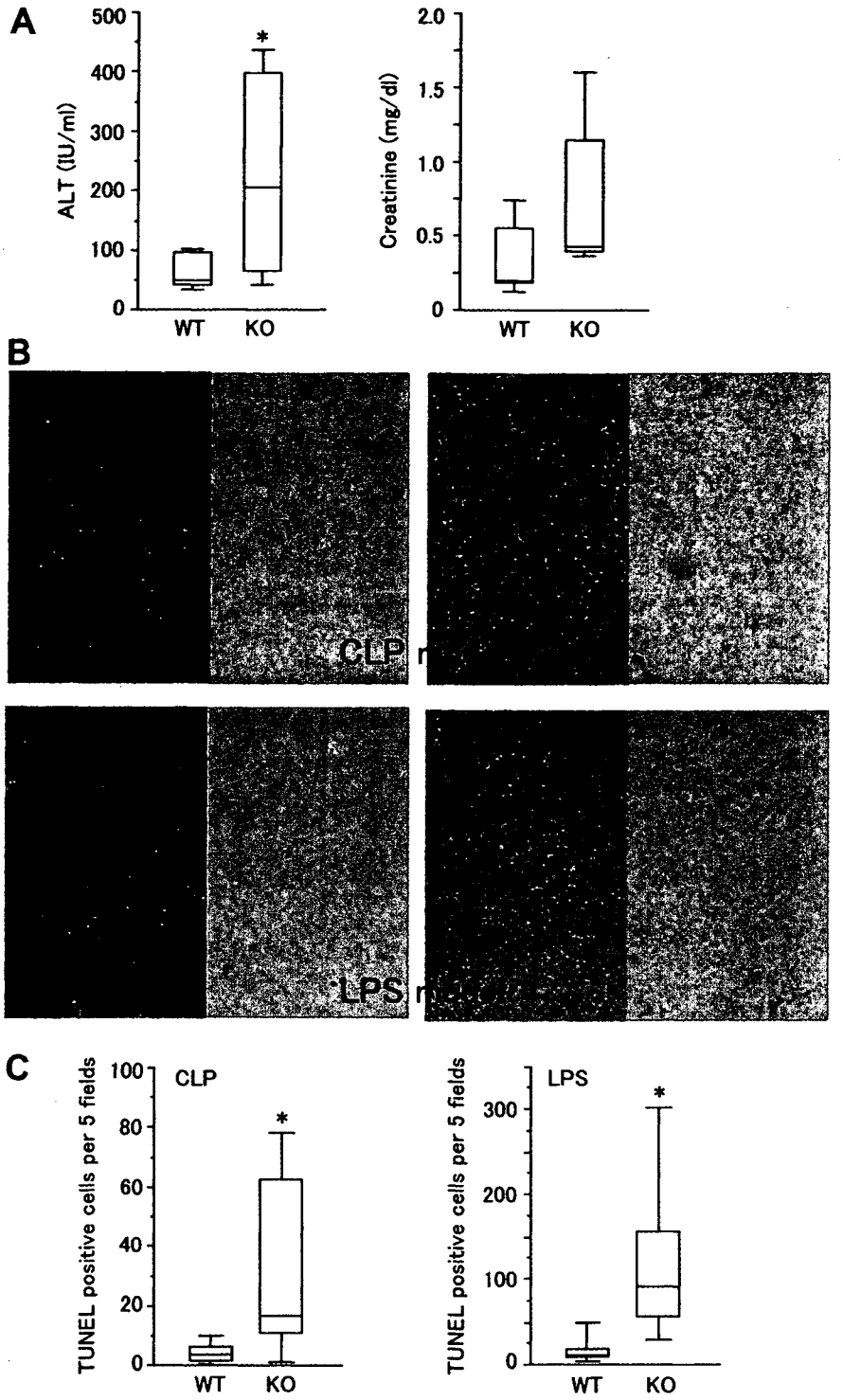

Fig. 3. Organ injury in septic mice. (A) Serum ALT and creatinine levels in L-STAT3 KO mice (KO) and control mice (WT) 24 hours after CLP. $*P < 0.05$. (B) Representative histology (left part of each panel) and TUNEL (right part of each panel) of liver sections 24 hours after CLP or LPS injection. (C) Comparison of TUNEL-positive hepatocytes for at least 9 mice in each group. $*P < 0.05$.

cytes. Furthermore, murine primary splenocytes produced IFN-γ upon LPS stimulation, and the production was also suppressed in the presence of conditional medium of hepatocytes. Again, IFN-γ production was significantly higher in splenocytes cultured with KO hepatocyte supernatant than in those with wild-type hepatocyte supernatant (Fig. 5D). These data indicate that soluble substances from hepatocytes suppressed activation of immune cells, which was critically dependent on STAT3.

Discussion

The present study clearly demonstrated that the absence of STAT3 in hepatocytes leads to high levels of circulating cytokines and increased mortality of CLP-in-

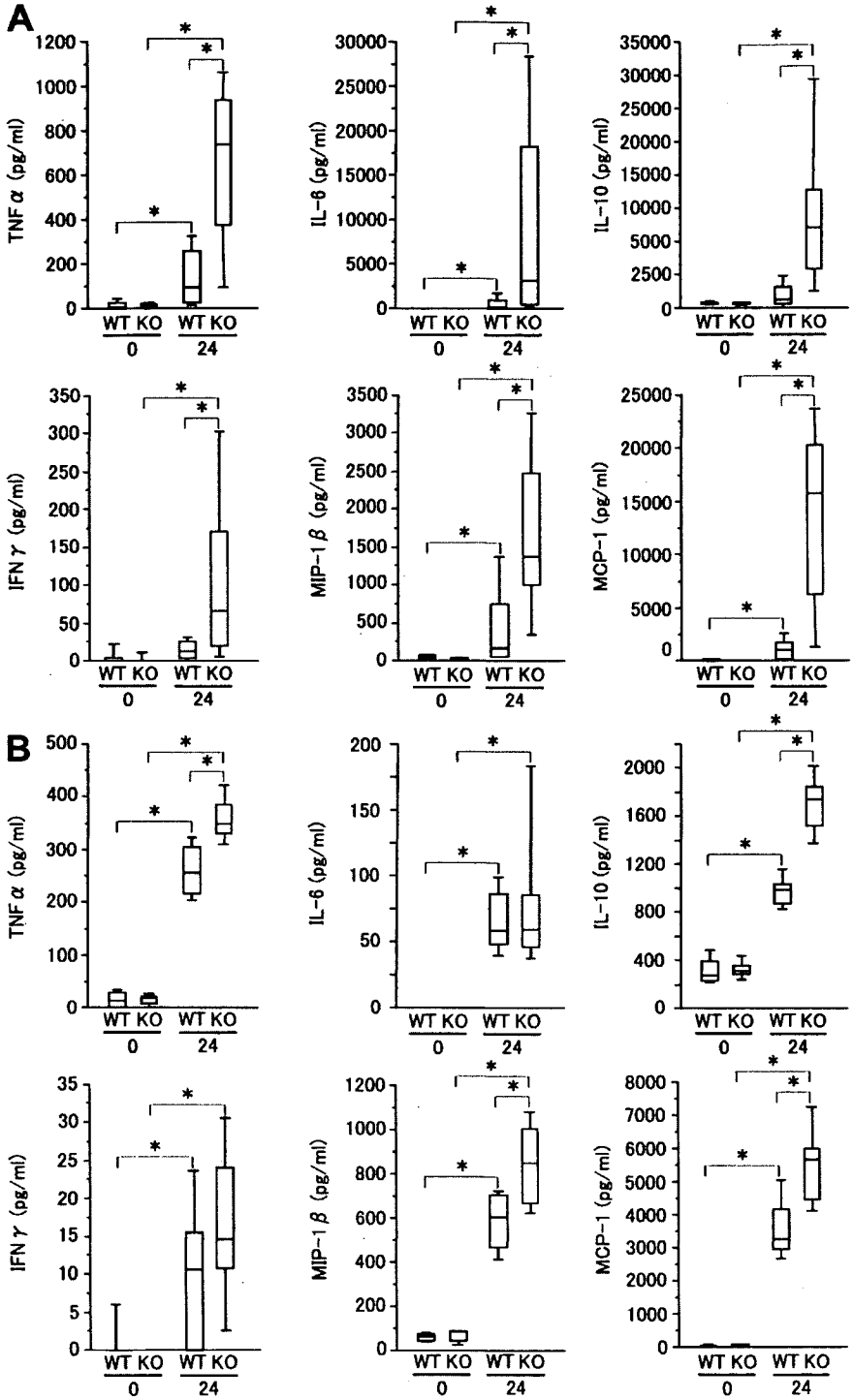

Fig. 4. Circulating cytokines before and after CLP or LPS injection. (A) L-STAT3 KO mice (KO) or wild-type littermates (WT) were treated with CLP (n = 8 in each group). Before and 24 hours after CLP, blood samples were obtained from mice and subjected to analysis of each cytokine indicated. $^*P < 0.05$. (B) Mice were injected with 4 mg/kg of LPS (n = 8 in each group). Before and 24 hours after LPS injection, blood samples were obtained and subjected to the analysis of each cytokine indicated. $^*P < 0.05$.

duced septic mice without affecting bacterial infection. L-STAT3 KO mice produced high levels of cytokines when injected with LPS, confirming that the absence of STAT3 signaling within hepatocytes induces a hyperinflammatory response even if the extent of the input stimuli remains constant. This phenomenon is similar to a previous report of macrophage-specific disruption of STAT3 in which serum cytokines such as TNF-α, IL-6, and IL-10 increased upon LPS stimulation.[22] In those mice, immune cells could not respond to IL-10, which potentially inhibit TNF-α production via STAT3 signaling, and thus produced high levels of TNF-α. Further study revealed those mice to be vulnerable to CLP-induced sepsis.[23,24] However, in our L-STAT3 KO mice, the levels of STAT3 in macrophage did not differ from control mice and produced the same amount of TNF-α in response to LPS (Fig. 1C-D). Thus, suppression of the inflammatory response in wild-type mice was critically

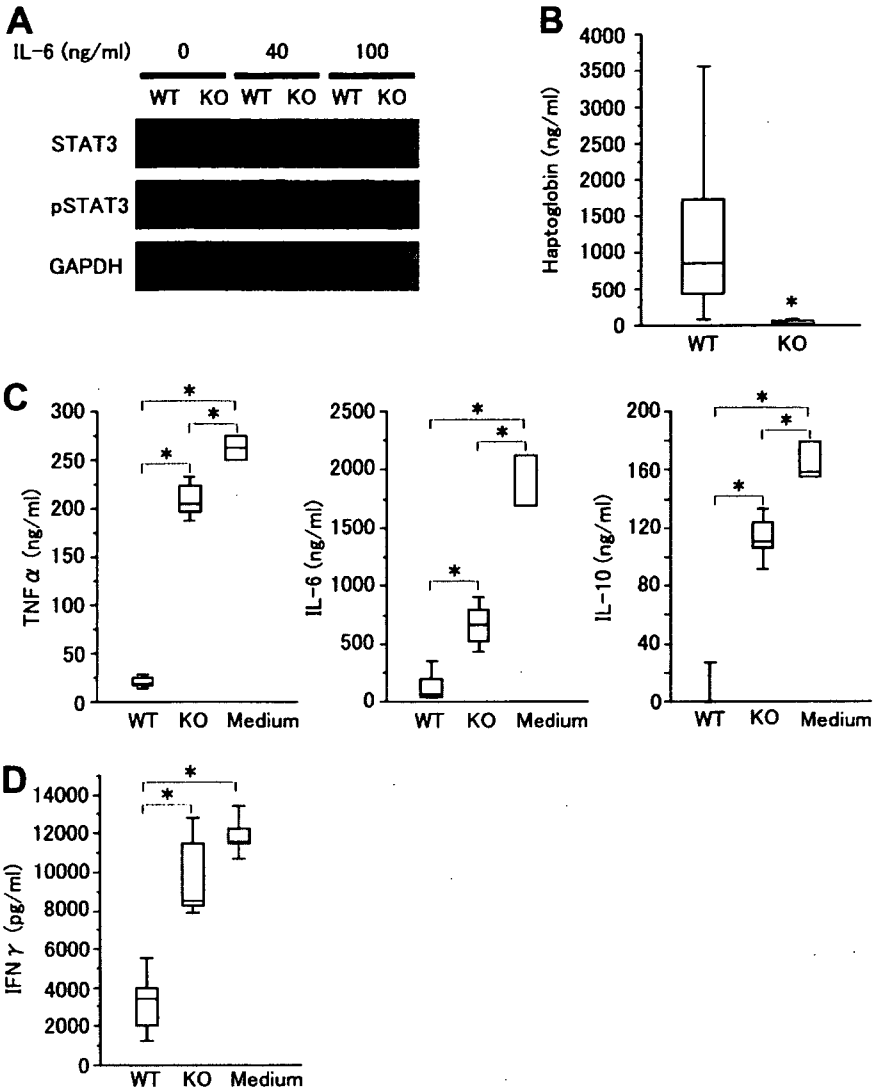

Fig. 5. Suppression of cytokine production from immune cells by hepatocyte culture supernatant. Hepatocytes were isolated from L-STAT3 KO (KO) or wild-type (WT) mice and cultured in the presence or absence of IL-6 for 2 hours (for western blot analysis) or 48 hours (for collection of culture supernatants). (A) STAT3 phosphorylation (pSTAT3) and STAT3 expression of hepatocytes via western blot analysis. GAPDH expression served as a control. Representative blots are shown. (B) Haptoglobin production from primary hepatocytes. Haptoglobin concentration was determined in the hepatocyte supernatants via ELISA. Comparison of haptoglobin production between knockout hepatocytes and wild-type hepatocytes ($n = 5$ mice/group) cultured in the absence of IL-6. $^*P < 0.05$. (C,D) Suppression of cytokine production in RAW cells or splenocytes by the hepatocyte supernatants. Hepatocytes were cultured for 48 hours. RAW cells (C) or splenocytes freshly isolated from wild-type mice (D) were cultured in the presence (KO or WT) or absence (Medium) of hepatocyte supernatants for 24 hours and then stimulated with LPS for another 24 hours. TNF-α, IL-6, IL-10, and IFN-γ production was determined via ELISA. $^*P < 0.05$.

dependent on hepatic STAT3 signaling. Indeed, in vitro analysis revealed that soluble factors from hepatocytes repress cytokine production from activated macrophage and splenocytes in a hepatic STAT3-dependent manner. Whereas research has established that STAT3 mediates a variety of effects on hepatocytes, including proliferation,[5] apoptosis protection,[6] and glucose metabolism,[7] the present study reveals that hepatic STAT3 has an important extrahepatic effect. This effect is activated by a variety of cytokines produced from immune cells such as IL-6 but, in turn, suppresses immune cell activation via production of soluble factors, providing a negative feedback loop. Thus, the present study describes a role of hepatic STAT3 in maintaining host homeostasis by negatively regulating the immune system.

APPs are liver plasma proteins whose levels of expression are either positively or negatively regulated by cytokines during inflammation. It has been established that STAT3 regulates the expression of most, if not all, APPs in the liver.[19] Consistent with this, L-STAT3 KO mice displayed impaired production of APPs in response to CLP (Fig. 2B). Some APPs such as C-reactive protein,[25] serum amyloid P,[26] and α2-macroglobulin[27] have been shown to bind bacteria and to positively or negatively affect their eradication. Several reports also suggest that APPs exert proinflammatory as well as anti-inflammatory effects.[25,28] C-reactive protein binds to the phosphocholine of some foreign pathogens as well as phospholipid constituents of damaged cells and can activate the complement system, whereas the antioxidants haptoglobin and hemopexin protect against reactive oxygen species. Thus, each APP has a unique role in the complex mechanism controlling infection-induced inflammation. The L-STAT3 KO mice used in the present study offer a unique model for identifying the net effect of STAT3-regulated APPs during the septic condition. Our work has revealed that the most prominent effect of STAT3-regulated APPs is suppression of the hyperinflammatory re-

sponse and lethality without an effect on bacterial infection. The soluble factors from hepatocytes that suppress cytokine production from immune cells are still unknown. Although there may be several substances involved in this phenomenon, one candidate might be haptoglobin, which was recently demonstrated to suppress TNF-α, IL-12, and IL-10 from human peripheral blood mononuclear cells in vitro.[29] We also obtained a similar finding that RAW cells produced a lesser amount of TNF-α upon LPS stimulation in the presence of haptoglobin (Supplementary Fig. 2). Identification of these substances may have important therapeutic implications for controlling the hyperinflammatory condition. Further study is needed to clarify this point.

The liver is one of the target organs of sepsis-induced multiple organ dysfunction syndrome. Evidence for this comes from the fact that CLP mice or LPS mice showed liver injury as evidenced by increases in serum ALT and TUNEL-positive hepatocytes scattered in the liver lobule. Furthermore, L-STAT3 KO mice displayed more hepatocyte apoptosis in mice subjected to CLP or LPS injection. Previous research has indicated that the absence of hepatic STAT3 renders hepatocytes more vulnerable to Fas-mediated apoptosis.[6] It is possible that STAT3-null hepatocytes are more vulnerable to apoptosis in the septic model. However, at the same time, L-STAT3 KO mice showed higher levels of proinflammatory cytokines such as TNF-α, which is a direct inducer of hepatocyte apoptosis. In our model, it is difficult to differentiate which contributed more to increased liver injury: the decrease in apoptosis resistance or the increase in proinflammatory cytokine. It can be said that the increase of proinflammatory cytokines is presumably one of the causes, but not a result, of liver injury. In addition, as discussed in the Results section, liver injury was relatively modest and probably not a direct cause of animal death.

In the present study, the lack of hepatic STAT3 caused increased mortality in CLP mice. Although we did not address the direct link between hypercytokinemia and animal death, accumulating evidence suggests that an increase in a variety of cytokines is involved in lethality in CLP mice. For example, it was shown that IL-6 plays an important role in the increased expression of the C5a receptor in the lung, liver, kidney, and heart during the development of sepsis in CLP mice and that interception of IL-6 leads to reduced expression of the C5a receptor and improved survival.[30] In addition, enforced expression of the IL-6 gene in wild-type mice led to high mortality (unpublished data). TNF-α and other cytokines increase expression of inducible nitric oxide synthase, and increased production of nitric oxide causes further vascular instability and may also contribute to the direct myocardial depression that occurs in sepsis.[31] Thus, dysregulation of cytokines may be harmful for host organs and is probably linked to animal death.

The present study revealed an important role of hepatocytes in repressing the hyperinflammatory response in pathologic conditions. This raises the possibility that hyperinflammation may be ill-controlled when liver function is severely impaired. Although sepsis itself is not a frequent cause of liver failure, it is a serious complication of acute or chronic liver failure. Systemic inflammatory response syndrome is an important determinant of prognosis in fulminant hepatitis.[12] Sepsis originating from spontaneous bacterial peritonitis or renal infection is one of the causes of patient death with decompensated cirrhosis.[13] In patients with limited function of the liver, possible impairment of STAT3-regulated hepatocyte function may be involved in their poor prognosis when complicated with severe inflammation. Careful liver-supporting therapy or early liver transplantation should be considered not only for maintaining liver function but also from the aspect of controlling dysregulated hyperinflammatory responses.

In conclusion, hepatic STAT3 represses systemic hyperinflammatory response by stimulating hepatic production of soluble substances that can attenuate immune cell overactivation and also improves host survival during septic condition. This sheds light on hepatocytic STAT3 as a negative regulator for immune cell overactivation and its role in host defense during systemic severe inflammation.

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Original Article

Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C

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Aim: Ribavirin, used to treat chronic hepatitis C, can induce hemolytic anemia, forcing the discontinuance of treatment. To establish a predictive measure to help circumvent this, we evaluated the relationship of hemoglobin (Hb) decline with the discontinuance of treatment during the progression of ribavirin-induced anemia.

Methods: One hundred and sixteen patients (71% male) with genotype 1 chronic hepatitis C were treated with pegylated interferon (PegIFN) α-2b and ribavirin. The mean age was 50.6 years and 55% were IFN naïve. A decline of Hb concentration by 2 g/dL at two weeks from the start of the treatment ("2 by 2" standard) was adopted as the predictive factor for the progression of anemia.

Results: By applying the "2 by 2" standard, with $\Delta Hb \geq 2$ g/dL (34%, $n = 39$), treatment was discontinued in 12 cases (31%), three of which (8%) because of severe anemia. For $\Delta Hb < 2$ g/dL (64%, $n = 76$), treatment was discontinued in 11 (14%) cases; none due to severe anemia. Ten percent (4/39) of patients showed the minimum $Hb \leq 8.5$ g/dL in the $\Delta Hb \geq 2$ g/dL group, with none in the $\Delta Hb < 2$ g/dL group ($P = 0.001$). Furthermore, the patients with minimum $Hb \leq 8.5$ g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Conclusion: Monitoring the Hb decline using the "2 by 2" standard can identify patients who are prone to developing severe anemia. Further prospective studies are needed using ribavirin reduction based on the "2 by 2" standard.

Key words: "2 by 2" standard, chronic hepatitis C, pegylated interferon and ribavirin combination therapy, progression of anemia

INTRODUCTION

THE AIM OF antiviral therapy for hepatitis C virus (HCV) is to obtain a sustained viral response (SVR) and to reduce the occurrence rate of hepatocellular carcinoma or hepatic disease-related mortality.[1,2] The current optimal therapy for patients with chronic hepatitis C is a combination of pegylated interferon (PegIFN) and ribavirin. This combination can significantly improve the SVR rate and is recommended as a standard regimen worldwide.[3–8] However, the SVR rates for the combination therapy of ribavirin with PegIFN for naïve patients with HCV genotype 1 has been reported to be 42–52%,[6,9,10] which means that eradication of HCV is not complete in approximately half of these patients. Recently, long-term treatment[11] and a higher dosage

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of drugs[12,13] have been used to try to raise the SVR rate for patients with HCV genotype 1. However, it remains to be established what constitutes satisfactory efficacy. In this study we focused on a treatment strategy to enable the prediction of severe side-effects in order to avoid the need to discontinue treatment and raise the SVR rate by PegIFN and ribavirin combination therapy. It is important that ribavirin, the key drug for eradicating HCV, is continued until the end of treatment in order to attain the maximum SVR rate. Hemolytic anemia induced by ribavirin is known as one of the most important adverse effects in the combination therapy of PegIFN and ribavirin.[14–17] To decrease the discontinuance rate of ribavirin due to severe anemia, epoetin alfa has been used for patients with progressing anemia, which can maintain the dose level of ribavirin as well as the quality of life of the patients.[18–20] However, from a cost-effectiveness standpoint, it would be difficult for this treatment strategy to become standard. Also, side-effects other than anemia arising from an overload of ribavirin mainly due to renal dysfunction cannot be avoided by the additional administration of epoetin alfa.

Hemolysis induced by ribavirin has been suggested to be related to a high plasma concentration of ribavirin.[21] The apparent clearance of ribavirin (CL/F), which reflects its plasma concentration at four weeks after the start of combination therapy, has been used as a predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[22–24] However, the progression of hemolytic anemia occurs due not only to hemolysis, but also impaired hematogenous function. On the other hand, hemoglobin (Hb) dynamics directly reflect the degree of progression of anemia. We have reported that the early decline of Hb correlates with the progression of anemia during IFN and ribavirin combination therapy.[25] It is necessary to verify that a similar early predictor for the progression of anemia can be adopted in PegIFN and ribavirin combination therapy, since PegIFN is known to induce less depression of bone marrow function than usual IFN.

In this study, we evaluated the utility of the early decline of Hb in comparison with the CL/F to predict the progression of anemia in the combination therapy of PegIFN and ribavirin.

METHODS

Patients

THIS STUDY WAS conducted at 12 institutions in Japan. A total of 116 patients with chronic hepatitis C were enrolled and treated with a combination of PegIFN and ribavirin. All patients were anti-hepatitis C virus antibody positive, had HCV-RNA detectable in their serum by the polymerase chain reaction (PCR) method, and showed elevated serum alanine transaminase (ALT) (above the upper limit of the normal), serum Hb concentration ≥12 g/dL, neutrocytes ≥1500/mm^3 and platelets ≥10^5/mm^3 within six months before the treatment. Exclusion criteria were the presence of hepatitis B surface antigen, antihuman immunodeficiency virus antibody and other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis).

Table 1 Patient characteristics

Age (years)	50.6 ± 10.1 (24–70)
Gender (male/female)	82/34 (male 70.7%)
Body weight (kg)	64.5 ± 11.1
Previous IFN therapy (naïve/relapser/no responder)	64/38/14
HCV-RNA level (KIU/L) (<500/500–850/850<)	18/27/71
ALT (IU/L)	110 ± 60 (33–76)
Cmn (mg/dL)	0.9 ± 0.2
Liver histology	
Fibrosis (F1/F2/F3/unknown)	35/49/31/1
Activity (A1/A2/A3/A4)	15/33/56/12
WBC (/mm^3)	5317 ± 1207
Neutrocytes (/mm^3)	2778 ± 902
Platelets (×10^4/mm^3)	17.4 ± 4.0
RBC (×10^4/mm^3)	459 ± 41
Hemoglobin (g/dL)	14.5 ± 1.2

Data are given as the mean ± SD.
ALT, alanine transaminase; RBC, red blood cells; WBC, white blood cells.

The baseline characteristics of the patients are shown in Table 1. The mean age was 50.6 ± 10.1 years, and 71% (82 patients) were male. All patients had HCV-RNA with genotype 1 and high viral loads (more than 10^5 copies/mL serum by Amplicor-HCV monitor assay). The mean ALT level was 110 ± 60 IU/L. Sixty-four patients (55%) were IFN naïve and the others were undergoing retreatment.

Treatment schedule

All patients were treated with a combination of PegIFN α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough) for 48 weeks. PegIFN was administered at a mean of 1.5 μg/kg body weight subcutaneously once a week. Ribavirin was given orally twice a day for the total dose. Dosages of both medications were decided based on the

body weight of the patients: those with a body weight of 40–60 kilograms (kg) were given PegIFN 75 μg/body and ribavirin 600 mg/day, those with a body weight of 60–80 kg were given PegIFN 105 μg/body and ribavirin 800 mg/day, and those with a body weight of 80–100 kg were given PegIFN 135 μg/body and ribavirin 1000 mg/day. The PegIFN dose was reduced by 50% if the neutrocyte count was below 750/mm^3 or the platelet (Plt) count was below 8×10^4/mm^3. The PegIFN was discontinued if the neutrocyte count was below 500/mm^3 or the Plt count was below 5.0×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dL and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dL, in accordance with the drug information for ribavirin. No ferric medicine or erythropoietin to prevent anemia was administered.

Patients with persistently undetectable HCV-RNA six-months after the end of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level, hematological and biochemical tests just before therapy, at the end of week 2 and every four weeks during the treatment. When the treatment was completed, the patients were assessed every four weeks up to 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar *et al.*, CL/F at the start of the treatment was calculated as follows: CL/F (L/h) = 32.3 × BW × (1 − 0.0094 × age) × (1 − 0.42 × sex)/Scr (BW, body weight; sex = 0 for male and 1 for female; Scr = serum creatinine).[17]

Definition of "severe anemia" leading to the discontinuance of ribavirin

In this study, the "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dL or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dL from the start of the combination therapy.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, white blood cell count, red blood cell count, Hb concentration, Plt, serum ALT levels and serum creatinine are expressed as mean ± SD. The SVR rate was evaluated using the intention-to-treat analysis (ITT analysis). The differences in proportions were tested by the χ^2-test and Mantel-Haenszel χ^2-test. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. All calculations were performed by SAS program 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Frequency and reasons for dose reduction or discontinuance of PegIFN and/or ribavirin

OF THE 116 patients, 92 completed 48 weeks of therapy, but 24 patients (21%) had to discontinue both PegIFN and ribavirin. Thirty-nine patients (34%) completed the entire treatment schedule without reduction or discontinuance of either drug. The ribavirin dose was decreased for 39 patients (34%) and the PegIFN dose was decreased for 33 patients (28%), including 19 patients for whom both drugs had to be reduced. The reasons for discontinuance of both drugs included anemia, thyroid dysfunction, skin eruption and neutropenia, with the major reasons being anemia (17%) and thyroid dysfunction (17%).

Efficacy of the combination therapy with dose reduction or discontinuance of PegIFN and/or ribavirin

The SVR rate was 57% (66/116) for all according to ITT analysis. According to the category of response to previous IFN therapy, the SVR rates were 43% (6/14) in

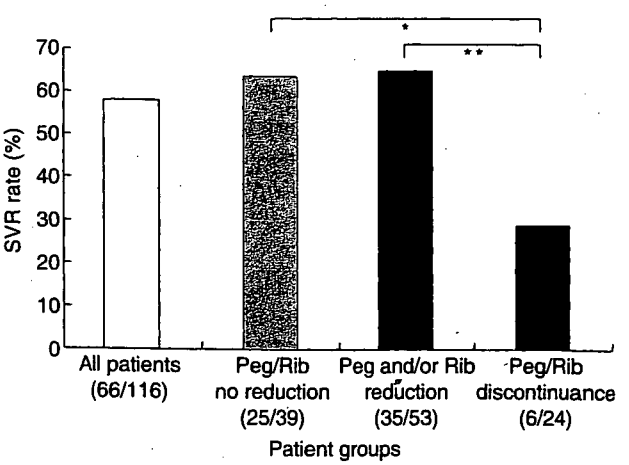

Figure 1 SVR rate due to PegIFN/ribavirin dose reduction or discontinuance. (□), All patients; (▨), patients without dose reduction; (■), patients with dose reduction; (■), patients with drug discontinuance. Significant levels: *$P = 0.003$; **$P = 0.001$.

Table 2 Rate of the ribavirin reduction or discontinuance due to adverse effects according to CL/F level

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
$20 \leq$ CL/F ($n = 12$)	67% (8/12)	25% (3/12)	8% (1/12)	0
$15 \leq$ CL/F < 20 ($n = 23$)	57% (13/23)	30% (7/23)	13% (3/23)	0
$10 \leq$ CL/F < 15 ($n = 39$)	46% (18/39)	31% (12/39)	23% (9/39)	5% (2/39)
CL/F < 10 ($n = 42$)	33% (14/42)	40% (17/42)	26% (11/42)	5% (2/42)

$P = 0.031$ (Mantel–Haenszel χ^2-test).

Table 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy according to CL/F level

	10 g/dL < Hb	8.5 < Hb ≤ 10 g/dL	Hb ≤ 8.5 g/dL
$20 \leq$ CL/F ($n = 12$)	92% (11/12)	12% (1/12)	0
$15 \leq$ CL/F < 20 ($n = 23$)	83% (19/23)	17% (4/23)	0
$10 \leq$ CL/F < 15 ($n = 39$)	72% (28/39)	23% (9/39)	5% (2/39)
CL/F < 10 ($n = 42$)	50% (21/42)	43% (18/42)	7% (3/42)

$P = 0.009$ (Mantel–Haenszel χ^2-test).

non-responders, 61% (23/38) in relapsers, and 58% (37/64) in naïve patients. The relationship between dose reduction or discontinuance of PegIFN and ribavirin and the SVR rate on ITT analysis is shown in Figure 1. Similar SVR rates were obtained in the groups without dose reduction of PegIFN and ribavirin (64%, 25/39) and with reduction of PegIFN and/or ribavirin (66%, 35/53); in detail, the SVR rate was 79% (11/14) in the group with reduction of only PegIFN, 55% (11/20) with reduction of only ribavirin, and 63% (12/19) with reduction of both PegIFN and ribavirin. In the group where both drugs were discontinued, the SVR rate was 25% (6/24), significantly lower than the group without reduction of both drugs ($P = 0.003$), and the group with reduction of PegIFN and/or ribavirin ($P = 0.001$).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients showed a median of 12.6 L/h (range 4.5–27.9). At the start of the treatment, 36% (42/116) were under 10 L/h, 34% (39/116) were 10–15 L/h, 20% (23/116) were 15–20 L/h and 10% (12/116) were 20 L/h or more.

The rate of dose reduction or discontinuance of ribavirin is shown in Table 2 for different levels of CL/F. The rate of discontinuance of ribavirin in all cases was 8% (1/12) for the CL/F ≥ 20, 13% (3/23) for the $15 \leq$ CL/F < 20, 23% (9/39) for the $10 \leq$ CL/F < 15, and 26% (11/42) for the CL/F < 10 group. Ribavirin did not have to be discontinued due to severe anemia among patients with $15 \leq$ CL/F, but did for the 18% (2/11) of those with CL/F < 10 and 22% (2/9) of those with $10 \leq$ CL/F < 15. The rate of reduction and discontinuance of ribavirin correlated significantly with the CL/F level.

CL/F and minimum hemoglobin level during treatment

To examine the relationship between anemia and the cessation of ribavirin in further detail, we evaluated the minimum hemoglobin level during treatment. Table 3 presents the different levels in relation to CL/F. The patients with minimum Hb ≤ 8.5 g/dL, the criterion for discontinuance of ribavirin, accounted for 7% (3/42) of the group of CL/F < 10, and 5% (2/39) of the group of $10 \leq$ CL/F < 15. No patients of the group of CL/F ≥15 showed minimum Hb ≤ 8.5 g/dL.

Early decline of Hb and progression of anemia during combination therapy

Following the initiation of combination therapy, the Hb concentration decreased rapidly until the end of four-weeks. At the end of two weeks, Hb had decreased by 1.1 ± 1.0 g/dL among the patients without dose reduction of ribavirin ($n = 53$), 1.6 ± 1.2 g/dL among those with dose reduction ($n = 39$), and 1.8 ± 1.0 g/dL among

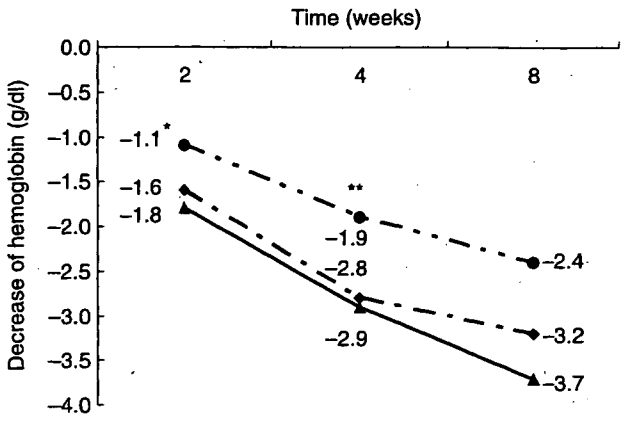

Figure 2 Course of ΔHb in the initial phase. (—··—), No reduction; (—·—·), reduction; (———), discontinuance. *Significantly different between patients with discontinuance and patients with no reduction ($P = 0.04$). **Significantly different between patients with discontinuance and patients with no reduction ($P = 0.008$), and between patients with discontinuance and patients with reduction ($P = 0.003$).

those who had discontinued ribavirin ($n = 24$). It was significantly different between the patients with no reduction and those with discontinuance of therapy ($P = 0.04$). At the end of four weeks, Hb had decreased by 1.9 ± 1.2 g/dL among the patients without dose reduction of ribavirin, 2.8 ± 1.2 g/dL among those with dose reduction, and 2.9 ± 1.2 g/dL among those who had discontinued ribavirin. Hb decline at the end of four weeks was significantly greater in the patients who had discontinued treatment and those who had reduced it, than in those with no reduction ($P = 0.008$, $P = 0.003$, respectively) (Fig. 2).

In this study, we selected the Hb decrease at the end of two weeks as the predictive factor for anemia progression. This is because the judgment of Hb decrease at the end of four weeks is too late to prevent progression of anemia or to perform appropriate counter-measures, such as the administration of epoetin or reduction of ribavirin. Next, we tried to use two borderlines of ΔHb: ΔHb 2.0 indicates a 2 g/dL Hb decrease at the end of two weeks and ΔHb 1.5 indicates a 1.5 g/dL Hb decrease. When ΔHb 2.0 was adopted, the rate of discontinuance of drugs was 31% (12/39) in the ΔHb ≥ 2.0 and 14% (11/76) in the ΔHb < 2.0. When ΔHb 1.5 was adopted, it was 23% (14/60) in the ΔHb ≥ 1.5 and 16% (9/55) in the ΔHb < 1.5. Comparison of the ΔHb 2.0 and ΔHb 1.5 standards showed the sensitivity to be 52% (12/23) and 61% (14/23), and the specificity to be 71% (65/92) and 50% (46/92), respectively. With respect to discontinuance due to anemia, both ΔHb 2.0 and ΔHb 1.5 gave 100% sensitivity (3/3), and the specificities were 68% (76/112) using ΔHb 2.0 and 49% (55/112) using ΔHb 1.5. We decided to adopt the standard of ΔHb 2 g/dL at the end of two weeks from the start of the pegylated IFN and ribavirin combination therapy as the predictive factor for anemia progression ("2 by 2" standard), which has been taken as a predictive factor for anemia in the IFN and ribavirin combination therapy.[25]

Applying the "2 by 2" standard to PegIFN plus ribavirin combination therapy, the rate of reduction or discontinuance of the ribavirin dose was examined with respect to the Hb decrease level (Table 4). Only one patient was excluded from this study, because the treatment was discontinued on the 11th day. In the group of ΔHb (the decrease in Hb concentration at two weeks from the baseline) ≥ 2 g/dL ($n = 39$), the doses were reduced for 18 patients (46%) and discontinued for 12 (31%), three of whom (8%) had severe anemia. For the group of ΔHb < 2 g/dL (76 patients), the doses were reduced for 21 patients (28%) and discontinued for 11 (14%); none due to severe anemia.

Early decline of Hb and minimum hemoglobin level during treatment

As in the case of ΔHb, we evaluated the minimum hemoglobin level during treatment, as shown in Figure 3. The patients with minimum Hb ≤ 8.5 g/dL accounted for 10% (4/39) of the group of ΔHb ≥ 2 g/dL, and there was no patient with minimum Hb ≤ 8.5 g/dL

Table 4 Rate of the ribavirin reduction or discontinuance due to adverse effects according to Hb decrease levels

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
ΔHb < 2 g/dL ($n = 76$)	58% (44/76)	28% (21/76)	14% (11/76)	0
ΔHb ≥ 2 g/dL ($n = 39$)	23% (9/39)	46% (18/39)	31% (12/39)	8% (3/39)

$P = 0.004$ (Mantel–Haenszel χ^2-test).