Farnesyltransferase Inhibitor FTI-277 Reduces Mortality of Septic Mice along with Improved Bacterial Clearance^[S]

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ABSTRACT

Treatment with statins, inhibitors of HMG-CoA reductase, extends the survival of septic mice. However, the molecular mechanisms underlying the cholesterol-lowering, independent beneficial effects of statins in sepsis are poorly understood. The inhibition of protein isoprenylation, namely farnesylation and geranylgeranylation, has been proposed as a mediator of the pleiotropic protective effects of statins, although direct evidence is lacking. Major features of sepsis-induced immune suppression include T-cell dysfunction, which is characterized by apoptosis of splenic T cells, increased CD4⁺Foxp3⁺ regulatory T cells (Tregs), and suppression of type 1 helper T-cell response [e.g., interferon- γ (IFN- γ) secretion] in mice. Here, we show that the induction of sepsis by cecal ligation and puncture (CLP) resulted in increases in farnesyltransferase activity and farnesylated proteins in the spleen relative to sham operation. Treatment with farnesyltransferase inhibitor *N*-[4-[2(*R*)-amino-

3-mercaptopropyl]amino-2-phenylbenzoyl]methionine methyl ester trifluoroacetate salt (FTI-277) (25 mg/kg b.wt. i.p.) at 2 h after CLP blocked the increase in farnesylated proteins and improved survival and bacterial clearance of septic mice. FTI-277 reverted to or mitigated sepsis-induced apoptosis in spleen and thymus, increased splenic CD4⁺Foxp3⁺ Tregs, and suppressed IFN- γ secretion and proliferation of splenocytes in response to anti-CD3+CD28 antibodies in mice. Moreover, FTI-277 promoted macrophage phagocytotic activity in septic mice. These results indicate that elevation in protein farnesylation plays a role in derangements in immune function and mortality of septic mice. These findings suggest that prevention of immune dysfunction might contribute to FTI-277-induced improvement in survival of septic mice. These data highlight protein farnesyltransferase as a novel potential molecular target to reduce the mortality of patients with sepsis.

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Introduction

Sepsis is the systemic inflammatory response syndrome that occurs during severe infection and a major health problem of growing concern in the United States and worldwide. Sepsis remains the leading cause of death of critically ill patients, despite advancements in antimicrobial therapy (Dombrovskiy et al., 2007). It is responsible for more than 200,000 deaths a year in the United States (Levy et al., 2010). Mortality rates in patients with severe sepsis are in the 30 to

ABBREVIATIONS: FTI-277, *N*-[4-[2(*R*)-amino-3-mercaptopropyl]amino-2-phenylbenzoyl]methionine methyl ester trifluoroacetate salt; FTI, farnesyltransferase inhibitor; LPS, lipopolysaccharide; CLP, cecal ligation and puncture; Tregs, regulatory T cells; TCR, T-cell receptor; Th1, type 1 helper T cell; PD-1, programmed death-1; PD-L1, PD-1 ligand-1; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; IFN- γ , interferon- γ ; IL-4, interleukin-4; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; ELISA, enzyme-linked immunosorbent assay; LV, left ventricular; LVIDs, left ventricular end-systolic diameter; Th2, type 2 helper T cell; HMGB1, high-mobility group protein box 1; RT-PCR, reverse transcription-polymerase chain reaction; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

40% range (Levy et al., 2010). Moreover, the incidence and mortality of patients with sepsis appear to be increasing in the United States (Dombrovskiy et al., 2007). Therefore, to reduce the mortality of patients with sepsis, new strategies need to be developed.

Recently, the protective effects of statins, inhibitors for HMG-CoA reductase, have been highlighted in sepsis. Observational studies have shown that patients taking statin therapy for hypercholesterolemia or ischemic heart disease have reduced incidence and mortality from sepsis compared with those who were not treated with statins (Almog et al., 2004, 2007). Likewise, previous studies in mice have shown that statin treatment extends survival after the induction of sepsis, infection, and endotoxin challenge (Merx et al., 2004, 2005; Yasuda et al., 2006; Ayyadurai et al., 2010; Rosch et al., 2010; Shinozaki et al., 2010; Takano et al., 2011). Recent meta-analyses of small-scale intervention studies have concluded that statins are efficacious in improving the outcome of patients with infection (Tleyjeh et al., 2009; Janda et al., 2010). Large-scale, randomized, placebo-controlled clinical trials in patients with sepsis are underway to evaluate the therapeutic efficacy and safety of statins.

The beneficial effects of statins in sepsis are independent of the cholesterol-lowering-effects of statins. To date, however, the molecular mechanisms by which statins exert protective effects against sepsis are poorly understood. The inhibition of HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway, by statins leads to reductions not only in cholesterol but also in precursors of cholesterol, such as farnesyl pyrophosphate, and its derivatives, including geranylgeranyl pyrophosphate. The cholesterol-lowering, independent pleiotropic effects of statins have been proposed to be mediated by the inhibition of protein isoprenylation, namely farnesylation and geranylgeranylation (Sugita et al., 2007), although there is no direct evidence. We have recently shown that both statin and farnesyltransferase inhibitor reduce mortality after lipopolysaccharide (LPS) challenge in mice (Shinozaki et al., 2010). However, it is unknown whether farnesyltransferase inhibitor can improve survival of septic mice, where the immune system has a greater role in the outcome than in LPS model.

Protein farnesylation, a lipid modification of cysteine residues in the CAAX motif located in the carboxyl terminus of proteins (where C is cysteine, A is aliphatic amino acid, and X is any amino acid, but usually serine, methionine, glutamine, or alanine). Farnesyltransferase, which consists of α - and β -subunits, catalyzes the covalent attachment of farnesyl pyrophosphate, a precursor of cholesterol, via a thioester linkage to cysteine residues in the CAAX box (Gelb et al., 2006). In many proteins, farnesylation serves as a critical regulatory mechanism of protein function, such as maturation, activity, protein-protein interaction, and membrane localization of proteins, including the Ras family small G-proteins, lamin A, the nuclear protein, and the centromeric protein.

Recently, immune suppression in the early stage of sepsis has emerged as the major pathological state affecting prognosis of patients with sepsis (Kellum et al., 2007; Huang et al., 2009; Hoogerwerf et al., 2010). Anti-inflammatory immunosuppressive response observed on the day of diagnosis predicts mortality of patients with severe sepsis (Huang et al., 2009; Hoogerwerf et al., 2010). In mouse model of severe

sepsis, both pro- and anti-inflammatory cytokine responses begin simultaneously in the early phase within 6 h after the induction of sepsis by cecal ligation and puncture (CLP) (Osuchowski et al., 2006). Major components of sepsis-induced immune suppression include increase in immunosuppressive CD4⁺Foxp3⁺ regulatory T cells (Tregs), anergy of T cells [hypoproliferative response to stimulation of T-cell receptor (TCR) and its coreceptor with anti-CD3 and anti-CD28 antibodies], and suppression of Th1 response (e.g., IFN- γ secretion). Moreover, several lines of evidence indicate the important role of the inhibitory signaling molecules in immune cells, especially the programmed death ligand-1 (PD-L1)/programmed death-1 (PD-1) receptor pathway, in sepsis-associated immune suppression (Huang et al., 2009). Recent studies have shown that PD-L1 and PD-1 expression in T cells and monocytes are increased in patients with sepsis (Guignant et al., 2011).

Previous studies have shown that statins ameliorate cardiac dysfunction in septic mice (Merx et al., 2004, 2005). In contrast, the effects of statins or farnesyltransferase inhibitor on immune function have not yet been studied. Therefore, we were motivated to examine the effects of farnesyltransferase inhibitor on splenic T cells as well as cardiac function in septic mice.

In this study, we show that CLP-induced sepsis resulted in increases in farnesyltransferase activity and farnesylated proteins in the spleen compared with sham operation and that farnesyltransferase inhibitor improved survival and bacterial clearance in septic mice compared with vehicle alone.

Materials and Methods

Animals. Male C57BL/6 mice at 7 weeks of age (The Jackson Laboratory, Bar Harbor, ME) were used for this study. The study protocol was approved by the Institutional Animal Care Committee of Massachusetts General Hospital. The animal care facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were housed in a pathogen-free animal facility with 12-h light/dark cycles at 25°C. *N*-[4-[2(*R*)-amino-3-mercaptopropyl]amino-2-phenylbenzoyl]methionine methyl ester trifluoroacetate salt (FTI-277) (25 mg/kg b.wt. i.p.; Sigma-Aldrich, St. Louis, MO) or vehicle [phosphate-buffered saline (PBS)] was given to mice at 2 h after the performance of CLP or sham operation. FTI-277 was dissolved in sterile distilled water and diluted by PBS just before injection. The effects of FTI-277 on survival were observed up to 7 days after CLP.

Cecal Ligation and Puncture. Sepsis was induced by CLP. In brief, mice were anesthetized with pentobarbital sodium (50 mg/kg b.wt. i.p.). After shaved, the ventral trunk was prepared with 70% ethanol. A midline incision (~1 cm) in the abdomen was made to expose the cecum. The cecum was ligated at 1 cm from the apex with a 5-0 silk suture and perforated by a single through-to-through puncture midway between the ligation and the tip of the cecum with an 18-gauge needle. The abdominal incision was closed in two layers with 5-0 suture. In sham-operated mice, the cecum was located and mobilized as described above but was neither ligated nor punctured. Immediately after the surgery, animals were resuscitated by subcutaneously injecting prewarmed normal saline (0.1 ml/g b.wt.). The mice were humanely sacrificed at 16 h after CLP or sham procedure to collect tissues for immunohistochemistry, biochemical analyses, and flow cytometry. The mice were euthanized by an overdose of pentobarbital sodium (200 mg/kg b.wt. i.p.) or exsanguination under anesthesia with pentobarbital sodium (50 mg/kg b.wt. i.p.).

Evaluation of Bacterial Clearance. Bacterial loads were determined in blood and peritoneal lavage. In brief, the mice were anesthetized with pentobarbital sodium at 16 h after CLP. The peritoneal cavities were washed with 1 ml of sterile PBS, and the peritoneal lavage fluids were collected under sterile condition. One hundred microliters of peritoneal lavage fluids or blood samples were placed on ice and serially diluted with sterile PBS. Thirty microliters of each diluted sample was placed on trypticase soy agar plates with 5% sheep blood (BD Biosciences, San Diego, CA) and incubated at 37°C for 24 h. The numbers of bacterial colonies were then counted and expressed as colony-forming units per 100 μ l of blood and peritoneal lavage.

Histology and Immunohistochemistry. Spleen and thymus were harvested at 16 h after CLP. After a rinse in PBS, the specimens were immediately fixed in 4% paraformaldehyde phosphate-buffered solution. The fixed specimens were then dehydrated, cleared, and embedded in paraffin. The 3- μ m thick sections were taken from these blocks by rotary microtome and put onto polypropylene microscope slides (Thermo Fisher Scientific, Waltham, MA). To assess apoptosis, paraffin-embedded sections of spleen and thymus were stained for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) as described previously (Yamada et al., 2010). The numbers of TUNEL-positive nuclei were normalized to those of all of the nuclei visualized with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). To further confirm the results obtained by fluorometric TUNEL staining, the sections were also stained with an immunoperoxidase TUNEL system (Apotag plus peroxidase in situ apoptosis kit; Millipore, Temecula, CA) and counterstained with methyl green (Sigma-Aldrich), which dyes the chromatin. Paraffin-embedded sections were also stained with polyclonal antibodies for farnesylated proteins (AB4073; Millipore), farnesyltransferase- α , or farnesyltransferase- β (SC-136, SC-137; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and visualized using diaminobenzidine substrate kit for peroxidase (Vector Laboratories). Staining with normal IgG in place of antifarnesylated protein antibody served as a negative control. The sections were counterstained by Mayer's hematoxylin solution (Sigma-Aldrich).

Splenocytes Preparation. Spleens were removed aseptically at 16 h after CLP. Newly harvested spleens were immediately placed in 15 ml of ice-cold PBS. Splenocytes in single cell suspension were obtained by passing through a 70- μ m cell strainer (BD Biosciences). Red blood cells were lysed with the red blood cell lysis reagent (eBioscience, San Diego, CA). The cells were washed twice with PBS and resuspended in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, and 10 mM HEPES, pH 7.5. After the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air for 2 h, the floating cells were collected for proliferation and cytokine secretion assays.

T-Cell Proliferation Assay. Isolated splenocytes were seeded at the cell density of 2×10^6 /ml in 100 μ l of the complete RPMI 1640 medium in 96-well plates precoated with or without anti-CD3 antibody (1 μ g/well) (53057, Isotype: Armenian Hamster IgG1, κ ; Clone: 145-2C11; BD Biosciences) and incubated in the presence and absence of soluble anti-CD28 antibody (1 μ g/well) (553294, Isotype: Syrian Hamster IgG2, λ 1; Clone: 37.51; BD Biosciences) at 37°C in a humidified atmosphere of 5% CO₂-95% air for 24 h. To evaluate proliferative response, cell viability was measured using resazurin dye solution (Sigma-Aldrich) according to the manufacturer's instructions. At 24 h after the cell inoculation, resazurin dye solution (Sigma-Aldrich) was added to each well, and after an additional 6-h incubation, fluorescence was measured by a fluorescence microplate reader (excitation: 544 nm; emission: 595 nm).

Cytokine Measurements. In 24-well plates precoated with or without anti-CD3 antibody (6.6 μ g/well), isolated splenocytes (10^6 /well) were incubated in 500 μ l of the complete RPMI 1640 medium with or without soluble anti-CD28 antibody (6.6 μ g/well) at 37°C in a humidified atmosphere of 5% CO₂-95% air for 24 h. The concen-

trations of IFN- γ and IL-4 in the supernatants of the cultured splenocytes were measured by mouse IFN- γ and IL-4 enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences) according to the manufacturer's instructions. The lowest detection limits for IFN- γ and IL-4 were 3.1 and 7.8 pg/ml, respectively.

Farnesyltransferase Activity Assay. Farnesyltransferase activity was evaluated as described previously (Goalstone et al., 2010) with minor modifications. Spleens were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 5 mM dithiothreitol (DTT), 0.5% Nonidet P-40, 0.05% SDS, 1 mM PMSF, 1 mM sodium phosphate, 2 mM sodium vanadate, and protease inhibitor cocktail). In vitro farnesylation assay was performed in farnesylation reaction buffer [50 mM HEPES, pH 7.5, 5 mM MgCl₂, 5 mM DTT, 0.15 μ Ci of [³H]farnesyl pyrophosphate (American Radiolabeled Chemicals, St. Louis, MO)] using a peptide containing COOH-terminal farnesylation motif in mouse Ras protein (KLNPPDESGPGCMSCVCVLS) as a substrate in the presence and absence of FTI-277 (200 μ M) in duplicate. Incorporation of [³H] into the peptide was measured by a scintillation counter. Farnesyltransferase activities were calculated by subtracting the peptide-associated radioactivities in the samples incubated with FTI-277 from those without FTI-277.

ELISA for Farnesylated Proteins. The amounts of farnesylated proteins were measured in triplicate using a sandwich ELISA protocol. The spleen was lysed in lysis buffer [50 mM HEPES, pH 8.0, 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM PMSF, 10% glycerol, 2 mM EDTA, 2 mM sodium vanadate, 1 mM DTT, 1% Nonidet P-40, 0.1% SDS, and protease inhibitor cocktail (Sigma-Aldrich)]. After 96-well plates were coated with the spleen lysate (50 μ g/well) at 4°C overnight, antifarnesylated protein antibody (0.25 μ g/well) was added to each well. Unbound antibody was removed by washing with PBS containing 0.05% Tween 20, and the antigen-antibody complex was incubated with alkaline phosphatase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) for 30 min. At 20 min after the addition of the substrate [p-nitrophenyl phosphate disodium salt (Santa Cruz Biotechnology, Inc.) diluted in diethanolamine], the absorbance at 405 nm was measured by a microplate reader, using 490 nm as a reference wavelength, according to the protocol of the manufacturer (Santa Cruz Biotechnology, Inc.) for sandwich ELISA. The peptide containing farnesylation motif in mouse Ras protein (3 μ M) was incubated with recombinant active farnesyltransferase protein (1.58 μ g/ml) (Axxora, San Diego, CA) in farnesylation reaction buffer for 1 h in the presence of farnesyl pyrophosphate (10 μ M) (Sigma-Aldrich) to obtain farnesylated peptide, which we used as a standard. The lowest detection limit for farnesylated peptide was 0.82 μ mol/g protein (or 0.21 μ M). When the peptide was incubated with recombinant active geranylgeranyl transferase protein (1.67 μ g/ml) (Axxora) and geranylgeranyl pyrophosphate (10 μ M) (Sigma-Aldrich), the ELISA did not show cross-reactivity with the geranylgeranylated peptide up to 8.2 μ mol/g protein (or 2.1 μ M), indicating the specificity of the assay.

Flow Cytometry. Isolated splenocytes were stained with APC-Cy7-conjugated anti-CD4 (552051, Isotype: Rat IgG2b, κ ; Clone: GK1.5), Alexa Fluor 647-conjugated anti-Foxp3 (560401, Isotype: Rat IgG2b; Clone: MF23), Alexa Fluor 647-conjugated anti-F4/80 (514801, Isotype: Rat IgG2a, κ ; Clone: BM8), phycoerythrin-conjugated anti-PD-1 (11-9985, Isotype: Armenian Hamster IgG; Clone: J43), and phycoerythrin-conjugated anti-PD-L1 (12-5982, Isotype: Rat IgG2a, λ , Clone: MIH5) antibodies (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. To evaluate phagocytic activities of macrophages, peritoneal lavage was harvested at 16 h after CLP, and the cells were incubated with fluorescent microspheres (Invitrogen, Carlsbad, CA) for 30 min according to the manufacturer's instructions. Macrophages then were stained with Alexa Fluor 647-conjugated anti-F4/80 antibody. Flow cytometry was performed using a FACSCalibur (BD Biosciences), and the data were analyzed using BDiva software (BD Biosciences).

Evaluation of Cardiac Function. Mice were anesthetized with 1 to 2% isoflurane mixed with room air and placed in a supine

position on a heated pad. Transthoracic echocardiography was performed using a transducer centered on 30 MHz (RMV707B, Vevo 770; Visualsonics, Toronto, ON, Canada) as described previously (Thibault et al., 2010). M-mode images were derived from a parasternal short axis view at the level of the papillary muscles. Left ventricular (LV) end-diastolic diameter (LVIDd) and left ventricular end-systolic diameter (LVIDs) were measured. Fractional shortening was defined as $[(LVIDd - LVIDs)/LVIDd] \times 100$ (%). Cardiac output was calculated as the product of the stroke volume (derived from the M-mode images) and heart rate (Tournoux et al., 2011). All measurements were averaged on three cardiac cycles and obtained by an echocardiographer who was blinded to the experimental design. Echocardiography was performed 1 week before CLP to assess basal cardiac function under naive condition and repeated at 14 h after CLP.

In Vivo Hemodynamics. Mice were anesthetized with isoflurane at 16 h after CLP and ventilated with a rodent ventilator (Harvard Apparatus, Holliston, MA) via tracheal intubation. To evaluate LV function, the LV apex was exposed via a thoracotomy, and a 1.4-Fr pressure-volume catheter (SPR 839; Millar Instruments, Houston, TX) was inserted through the apex to lie along the longitudinal axis (Minamishima et al., 2009). To ensure proper placement, pressure-volume tracings were evaluated in real time to adjust the catheter as necessary. Data were recorded and analyzed with LabScribe2 software (iWorx Systems, Dover, NH).

Measurement of Wet and Dry Lung Weights. To measure wet and dry weights, lungs were excised at 16 h after CLP or sham operation and weighed for determination of wet lung weight. The lungs then were dried in an oven at 60°C for 5 days and weighed to determine wet-to-dry weight ratios.

Immunoblotting. Spleen samples were homogenized as described previously (Sugita et al., 2005) with minor modifications. In brief, spleen was homogenized in ice-cold homogenization buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 2 mM EDTA, 7.5% lithium dodecyl sulfate, 2% CHAPS, 10% glycerol, 10 mM sodium fluoride, 2 mM sodium vanadate, 1 mM PMSF, 10 mM sodium pyrophosphate, 1 mM DTT, and protease inhibitor cocktail). After being incubated at 10°C for 30 min, the homogenized samples were centrifuged at 14,000 rpm for 30 min at 10°C. Immunoblotting with rat polyclonal antibodies for farnesyltransferase- α , farnesyltransferase- β (SC-136, SC-137; Santa Cruz Biotechnology, Inc.), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2275-PC; Trevigen, Gaithersburg, MD) and mouse monoclonal antibody for β -actin (A5316, Isotype: mouse IgG2a; Clone: AC-74) (Sigma-Aldrich) was performed as described previously (Sugita et al., 2005). The blots were visual-

ized with ECL advance reagent (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Bands of interest were scanned by the use of ScanMaker (Microtek, Carson, CA) and quantified by NIH Image 1.62 software (National Technical Information Service, Springfield, VA).

Quantitative RT-PCR. Total RNA was isolated from spleen samples with an RNeasy Mini kit (QIAGEN, Valencia, CA). The first-strand cDNA was synthesized from 1 μ g of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Real-time RT-PCR analyses were performed as described previously (Yamada et al., 2010) using 10 ng of cDNA and TaqMan probes (Applied Biosystems) for farnesyltransferase- α and - β , and 18S, conducted with Mastercycler ep realplex (Eppendorf North America, New York, NY). mRNA expression levels of 36B4 and GAPDH were evaluated with SYBR Green (Applied Biosystems).

Measurement of High-Mobility Group Protein Box 1. Blood samples were collected from the heart under anesthesia with pentobarbital sodium (50 mg/kg b.wt. i.p.). Serum concentrations of high-mobility group protein box 1 (HMGB1) were measured by ELISA kit (Chondrex, Redmond, WA) according to the manufacturer's instructions.

Statistical Analysis. The data were compared with one-way analysis of variance followed by Newman-Keuls comparison or Student's *t* test. The effect of FTI-277 on survival of septic mice was analyzed by Kaplan-Meier survival curve with log-rank and χ^2 tests. A value of $p < 0.05$ was considered statistically significant. All values are expressed as mean $\pm$ S.E.M.

Results

Farnesyltransferase Inhibitor Improved Survival and Bacterial Clearance in Septic Mice. A single injection of farnesyltransferase inhibitor (25 mg/kg b.wt. FTI-277) at 2 h after CLP prolonged survival time of septic mice compared with vehicle alone. Kaplan-Meier survival curve analysis showed statistically significant beneficial effects of FTI-277 compared with vehicle alone ($p < 0.0001$) (Fig. 1A). χ^2 test also revealed that FTI-277 significantly reduced mortality after CLP in mice ($p = 0.001$). Vehicle-treated septic mice (14 of 15) died after CLP. In contrast, only five of 15 FTI-277-treated septic mice died. In naive mice, neither FTI-277 nor vehicle alone caused mortality (data not shown).

Bacterial loads in the circulation and peritoneal cavity

Fig. 1. Farnesyltransferase inhibitor, FTI-277, reduced the mortality of septic mice along with improved bacterial clearance and reversal of elevated serum HMGB1 concentration. Mice were treated with farnesyltransferase inhibitor FTI-277 (25 mg/kg b.wt.) or vehicle at 2 h after the induction of sepsis by CLP. A, FTI-277 reduced the mortality of septic mice compared with vehicle alone. B, bacterial loads in the circulation and peritoneal cavity were assessed by bacterial colony formation assay. $n = 6$ per group. *, $p < 0.05$, **, $p < 0.01$ versus vehicle. C, serum HMGB1 concentrations were markedly elevated at 16 h after CLP in vehicle-treated animals. FTI-277 almost completely blocked increase in serum HMGB1 concentration in septic mice. *, $p < 0.05$ versus sham and CLP with FTI. $n = 4$ per group.

Fig. 2. Sepsis-induced apoptosis was prevented by farnesyltransferase inhibitor FTI-277 in mouse spleen and thymus. At 16 h after CLP, TUNEL-positive apoptotic nuclei were increased in spleen (A) and thymus (B). Farnesyltransferase inhibitor FTI-277 significantly decreased the percentage of TUNEL-positive nuclei in spleen and thymus of septic mice compared with vehicle alone (PBS). $n = 3$ per group. *, $p < 0.05$ versus vehicle.

were significantly ameliorated in FTI-277-treated septic mice compared with vehicle alone at 16 h after CLP (Fig. 1B). None of the mice died within 16 h after CLP regardless of treatments. CLP resulted in a marked increase in serum HMGB1 concentration, a proposed predictor of the outcome of patients with severe sepsis (Karlsson et al., 2008), in vehicle-treated animals, as shown previously (Yang et al., 2004). Consistent with improved survival and bacterial clearance by FTI-277, FTI-277 almost completely reversed increased HMGB1 concentrations in septic mice (Fig. 1C).

Improved bacterial clearance and reversal of elevated cir-

culating HMGB1 by FTI-277 were accompanied by attenuation of sepsis-induced apoptosis in spleen and thymus of septic mice relative to vehicle. TUNEL-positive apoptotic cells were markedly increased in spleen and thymus of septic mice. FTI-277 significantly attenuated TUNEL-positive cells in spleen and thymus of septic mice (Fig. 2). Sham operation did not increase apoptosis in spleen and thymus compared with naive animals (data not shown).

Sepsis Increased Farnesylated Proteins and Farnesyltransferase Activity in Mouse Spleen. Farnesylated proteins were increased in spleen at 16 h after CLP compared

Fig. 3. Sepsis increased farnesylated proteins and farnesyltransferase activity in spleen. A and B, amounts of farnesylated proteins were evaluated by immunohistochemical analysis (A) and ELISA (B). $n = 4$ per group. C, farnesyltransferase (FTase) activity was assessed by in vitro farnesylation assay. $n = 4-5$ per group. D, the protein expression of farnesyltransferase (FT)- α and - β was evaluated by immunoblotting.

Fig. 4. Sepsis-induced increase in CD4⁺FcγR3⁺ Tregs was attenuated by farnesyltransferase inhibitor, FTI-277. A and B, at 16 h after CLP or sham operation, splenocytes were analyzed by flow cytometry to evaluate the population of CD4⁺FcγR3⁺ Tregs. The numbers in parenthesis in A indicate the percentages of the numbers of CD4⁺FcγR3⁺ cells in total CD4⁺ cells. *n* = 8 per group. C, the number of total CD4⁺ splenocytes was decreased in septic mice relative to sham animals. *n* = 8 per group.

with sham-operated mice, as judged by immunohistochemistry and ELISA (Fig. 3, A and B). Elevated protein farnesylation in septic mice was reverted by FTI-277, although FTI-277 did not significantly decrease the content of farnesylated proteins in sham animals. Consistently, farnesyltransferase activity was significantly greater in the spleen of vehicle-treated septic mice than that of sham-operated animals (Fig. 3C). FTI-277 attenuated farnesyltransferase activity in septic mice. However, the protein and mRNA expression of farnesyltransferase and GAPDH were not significantly altered by CLP or FTI-277 as judged by immunoblotting, immunohistochemistry, and real-time RT-PCR (Fig. 3D; Supplemental Figs. 1–5).

FTI-277 Reverted to Sepsis-Induced Increase in CD4⁺FcγR3⁺ Tregs in Spleen. CD4⁺FcγR3⁺ Tregs plays an important role in immune suppression in various disease conditions, including sepsis (Fontenot et al., 2003). Therefore, we investigated the effects of FTI-277 on CD4⁺FcγR3⁺ Tregs in splenocytes. In vehicle-treated animals, the population of CD4⁺FcγR3⁺ Tregs increased after CLP relative to sham operation, in agreement with previous studies (Bomhardt et al., 2004). FTI-277 partially but significantly inhibited sepsis-induced increase in splenic CD4⁺FcγR3⁺ Tregs (Fig. 4, A and B). Sepsis decreased the number of total CD4⁺ splenocytes compared with sham animals. Unlike CD4⁺FcγR3⁺ cells, the total CD4⁺ cell numbers were not affected by FTI-277 in sham and septic mice (Fig. 4C).

Sepsis-Associated Suppression of IFN-γ Secretion and Proliferative Response of Splenic T cells Were Ameliorated by FTI-277. Sepsis-associated increase in CD4⁺FcγR3⁺ Tregs is a major contributor to T-cell dysfunction, such as suppression of Th1 cytokine release and hypoproliferative response to anti-CD3+CD28 antibodies (Fontenot et al., 2003). Therefore, we examined the effects of FTI-277 on sepsis-induced splenic T-cell function. In vitro secretion of IFN-γ, a proinflammatory Th1 cytokine, in response to stimulation of TCR and its coreceptor with anti-

CD3+CD28 antibodies was significantly attenuated in cultured splenocytes from vehicle-treated septic mice compared with sham animals. FTI-277 treatment restored IFN-γ secretion in septic mice to the levels in sham animals (Fig. 5A). FTI-277 seemed to decrease IFN-γ secretion in sham animals, but there was no significant difference (*p* > 0.10).

In contrast to the effects on IFN-γ, FTI-277 treatment in sham mice resulted in significantly increased secretion of IL-4, an anti-inflammatory type 2 helper T-cell (Th2) cyto-

Fig. 5. Effects of FTI-277 treatment on secretion of IFN-γ and IL-4, ratio of IFN-γ to IL-4 secretion, and proliferative response in anti-CD3+CD28 antibodies-stimulated splenocytes. Mice were treated with farnesyltransferase inhibitor FTI-277 or vehicle at 2 h after CLP or sham operation. Splenocytes were isolated at 16 h after CLP or sham operation and incubated with and without plate-coated anti-CD3 antibody and soluble anti-CD28 antibody. IFN-γ (A) and IL-4 (B) concentrations in the supernatants were measured by ELISA. *n* = 10 per group. C, the ratio of IFN-γ to IL-4 secretion by cultured splenocytes was altered by CLP and FTI-277. *n* = 10 per group. D, cell viability was measured in isolated splenocytes cultured in the presence and absence of anti-CD3+CD28 antibodies. *n* = 10 per group.

kine, by anti-CD3+CD28 antibodies-stimulated splenocytes compared with vehicle alone (Fig. 5B). Without FTL-277 treatment, no significant difference was found in IL-4 secretion between sham and septic mice. To assess shifts to Th1 or Th2 response, we calculated the ratio of IFN- γ (Th1 cytokine) to IL-4 (Th2 cytokine) secretion, which has been used as a surrogate indicator of Th1/Th2 balance.

When treated with vehicle alone, CLP resulted in significantly decreased IFN- γ to IL-4 ratio in anti-CD3+CD28-stimulated splenocytes. Decreased IFN- γ to IL-4 ratio in septic mice was reversed by FTL-277 (Fig. 5C). To the contrary, FTL-277 significantly decreased IFN- γ to IL-4 ratio in sham animals. When unstimulated with anti-CD3+CD28 antibodies, the concentrations of IFN- γ and IL-4 in the supernatants of cultured splenocytes were below the detection limits regardless of sham, CLP, or treatments.

Stimulation with anti-CD3+CD28 antibodies induced proliferative response of splenocytes isolated from both sham and septic mice. Proliferative response of splenocytes was suppressed by CLP when the mice were treated with vehicle alone. FTL-277 significantly up-regulated proliferative response of splenic T cells in septic mice (Fig. 5D). However, FTL-277 treatment did not affect it in sham animals. Without stimulation with anti-CD3+CD28 antibodies, neither CLP nor FTL-277 significantly altered the cell viability of cultured splenocytes.

Effects of FTL-277 on PD-L1 and PD-1 Expression in CD4⁺ T Cells and Macrophages in the Spleen of Septic Mice. CLP significantly increased PD-L1 and PD-1 expression in splenic CD4⁺ T-cells and macrophages in vehicle-treated mice (Fig. 6). FTL-277 treatment decreased PD-L1 and PD-1 expression in CD4⁺ T cells and macrophages of septic mice compared with vehicle alone. PD-L1 expression in CD4⁺ T cells and macrophages appeared to be increased in FTL-277-treated septic mice compared with sham animals, but there was no statistical significance ($p > 0.10$). On the

other hand, PD-1 expression was significantly greater in CD4⁺ T cells and macrophages of FTL-277-treated septic mice than sham animals. FTL-277 did not alter PD-L1 and PD-1 expression in CD4⁺ T cells or macrophages of sham mice.

Effects of FTL-277 on Phagocytotic Activities of Macrophages. A previous study has shown that the PD-L1/PD-1 pathway impairs macrophages/monocytes function (Huang et al., 2009). Therefore, we examined the effects of FTL-277 on phagocytosis by peritoneal F4/80⁺ macrophages. FTL-277 significantly increased phagocytotic activities of macrophages in septic mice, as judged by percentages of fluorescent microsphere-positive cells (Fig. 7A) and mean fluorescent intensity per cell (Fig. 7B). In sham mice, FTL-277 did not significantly affect phagocytotic activities of macrophages. CLP markedly increased the number of peritoneal macrophages. FTL-277 did not alter the numbers of macrophages in the peritoneal cavity in septic or sham animals.

Overt Cardiac Dysfunction Was Not Observed at 16 after CLP in Mice. We evaluated cardiac function by echocardiography and LV function test at 14 and 16 h after CLP, respectively. Echocardiography unexpectedly revealed that the septic mice did not show overt cardiac dysfunction regardless of treatment with FTL-277 or vehicle compared with naive state without CLP or sham operation (Supplemental Table 1). In addition, LV function did not differ between FTL-277- and vehicle-treated septic mice (Supplemental Table 2). These observations indicate that improved bacterial clearance and attenuated sepsis-induced apoptosis in spleen and thymus by FTL-277 were found in the early stage of sepsis before overt cardiac dysfunction became apparent in septic mice. The ratio of wet to dry lung weights was slightly but significantly increased at 16 h after CLP relative to sham operation, indicating modest pulmonary edema in septic mice. However, FTL-277 did not alter the wet to dry lung weight ratio (Sham+Vehicle: 4.8 ± 0.2 ; Sham+FTL: $4.7 \pm$

Fig. 6. FTL-277 inhibited CLP-induced increased PD-L1 and PD-1 expression in CD4⁺ T-cells and F4/80⁺ macrophages. At 16 h after CLP or sham operation, PD-L1 (A) and PD-1 (B) expression was evaluated in isolated splenocytes by flow cytometry using fluorescence-conjugated antibodies. $n = 8$ per group. *, $p < 0.05$ versus sham with and without FTL.

Fig. 7. FTL-277 increased phagocytotic activities of macrophages in septic mice. A and B, phagocytotic activities of peritoneal macrophages were evaluated by uptake of fluorescein isothiocyanate (FITC)-conjugated microsphere in F4/80⁺ cells at 16 h after CLP or sham operation. The ratio of the numbers of FITC-positive macrophages to total macrophage population (A) and mean FITC-fluorescence intensity per F4/80⁺ macrophage (B) indicate that FTL-277 significantly increased phagocytotic activities in septic mice. $n = 8$ per group. C, CLP increased the numbers of peritoneal macrophages. $n = 8$ per group.

0.1; CLP+Vehicle: 5.2 ± 0.1 ; CLP+FTI: 5.4 ± 0.2 , $p < 0.05$ sham versus CLP, $n = 5$ per group).

Discussion

Our results clearly demonstrate that farnesyltransferase inhibitor FTI-277 improved survival of septic mice. The pro-survival effects of FTI-277 were associated with improvement in bacterial clearance (Fig. 1) and amelioration of apoptosis of splenocytes and thymocytes (Fig. 2), and inhibition of increased $CD4^+Foxp3^+$ Tregs (Fig. 4), hyporesponsiveness (anergy), attenuated IFN- γ secretion of splenic T cells (Fig. 5), increased PD-1 and PD-L1 expression in $CD4^+$ T cells and macrophages in septic mice (Fig. 6), and enhanced phagocytotic activities of macrophages (Fig. 7). The induction of sepsis by CLP, the clinically relevant, well established rodent model of polymicrobial sepsis, resulted in increases in farnesyltransferase activity and farnesylated proteins in the spleen, which were reverted by FTI-277 treatment (Fig. 3). These results indicate that FTI-277 exerts the beneficial effects by reverting to elevation in protein farnesylation in septic mice.

An early study has shown that LPS induces a farnesylated 65-kDa protein in cultured macrophages (Stickney and Buss, 2000). It remains unknown whether protein farnesylation and farnesyltransferase activity are modulated by sepsis. We found that farnesylated proteins and farnesyltransferase activity were increased in the spleen of septic mice compared with sham animals. These findings suggest that sepsis-induced increase in farnesylated proteins may play a role in apoptosis and dysfunction of splenocytes.

Farnesyltransferase expression was not increased at 16 h after CLP, whereas farnesyltransferase activity and farnesylated proteins were increased when treated with vehicle alone. It is possible that increased farnesyltransferase activity might be explained by posttranslational modifications, such as phosphorylation. However, controversial results have been reported on the impact of phosphorylation on farnesyltransferase activity. Farnesyltransferase- α activity was down-regulated by phosphorylation in a previous study (Kumar et al., 1996), but other studies have shown increased activity of the enzyme by phosphorylation (Goalstone et al., 1997). Modulation of farnesyltransferase activity by post-translational modifications is currently studied in our laboratory.

Consistent with previous data in humans and rodents (Bommhardt et al., 2004), we found that the induction of sepsis resulted in increased $CD4^+Foxp3^+$ Tregs and suppression of IFN- γ secretion, attenuated proliferation of splenic T cells in response to anti-CD3+CD28 antibodies, and increased expression of PD-1 and PD-L1 in $CD4^+$ T cells and macrophages (Figs. 4–6) compared with sham operation. FTI-277 treatment reverted to or ameliorated all of these changes in septic mice. These results clearly indicate that inhibition of farnesyltransferase prevented functional derangements in splenic T cells in the early stage of severe sepsis.

Most but not all of the previous studies have focused on anti-inflammatory effects of statins and inhibition of farnesylation in cultured cells and in vivo in rodents (Youssef et al., 2002; Dunn et al., 2006; Xue et al., 2006). A previous study has shown that in vitro treatment with atorvastatin

enhances IL-4 secretion and inhibits IFN- γ secretion by cultured T cells from normal mice (Dunn et al., 2006). It is noteworthy that the addition of farnesyl pyrophosphate reverts to the effects of atorvastatin (Dunn et al., 2006). These data suggest that under normal conditions statin may exert anti-inflammatory actions, at least in part, by promoting Th2 polarization via inhibition of protein farnesylation. Consistently, we found the anti-inflammatory effect of FTI-277 in sham animals. Treatment with FTI-277 significantly increased IL-4 secretion by cultured splenocytes from sham animals, relative to vehicle alone (Fig. 5B). There was a trend of decreased IFN- γ secretion from splenocytes from FTI-277-treated sham animals compared with vehicle-treated sham mice, but no significant difference was found. In a stark contrast, FTI-277 elicited immunostimulatory but not anti-inflammatory actions in septic mice. FTI-277 reverted to suppressed IFN- γ secretion in septic mice to the levels observed in sham animals (Fig. 5A). Taken together, these data argue that FTI-277 exerted differential effects on Th1 and Th2 cytokine responses toward opposite directions in sham versus septic mice. Our data indicate that FTI-277 promoted Th1/Th2 balance toward Th1 predominance in septic mice but shifted it to Th2 in sham animals, as reflected by the ratios of IFN- γ to IL-4 secretion.

Increased $CD4^+Foxp3^+$ Tregs plays a pivotal role in suppression of Th1 response in sepsis (Nascimento et al., 2010). In agreement with previous studies in patients and rodents with sepsis (Nascimento et al., 2010), splenic $CD4^+Foxp3^+$ Tregs increased in number after CLP relative to sham operation when treated with vehicle alone. FTI-277 treatment inhibited the increase in $CD4^+Foxp3^+$ Tregs in septic mice (Fig. 4). Therefore, one can speculate that FTI-277 might revert to splenic T-cell dysfunction, at least in part, by preventing increase in $CD4^+Foxp3^+$ Tregs. In addition, the important role of the PD-L1/PD-1 inhibitory cosignaling pathway has been recently shown in sepsis-associated dysfunction of both innate and adaptive immunity. The PD-L1/PD-1 pathway promotes the induction and maintenance of Tregs (Francisco et al., 2009) and impairs phagocytotic activities of macrophages (Huang et al., 2009). Blockade of PD-L1 or PD-1 by specific antibodies or gene disruption improves survival of septic mice (Huang et al., 2009). It is possible that the attenuation of CLP-induced increased PD-L1 and PD-1 expression by FTI-277 might contribute to improved bacterial clearance and phagocytotic activities of macrophages and prevention of increase in Tregs and Th2-skewed response in septic mice. In combination, these data argue that the inhibitor of farnesyltransferase improved survival of septic mice as an immune-stimulatory agent rather than as a simple anti-inflammatory drug.

In contrast to septic mice, FTI-277 did not alter splenic $CD4^+Foxp3^+$ Tregs in sham animals (Fig. 4). This result seems to be different from previous findings that statins promotes in vitro differentiation of $CD4^+Foxp3^+$ Tregs from $CD4^+$ T cells (Mausner-Fainberg et al., 2008; Kagami et al., 2009). It is noteworthy that a previous study has shown that simvastatin and geranylgeranyltransferase inhibitor, but not FTI-277, promotes differentiation of $CD4^+Foxp3^+$ Tregs from $CD4^+$ T cells in vitro (Kagami et al., 2009). Therefore, it is tempting to speculate that inhibition of farnesylation rather than geranylgeranylation may play an important role in the immunostimulatory actions of statins in septic mice. However, it should be noted that neither atorvastatin nor pravastatin

increases the number of Tregs in vivo in mice under normal conditions (Mausner-Fainberg et al., 2008). Hence, it is an open question about the in vivo biological relevance of the differential effects of farnesylation versus geranylgeranylation on Tregs.

The protective effects of FTI-277 on bacterial clearance and immune dysfunction were obvious at 16 h after CLP, although we did not find overt cardiac dysfunction in septic mice at this time point. Therefore, it is unlikely that the beneficial effects of FTI-277 on bacteremia, Tregs, splenic T cells, and macrophages in the early stage of severe sepsis were secondary to amelioration of cardiac dysfunction in septic mice. These data support the notion that the immune system may be a major target of the protective effects of farnesyltransferase inhibitor in the early stage of sepsis in mice. However, it should be noted that our data do not exclude the possibilities that organs other than immune cells, such as cardiovascular system, may be also important direct targets of farnesyltransferase inhibitor in sepsis.

In conclusion, our findings suggest that elevation in protein farnesylation may play a role in sepsis-induced immune dysfunction and mortality. It is reasonable to speculate that the inhibition of protein farnesylation might contribute to the pleiotropic beneficial effects of statins in sepsis. These findings identify farnesyltransferase as a novel potential molecular target to reduce the mortality of patients with sepsis and reverse sepsis-induced immune suppression.

Authorship Contributions

Participated in research design: Yang, Yamada, Tamura, Chang, Mao, Zou, Feng, Kida, Scherrer-Crosbie, Chao, Ichinose, Yu, Fischman, Tompkins, Yao, and Kaneki.

Conducted experiments: Scherrer-Crosbie, Yang, Yamada, Tamura, Chang, Zou, Feng, and Kida.

Performed data analysis: Yang, Yamada, Tamura, Chang, Mao, Zou, Feng, Kida, Scherrer-Crosbie, Chao, Ichinose, Yu, Fischman, Tompkins, Yao, and Kaneki.

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