nature medicine



# Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis

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**Suppressor of cytokine signaling (SOCS) 3 attenuates proinflammatory signaling mediated by the signal transducer and activator of transcription (STAT) family of proteins. But acute inflammation can occur after exposure to pathogen-derived inducers staphylococcal enterotoxin B (SEB) and lipopolysaccharide (LPS), or the lectin concanavalin A (ConA), suggesting that physiologic levels of SOCS3 are insufficient to stem proinflammatory signaling under pathogenic circumstances. To test this hypothesis, we developed recombinant cell-penetrating forms of SOCS3 (CP-SOCS3) for intracellular delivery to counteract SEB-, LPS- and ConA-induced inflammation. We found that CP-SOCS3 was distributed in multiple organs within 2 h and persisted for at least 8 h in leukocytes and lymphocytes. CP-SOCS3 protected animals from lethal effects of SEB and LPS by reducing production of inflammatory cytokines and attenuating liver apoptosis and hemorrhagic necrosis. It also reduced ConA-induced liver apoptosis. Thus, replenishing the intracellular stores of SOCS3 with CP-SOCS3 effectively suppresses the devastating effects of acute inflammation.**

Inflammation represents a fundamental response to microbial, chemical and physical injury. The production of inflammatory mediators depends on tightly regulated intracellular signaling by stress-responsive transcription factors as positive activators of the proinflammatory genetic program[1]. Concurrently, the genome can respond physiologically by eliciting a set of repressors that extinguish inflammation. SOCS1 and SOCS3 are rapidly induced and then degraded[2]. They block phosphorylation-dependent activation of STAT1 in response to interferon (IFN)-γ or STAT3 phosphorylation in response to interleukin (IL)-6, and target the IFN-γ receptor and/or IL-6 receptor signaling complexes for proteosomal degradation[3–5]. Paradoxically, despite the presence of physiologic regulators such as SOCS, the host defense systems can pathologically perpetuate inflammation by overproducing host mediators that cause collateral damage to multiple organs. In the well-established animal models of acute organ injury resulting from pathogen-derived inducers SEB and LPS or lectin ConA, inflammation and apoptosis of the liver depend on cytotoxic signaling by tumor necrosis factor (TNF)-α and IFN-γ[6–8] and Fas–Fas ligand interaction[9,10], respectively. These models of acute liver injury provide a well-defined *in vivo* system for inflammation-associated apoptosis, which is relevant to fulminant hepatitis caused by viral and nonviral agents. We reasoned that replenishment of SOCS proteins with recombinant, cell-penetrating versions may represent a powerful approach to treatment of these acute inflammatory disorders. To test this hypothesis, we developed cell-penetrating SOCS3 (CP-SOCS3) as the prototype for intracellular protein therapy and conducted experiments to determine whether exogenously administered SOCS proteins can compensate for the degradative loss of endogenous SOCS3 inhibitor and terminate noxious cytokine signaling during an acute inflammatory response.

We designed and developed recombinant mouse CP-SOCS3 proteins (**Fig. 1a**,**b**). A membrane-translocating motif (MTM) composed of 12 amino acids from a hydrophobic signal sequence from fibroblast growth factor 4 (ref. 11) was attached to either the N-terminal (HMS3) or C-terminal (HS3M) ends to mediate uptake into cells. We also constructed a control protein (His-SOCS3; HS3) lacking the MTM. Purity and yields of the recombinant SOCS3 proteins were comparable (**Fig. 1c**).

We detected the intracellular delivery of recombinant SOCS3 proteins in mouse macrophage RAW cells by confocal laser scanning microscopy. Fluorescein isothiocyanate (FITC)-labeled SOCS3 lacking MTM was not detectable in RAW cells. In contrast, the two MTM-bearing CP-SOCS3 proteins, His-SOCS3-MTM (HS3M) and His-MTM-SOCS3 (HMS3), were abundantly present in the cytoplasm of RAW cells (**Fig. 1c**). These cells were not fixed and the broad-range protease proteinase K was used after pulsing cells with FITC-labeled proteins to prevent background fluorescence from cell surface–absorbed SOCS3 proteins. Thus, the protease-resistant fluorescence indicates that only MTM-bearing SOCS3 proteins were able to penetrate cells.

To confirm that the CP-SOCS3 proteins could penetrate cells we tested their effect on intracellular STAT1 phosphorylation. Inducibly expressed endogenous SOCS1 and SOCS3 are known to block STAT1 phosphorylation by Janus kinases (JAK) 1 and 2, a key step in intracellular signaling induced by IFN-γ[3,12]. IFN-γ–induced phosphorylation of STAT1 was readily detected in cells exposed to HS3, which lacks the MTM motif required for membrane penetration (**Fig. 1d**). In contrast, both forms of CP-SOCS3, HS3M and HMS3, suppressed STAT1 phosphorylation in a dose-dependent manner, with a concentration causing 50% inhibition ($IC_{50}$) <2 μM (**Fig. 1e**). We confirmed the inhibitory effect of

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Published online 10 July 2005; doi:10.1038/nm1269

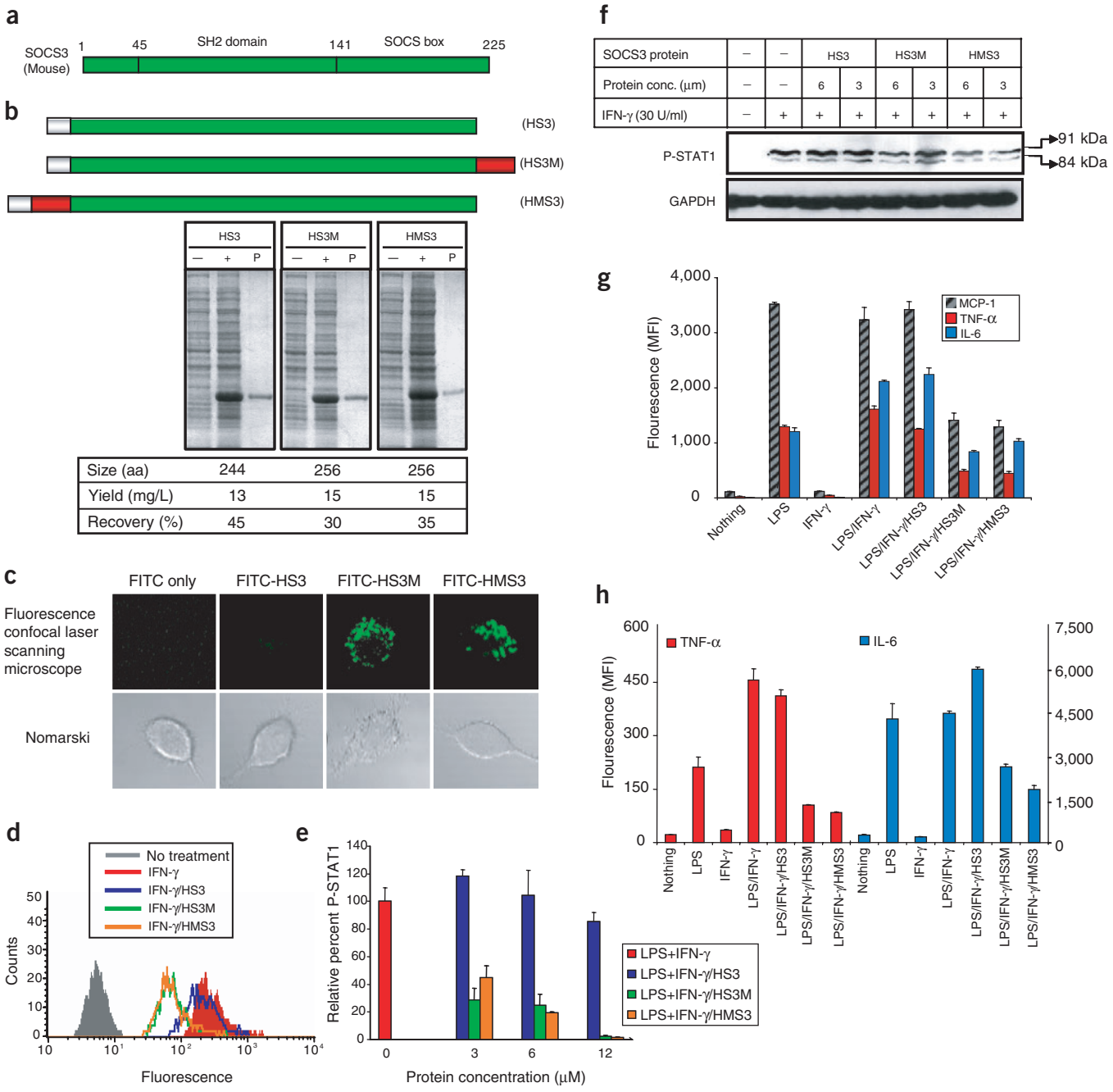


**Figure 1** Structure, design, expression, purification, intracellular delivery and inhibitory activity of recombinant SOCS3 proteins. (**a**) Structure of mouse SOCS3 protein. (**b**) Design of recombinant SOCS3 proteins that contained MTM (AAVLLPVLLAAP, red), histidine tag for affinity purification (MGSSHHHHHHSSLVPRGSH, white) and cargo (SOCS3, green). Expression of SOCS3 fusion proteins in *E. coli* before (–) and after (+) induction with IPTG monitored by SDS-PAGE and stained with Coomassie blue. The size (in amino acids), yield and recovery (in percent) of soluble form from denatured form are indicated. (**c**) Fluorescence confocal laser scanning microscopy shows intracellular localization of recombinant SOCS3 proteins (top). Nomarski image of the same cells (bottom). (**d**) Inhibition of STAT1 phosphorylation detected by cytometric bead array (CBA) method. The levels of phosphorylated STAT1 untreated and treated with IFN-γ were compared to the levels in IFN-γ–treated RAW cells that were pulsed with 10 μM of HS3, HS3M or HMS3. (**e**) Concentration-dependent inhibition of STAT1 phosphorylation detected by CBA method. (**f**) Inhibition of phosphorylation of STAT1 by CP-SOCS3 using immunoblotting analysis. (**g**) Inhibition of MCP-1, TNF-α and IL-6 expression by CP-SOCS3 in cultured AMJ2-C8 macrophages. (**h**) Inhibition of TNF-α and IL-6 expression by CP-SOCS3 in primary macrophages isolated from peritoneal exudates of C3H/HeJ mice. Error bars in **c**, **e** and **f** indicate the ± s.d. of the mean value derived from each assay done in triplicate.

CP-SOCS3 proteins on STAT1 phosphorylation using immunoblotting studies (**Fig. 1f**).

Treatment of macrophages with 10 μM HS3M or HMS3 inhibited the expression of TNF-α, IL-6 and monocyte chemoattractant protein (MCP)-1 by 55–75% during a subsequent incubation of 4 h. In contrast, expression of cytokines and chemokines in macrophages treated with a control SOCS3, HS3, was unchanged, indicating that recombinant SOCS3 without MTM was not inhibitory (**Fig. 1g**). Two CP-SOCS3

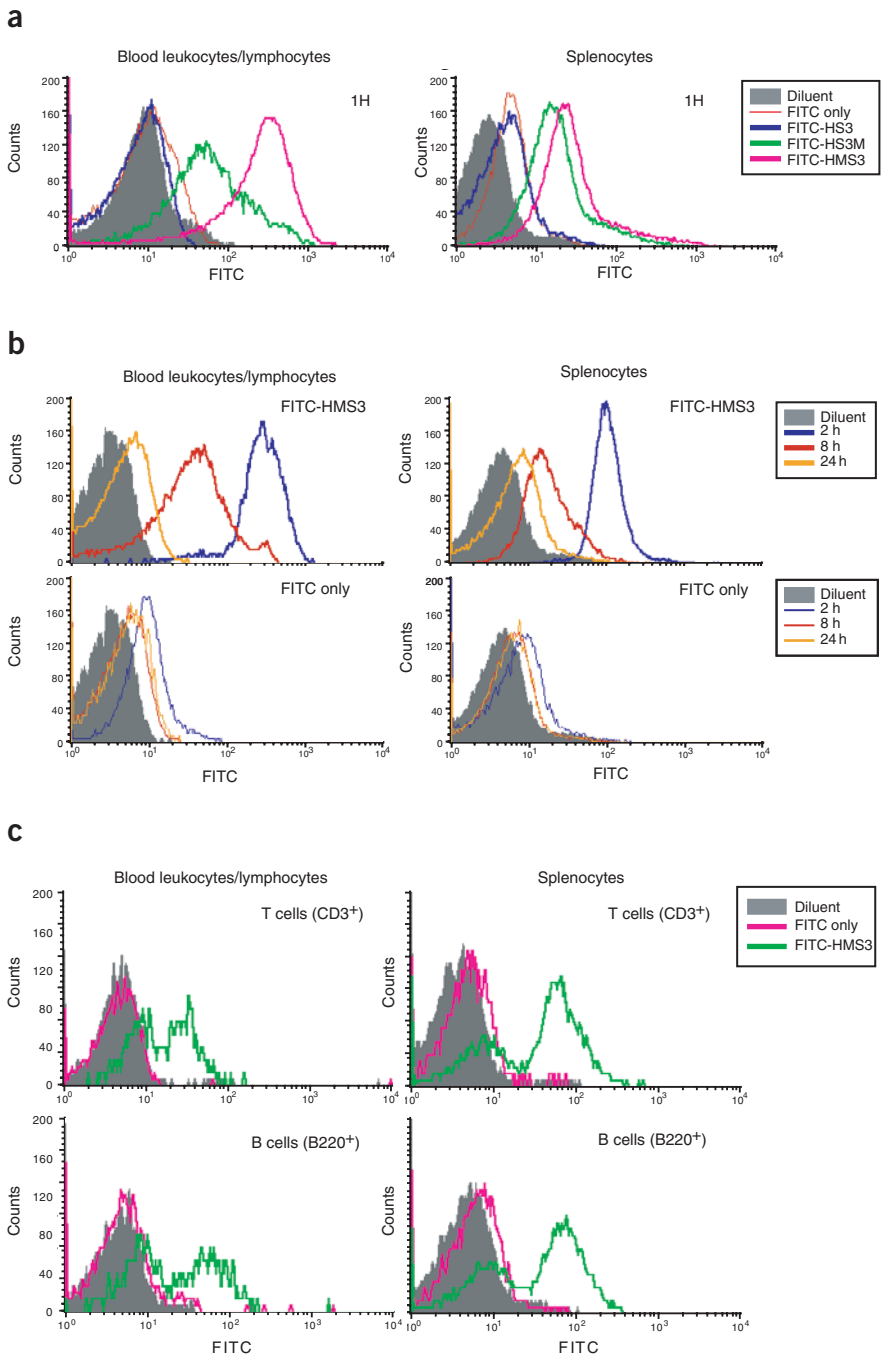


**Figure 2** *In vivo* delivery, intracellular persistence and tissue distribution of the CP-SOCS3 proteins. (**a**) FACS analysis of leukocytes and lymphocytes isolated from whole blood (blood leukocytes/lymphocytes) and splenocytes of C3H/HeJ mice 1 h after intraperitoneal injection of diluent, unconjugated free FITC (1 μM, FITC only) and FITC-conjugated SOCS3 proteins (1 μM, FITC-HS3, FITC-HS3M and FITC-HMS3). (**b**) Persistence of FITC-conjugated CP-SOCS3 in cells prepared from C3H/HeJ mice at different time points after intraperitoneal injection of FITC-conjugated CP-SOCS3 protein (1 μM, FITC-HMS3; 2, 8 and 24 h) and unconjugated free FITC (FITC only; 2, 8 and 24 h). FACS analysis was performed immediately after cell preparation without fixation and after treatment with proteinase K to degrade cell surface–bound SOCS3 proteins. (**c**) *In vivo* cellular uptake of CP-SOCS3 in T and B cells. Cells isolated from mice treated with diluent, FITC only or FITC-HMS3 were blocked with Fc-specific antibody followed by binding with cell-type specific anti-CD3 and anti-B220 antibodies. (**d**) Tissue distribution of CP-SOCS3 *in vivo*. Fluorescence microscopy shows CP-SOCS3 protein in various organs after intraperitoneal injection of FITC only or FITC-HMS3. Cryosections (15 μm) of organs were analyzed with fluorescence microscope.

proteins suppressed TNF-α and IL-6 expression induced by the combination of IFN-γ and LPS in primary peritoneal macrophages isolated from C3H/HeJ mice, which use the pathway depending on interaction of Toll-like receptor 3 (TLR3) with adaptor protein Trif (AW046014)[13,14]. In contrast, HS3 was inactive, reaffirming the inability of a recombinant SOCS3 without MTM to inhibit intracellular signaling (**Fig. 1h**).

We monitored *in vivo* delivery of CP-SOCS3 proteins, FITC-labeled HS3M and HMS3, after intraperitoneal injections into separate groups of mice. The blood leukocyte- and lymphocyte-rich fraction collected within 1 h of injection and analyzed by FACS showed a gain in fluorescence, indicative of the presence of FITC-labeled proteins as compared with control animals that received FITC-labeled HS3 or unconjugated FITC (**Fig. 2a**). One of the two CP-SOCS3 proteins, HMS3, showed a stronger intracellular signal in blood leukocytes and lymphocytes (**Fig. 2a**), prompting us to analyze its time-dependent kinetics in blood and spleen leukocytes and lymphocytes. Notably, FITC-labeled HMS3 was detectable, albeit in reduced amounts, at 8 h and even 24 h after intraperitoneal injection (**Fig. 2b**). In contrast, unconjugated free FITC at equimolar concentration (FITC only) did not produce any significant gain in fluorescence as compared with diluent (**Fig. 2b**). Thus, MTM enabled two CP-SOCS3 proteins (HS3M and HMS3) to

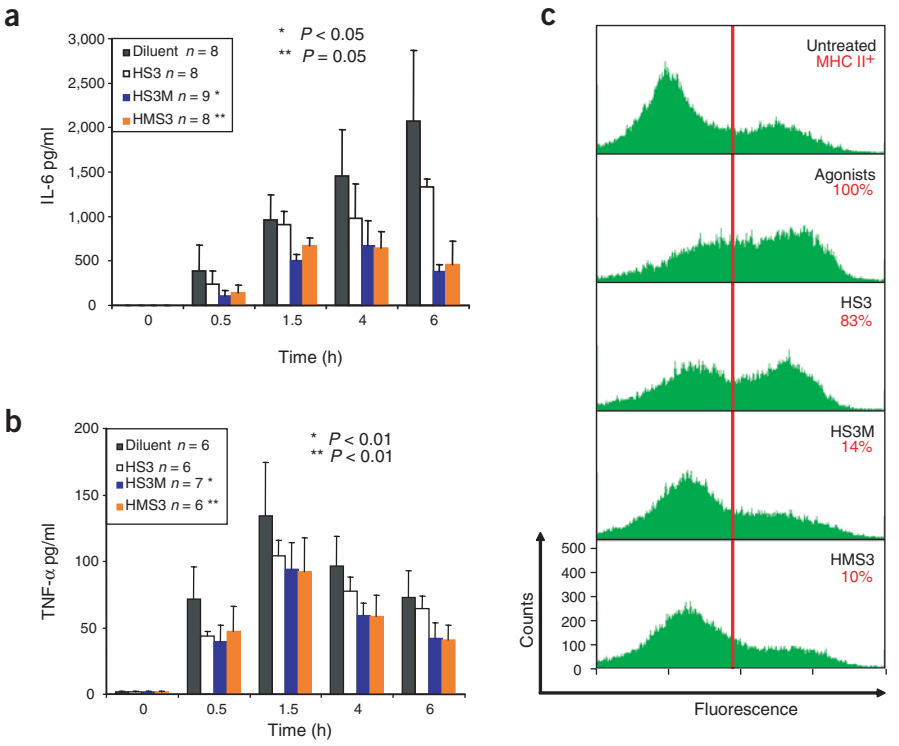


**Figure 3** CP-SOCS3 proteins inhibit the production of inflammatory cytokines IL-6 and TNF-α, and the cell-surface expression of MHC class II *in vivo*. (**a**,**b**) IL-6 and TNF-α were measured by a CBA in blood plasma obtained from saphenous vein of C3H/HeJ mice at indicated intervals after SEB+D-GAL challenge. Error bars indicate mean ± s.d. derived from each assay done in at least eight mice (**a**) or at least six mice (**b**). (**c**) Total splenocytes were isolated from the C3H/HeJ mice that survived 48 h after intraperitoneal administration of SEB+D-GAL. Cell surface–expressed MHC class II molecules on CD11b$^+$ cells from mice that were not challenged (untreated) or challenged with agonists (SEB+D-GAL) only (agonists), plus treated with SOCS3 proteins (HS3, HS3M or HMS3), were measured 48 h after challenge. The value of 100% represents the increment in the number of double positive (CD11b and I-A$^k$) cells between untreated and agonist only–treated mice. The inhibition of MHC-II in CD11b-positive cells treated with SOCS3 protein represents the percentage of double positive cells as compared to the 100% in agonist only–treated mice.



gain rapid (1 h) entry to blood and spleen leukocytes and lymphocytes, wherein persistence of HMS3 was observed for at least 8 h.

Further analysis of CP-SOCS3 delivery *in vivo* was extended to the leukocyte and lymphocyte subsets in blood, spleen and liver. White blood cell–rich fractions, total splenocytes and intrahepatic lymphocytes were isolated 2 h after a single intraperitoneal injection of FITC-conjugated HMS3, unconjugated FITC or diluent only. T and B cells from blood and spleen showed high levels of CP-SOCS3 uptake (**Fig. 2c**). Intrahepatic lymphocytes showed the highest uptake of CP-SOCS3 *in vivo* (**Supplementary Table 1** online). Moreover, liver macrophages (Kupffer cells) and NKT cells showed uptake of CP-SOCS3. These results indicate that CP-SOCS3 can be delivered to major leukocyte and lymphocyte subsets in blood, spleen and liver.

Whereas these cells are primary participants in the inflammatory response because they produce inflammatory cytokines and chemokines, other cells that comprise potential targets of inflammatory injury by cytokines (*e.g.*, hepatocytes) might benefit from delivery of CP-SOCS3. Therefore, we determined the distribution of fluorescently tagged CP-SOCS3 in different organs in cryosections analyzed by fluorescence microscopy (**Fig. 2d**). Predictably, liver showed the highest level of fluorescence resulting from CP-SOCS3 uptake because the intraperitoneal route of administration favors delivery of CP-SOCS3 through the portal circulation to this organ. Notably, kidney also showed a high level of protein uptake compared to other organs. CP-SOCS3 was detectable to a lesser degree in lung, spleen and heart. Results obtained in mice treated with diluent were identical to those treated with FITC (data not shown). We confirmed this direct fluorescence detection of CP-SOCS3 with indirect immunodetection using SOCS3-specific antibody (data not shown).

Superantigen SEB targets T cells, inducing cytokine-mediated systemic inflammation, and causes fulminant liver injury followed by rapid death of D-galactosamine–sensitized mice[6–8,15,16]. We used this well-established model to test the hypothesis that an *in vivo* balance in favor of proinflammatory intracellular transducers evoked by cytokines and chemokines induced by SEB can be shifted toward physiologic anti-inflammatory regulators by introduction of recombinant CP-SOCS3. Mice sensitized with D-galactosamine were intraperitoneally injected with SEB and treated with SOCS3 proteins according to Protocol A. Consistent with the *ex vivo* data (**Fig. 1h**), we observed strong suppression of IL-6 (**Fig. 3a**) and moderate attenuation of TNF-α production (**Fig. 3b**) by CP-SOCS3.

Proinflammatory signaling exemplified by IFN-γ–induced STAT1 phosphorylation leads to inducible expression of the major histocompatibility complex (MHC) class II molecules that contribute to superantigen SEB binding and pathogenity[16–18]. Treatment of mice with SEB plus D-galactosamine (SEB+D-GAL) increased the expression of MHC class II, which peaked at 48 h (**Fig. 3c**). This induction of MHC class II (calculated as 100%) was not significantly altered by HS3 (83%) administered intraperitoneally. In contrast, the induction of MHC class II was substantially reduced to 14% and 10% after *in vivo* administration of CP-SOCS3 proteins HS3M and HMS3, respectively. This hitherto unreported effect of SOCS3 underscores its negative regulatory role in induction of MHC class II *in vivo*, probably through inhibition of IFN-γ–induced activation of STAT1, which interacts with the type IV *C2ta* promoter for expression of MHC class II[19].

We next compared the *in vivo* effect of CP-SOCS3 using two treatment protocols, A and B, in the SEB+D-GAL model of acute liver injury. In Protocol A, mice were administered CP-SOCS3 before and after the challenge with SEB+D-GAL. In Protocol B, mice were administered CP-SOCS3 only after challenge with SEB+D-GAL. Of C3H/HeJ mice treated with intraperitoneal injections of diluent or HS3 according to Protocol A, 70–80% showed progressive signs of illness leading to death within 48 h after SEB+D-GAL challenge (**Fig. 4a**). In contrast, administration of a CP-SOCS3 (HS3M) produced a markedly protective effect (**Fig. 4b**). Thus, HS3M increased survival from 20% to 100% ($P < 0.001$), whereas HMS3-treated mice were protected to a lesser degree (75% survival, $P < 0.05$; **Fig. 4a**).

Administration of CP-SOCS3 proteins after challenge with SEB+D-GAL, according to Protocol B, produced results similar to those obtained

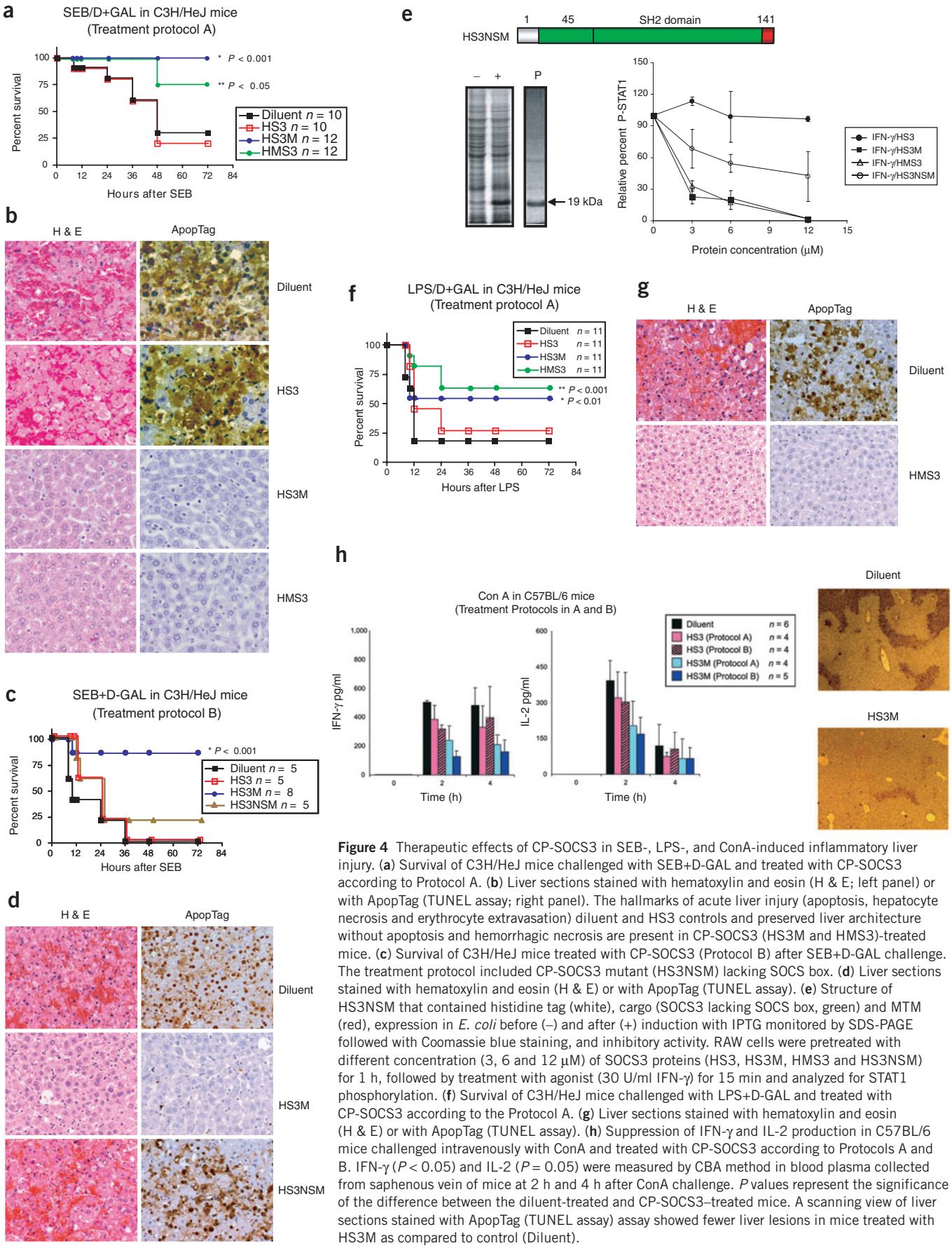


**Figure 4** Therapeutic effects of CP-SOCS3 in SEB-, LPS-, and ConA-induced inflammatory liver injury. (**a**) Survival of C3H/HeJ mice challenged with SEB+D-GAL and treated with CP-SOCS3 according to Protocol A. (**b**) Liver sections stained with hematoxylin and eosin (H & E; left panel) or with ApopTag (TUNEL assay; right panel). The hallmarks of acute liver injury (apoptosis, hepatocyte necrosis and erythrocyte extravasation) diluent and HS3 controls and preserved liver architecture without apoptosis and hemorrhagic necrosis are present in CP-SOCS3 (HS3M and HMS3)-treated mice. (**c**) Survival of C3H/HeJ mice treated with CP-SOCS3 (Protocol B) after SEB+D-GAL challenge. The treatment protocol included CP-SOCS3 mutant (HS3NSM) lacking SOCS box. (**d**) Liver sections stained with hematoxylin and eosin (H & E) or with ApopTag (TUNEL assay). (**e**) Structure of HS3NSM that contained histidine tag (white), cargo (SOCS3 lacking SOCS box, green) and MTM (red), expression in *E. coli* before (–) and after (+) induction with IPTG monitored by SDS-PAGE followed with Coomassie blue staining, and inhibitory activity. RAW cells were pretreated with different concentration (3, 6 and 12 μM) of SOCS3 proteins (HS3, HS3M, HMS3 and HS3NSM) for 1 h, followed by treatment with agonist (30 U/ml IFN-γ) for 15 min and analyzed for STAT1 phosphorylation. (**f**) Survival of C3H/HeJ mice challenged with LPS+D-GAL and treated with CP-SOCS3 according to the Protocol A. (**g**) Liver sections stained with hematoxylin and eosin (H & E) or with ApopTag (TUNEL assay). (**h**) Suppression of IFN-γ and IL-2 production in C57BL/6 mice challenged intravenously with ConA and treated with CP-SOCS3 according to Protocols A and B. IFN-γ ($P < 0.05$) and IL-2 ($P = 0.05$) were measured by CBA method in blood plasma collected from saphenous vein of mice at 2 h and 4 h after ConA challenge. *P* values represent the significance of the difference between the diluent-treated and CP-SOCS3–treated mice. A scanning view of liver sections stained with ApopTag (TUNEL assay) assay showed fewer liver lesions in mice treated with HS3M as compared to control (Diluent).

with Protocol A. HS3M increased survival by 88% as compared to mice treated with diluent (0%), HS3 (0%) or HS3NSM (20%; **Fig. 4c**). Histologic analysis of liver sections obtained from mice injected with HS3 according to Protocols A and B or treated with HS3NSM according to Protocol B showed diffuse hepatocellular injury marked by extensive apoptosis, which was characterized by chromatin condensation and DNA fragmentation (**Fig. 4b**,**d**). Moreover, hemorrhage and necrosis were prominent (**Fig. 4b**,**d**). In contrast, surviving mice treated with CP-SOCS3 had normal tissue architecture with no signs of apoptotic and/or necrotic liver injury. The dependence of CP-SOCS3's therapeutic effect on its 'cargo' rather than on an MTM-nonspecific effect was established by designing and testing CP-SOCS3 mutant protein engineered to have an SOCS3-derived amino terminal region and an SH2 domain fused to MTM, expressed and purified to homogeneity (**Fig. 4e**). This CP-SOCS3 mutant, denoted HS3NSM, lacked a SOCS box and was cell penetrating, but with considerable loss of function in a STAT1 phosphorylation assay ($IC_{50}$ >10 μM, **Fig. 4e**) as compared with the two CP-SOCS3 proteins that bear a full-length SOCS3 ($IC_{50}$ <2 μM).

The above studies of the therapeutic potential of CP-SOCS3 in the SEB+D-GAL model were extended to another pathogen-derived pro-inflammatory agent, LPS, which induces monocyte- and macrophage-mediated acute liver injury in D-galactosamine–sensitized mice[20]. We tested this well-established model of LPS lethality in C3H/HeJ mice using a high dose of LPS (130 μg)[20,21]. The C3H/HeJ mice maintain responsiveness to high concentration of LPS through TLR3-Trif pathway, despite a loss-of-function mutation of TLR4 (refs. 13,14,22). Administration of CP-SOCS3 according to Protocol A increased survival of mice to 64% ($P < 0.001$) and 55% ($P < 0.01$) in HMS3 and HS3M groups, respectively, as compared to diluent (18%; **Fig. 4f**). Histologic analysis documented the protective effect of CP-SOCS3 (HMS3) on LPS-induced massive liver apoptosis and hemorrhagic necrosis (**Fig. 4g**). Thus, CP-SOCS3 was effective in the second model of acute inflammation caused by pathogen-derived LPS.

Concanavalin A (ConA)-induced hepatitis represents a widely used model of inflammatory liver injury mediated by T cells and Fas ligand–Fas death receptor signaling[10,23–25]. *In vivo* ConA-induced production of T-cell cytokines such as IFN-γ and IL-2 was reduced in C57BL/6 mice that received CP-SOCS3 (HS3M) according to Protocols A and B as compared to the mice treated with diluent or HS3 (**Fig. 4h**). In the scanning view of the liver, we observed a marked reduction of ConA-induced apoptosis upon treatment with CP-SOCS3 (**Fig. 4h**).

Cumulatively, the *in vivo* cytoprotective effect of CP-SOCS3 proteins resulted in increased survival of mice challenged with SEB+D-GAL or LPS+D-GAL. Moreover, CP-SOCS3 showed similar effectiveness on suppression of inflammatory cytokines when administered using Protocols A and B. Two control recombinant SOCS3 proteins, non-CP-SOCS3 (HS3) lacking MTM and SOCS3 mutant (HS3NSM) that contains MTM but lacks functional SOCS box, did not influence any parameters studied in animal models of acute inflammation. Thus, the anti-inflammatory and antiapoptotic *in vivo* effects of CP-SOCS3 proteins depended on structurally intact SOCS3 molecule containing the SOCS box and MTM.

In summary, we have successfully enabled recombinant forms of the physiologic inhibitor SOCS3 to be delivered to immune cells, liver and other major organ systems. Replenishing the intracellular stores of SOCS3 with its cell-penetrating counterpart effectively suppresses cytokine-mediated signal transduction associated with acute inflammation and massive liver apoptosis and hemorrhagic necrosis induced by pathogen-derived agonists, leading to increased survival rates. Given their efficient delivery and therapeutic potential, cell-penetrating forms of conditionally labile inhibitors such as SOCS3 may represent a more favorable strategy for treating systemic and localized inflammatory syndromes than previously reported approaches using gene transfer technology[26].

## METHODS

**Preparation of CP-SOCS3 proteins.** The full-length cDNA for murine SOCS3 was provided by M. Shong (Chungnam National University, Korea). Fusion proteins comprising a full-length SOCS3 or its loss-of-function deletion mutant were engineered as described previously[27–29]. HS3, HS3M, HMS3 and His-SOCS3-Mutant-MTM (HS3NSM) were purified by metal-affinity chromatography under denaturing conditions (Qiagen) and reconstituted. Purified proteins contained 8–13 μg LPS/mg recombinant protein as determined by the Limulus chromogenic assay (Associates of Cape Cod). LPS content was greatly reduced by incubating the bacteria pellet with denaturing buffer containing 8 M urea with agitation (>12 h) before sonication and extensive dialysis of purified protein for 24 h with 300-fold (vol/vol) of DMEM. This method produced recombinant proteins with minimal LPS content (0.2–0.3 μg LPS/mg protein) albeit at lower yield (20–35% versus 30–45% recovery).

**Protein labeling and intracellular detection.** We labeled proteins with FITC (Pierce Chemical) according to the manufacturer's instructions. We analyzed FITC-labeled proteins for their intracellular localization in unfixed RAW 264.7 cells by confocal laser scanning microscopy using direct fluorescence. Cells were incubated with 1 μm FITC-labeled proteins (FITC-HS3, FITC-HS3M and FITC-HMS3) or an equimolar concentration of unconjugated FITC (FITC only). We treated cells with proteinase K to remove cell surface–absorbed proteins[18].

**STAT1 phosphorylation.** We measured phosphorylated STAT1 in RAW cell lysates using a cytometric bead array (CBA, Pharmingen). Cells were pretreated with different concentrations (3, 6 and 12 μM) of SOCS3 proteins (HS3, HS3M and HMS3) for 1 h, followed by treatment with agonists (10 U/ml IFN-γ + 100 ng/ml LPS) for 15 min. For western analysis, cells were pretrated with different concentrations (3 and 6 μM) of SOCS3 proteins (HS3, HS3M and HMS3) for 1 h, followed by treatment with agonists (30 U/ml IFN-γ + 100 ng/ml LPS) for 15 min. We prepared and analyzed denatured whole-cell lysates by using antibody against phosphorylated (pY701)-specific STAT1. We also visualized GAPDH as internal loading control.

**Cytokine and chemokine measurement.** We measured concentrations of TNF-α, IL-6 and MCP-1 in the culture supernatants of transformed (AMJ2-C8, ATCC) or primary macrophages using a CBA performed according to the manufacturer's instructions. Primary peritoneal macrophages were obtained from C3H/HeJ mice. We pretreated cells with 10 μM SOCS3 proteins and then stimulated them with LPS (1 μg/ml) and/or IFN-γ (100 U/ml) without the removal of SOCS3 proteins. We collected supernatants for analysis after 4 h or 24 h of stimulation.

**Cell and organ distribution of CP-SOCS3 proteins.** We prepared white blood cell–rich fractions from blood, total splenocytes from spleen and intrahepatic lymphocytes from liver from mice injected intraperitoneally with FITC-SOCS3 proteins (70 μg) or an equimolar concentration of FITC[20]. CP-SOCS3 in mixed cell populations from blood and spleen were analyzed by FACS without fixation[15]. We also analyzed cellular uptake of CP-SOCS3 in each cell type (T and B cells, macrophages, dendritic cells, NK or NKT cells) from blood, spleen or liver by FACS. We analyzed tissue distribution of CP-SOCS3 on cryosections (15 μm thickness) of organ tissues (liver, kidney, lung, spleen and heart) using fluorescence microscopy.

***In vivo* models of SEB+D-GAL–induced toxic shock, LPS+D-GAL–induced lethal shock and ConA-induced hepatitis.** We challenged C3H/HeJ mice with an intraperitoneal injection of SEB (280 μg/300 μl/mouse, Toxin Technology) and D-galactosamine (20 mg/200 μl/mouse, Sigma). Two protocols were designed for administration of SOCS3 proteins (0.25 μg/μl, 300 μl/injection/mouse). We intraperitoneally injected proteins or diluent (DMEM) into mice according to Protocol A 30 min before and after (30 min, 1.5 h, 2.5 h, 4.5 h and 6.5 h) challenge with inflammation inducer. Alternatively, we used Protocol B, in which we administered proteins or diluent only after the challenge at the same time intervals as

above. We intravenously injected C57BL/6 mice with ConA (15 mg/kg, Sigma) through the tail vein and then treated them with the proteins (six intraperitoneal injections of 75 μg each). In these experiments, CP-SOCS3 contained a negligible level of LPS (0.2–0.3 μg/mg SOCS3 protein). Animal handling and experimental procedures were performed in accordance with the American Association of Accreditation of Laboratory Animal Care guidelines and approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

***In vivo* cytokine assay in blood.** We collected blood samples taken from the saphenous vein (30 μl) before (30 min) and after SEB+D-GAL challenge at indicated intervals (0.5, 1.5, 4 and 6 h), and after ConA challenge at indicated time (2 h and 4 h). We measured the plasma level of cytokines using CBA according to the manufacturer's instructions.

**Measurement of MHC class II expression *in vivo*.** At 24 h and 48 h after SEB+D-GAL challenge, we prepared total splenocytes from C3H/HeJ mice untreated or treated with diluent or SOCS3 proteins. The doubly positive (Mac-1 and I-$A^k$) cells were analyzed by FACScalibur.

**Histologic analysis.** We stained formalin-fixed and paraffin-embedded sections of tissue samples (liver, kidney, lung, spleen and heart) with hematoxylin and eosin. Apoptosis of liver cells was evaluated by histology and by TUNEL assay using the ApopTag reagent (Chemicon) according to the manufacturer's instructions.

**Statistical analysis.** All experimental data obtained from cultured macrophages were expressed as mean ± s.d. A two-way repeated measure analysis of variance and a log-rank test were used to determine the significance of the difference in *in vivo* cytokine production and survival, respectively.

*Note: Supplementary information is available on the Nature Medicine website.*

ACKNOWLEDGMENTS

We thank D. Ballard and E. Ruley for critical reading the manuscript, X.-Y. Liu for experimental advice, M. Shong (Chungnam National University, Korea) for providing mouse SOCS3 cDNA with permission from D. J. Hilton (Royal Melbourne Hospital, Australia). We also thank A. M. Hernandez and K. Quarry for assistance in preparation of the manuscript. US National Institutes of Health grants HL 69542, HL 62356 and HL 68744 supported this work. The use of core facilities in this study was supported by US National Institutes of Health 2P30 CA 68485 to the Vanderbilt Ingram Cancer Center and by 5P30DK058404 to the Vanderbilt Digestive Disease Research Center.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

Received 9 May; accepted 2 June 2005
Published online at http://www.nature.com/naturemedicine/

1. Hawiger, J. Innate immunity and inflammation: a transcriptional paradigm. *Immunol. Res.* **23**, 99–109 (2001).
2. Alexander, W.S. Suppressors of cytokine signalling (SOCS) in the immune system. *Nat. Rev. Immunol.* **2**, 410–416 (2002).
3. Krebs, D.L. & Hilton, D.J. SOCS proteins: negative regulators of cytokine signaling. *Stem Cells* **19**, 378–387 (2001).
4. Yasukawa, H., Sasaki, A. & Yoshimura, A. Negative regulation of cytokine signaling pathways. *Annu. Rev. Immunol.* **18**, 143–164 (2000).
5. Zhang, J.G. *et al.* The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action in vivo. *Proc. Natl Acad. Sci. USA* **98**, 13261–13265 (2001).
6. Miethke, T. *et al.* T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. Exp. Med.* **175**, 91–98 (1992).
7. Pfeffer, K. *et al.* Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell* **73**, 457–467 (1993).
8. Car, B.D. *et al.* Interferon gamma receptor deficient mice are resistant to endotoxic shock. *J. Exp. Med.* **179**, 1437–1444 (1994).
9. Song, E. *et al.* RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **9**, 347–351 (2003).
10. Seino, K. *et al.* Contribution of Fas ligand to T cell-mediated hepatic injury in mice. *Gastroenterology* **113**, 1315–1322 (1997).
11. Hawiger, J. Noninvasive intracellular delivery of functional peptides and proteins. *Curr. Opin. Chem. Biol.* **3**, 89–94 (1999).
12. Yasukawa, H. *et al.* IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat. Immunol.* **4**, 551–556 (2003).
13. Hoebe, K. *et al.* Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* **424**, 743–748 (2003).
14. Fitzgerald, K.A. *et al.* LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J. Exp. Med.* **198**, 1043–1055 (2003).
15. Liu, D. *et al.* Suppression of Staphylococcal Enterotoxin B-induced Toxicity by a Nuclear Import Inhibitor. *J. Biol. Chem.* **279**, 19239–19246 (2004).
16. Arad, G., Levy, R., Hillman, D. & Kaempfer, R. Superantigen antagonist protects against lethal shock and defines a new domain for T-cell activation. *Nat. Med.* **6**, 414–421 (2000).
17. Cavaillon, J.M., Adib-Conquy, M., Fitting, C., Adrie, C. & Payen, D. Cytokine cascade in sepsis. *Scand. J. Infect. Dis.* **35**, 535–544 (2003).
18. Veach, R.A. *et al.* Receptor/transporter-independent targeting of functional peptides across the plasma membrane. *J. Biol. Chem.* **279**, 11425–11431 (2004).
19. O'Keefe, G.M., Nguyen, V.T., Ping Tang, L.L. & Benveniste, E.N. IFN-gamma regulation of class II transactivator promoter IV in macrophages and microglia: involvement of the suppressors of cytokine signaling-1 protein. *J. Immunol.* **166**, 2260–2269 (2001).
20. Liu, D. *et al.* Nuclear import of proinflammatory transcription factors is required for massive liver apoptosis induced by bacterial lipopolysaccharide. *J. Biol. Chem.* **279**, 48434–48442 (2004).
21. Yasuda, S., Nagaki, M. & Moriwaki, H. Staphylococcal enterotoxin B induces hepatic injury and lethal shock in endotoxin-resistant C3H/HeJ mice despite a deficient macrophage response. *J. Endotoxin Res.* **8**, 253–261 (2002).
22. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085–2088 (1998).
23. Tiegs, G., Hentschel, J. & Wendel, A.A. T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J. Clin. Invest.* **90**, 196–203 (1992).
24. Trautwein, C. *et al.* Concanavalin A-induced liver injury triggers hepatocyte proliferation. *J. Clin. Invest.* **101**, 1960–1969 (1998).
25. Hong, F. *et al.* Opposing roles of STAT1 and STAT3 in T cell-mediated hepatitis: regulation by SOCS. *J. Clin. Invest.* **110**, 1503–1513 (2002).
26. Shouda, T. *et al.* Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. *J. Clin. Invest.* **108**, 1781–1788 (2001).
27. Jo, D. *et al.* Cell cycle-dependent transduction of cell-permeant Cre recombinase proteins. *J. Cell. Biochem.* **89**, 674–687 (2003).
28. Jo, D. *et al.* Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase. *Nat. Biotechnol.* **19**, 929–933 (2001).
29. Lin, Q., Jo, D., Grebre-Amlak, K.D. & Ruley, H.E. Enhanced cell-permeant Cre protein for site-specific recombination in cultured cells. *BMC Biotechnol.* **4**, 25 (2004).