Original Paper

Novel Role of p53 in Septic Immunosuppression: Involvement in Loss and Dysfunction of CD4⁺ T Lymphocytes

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Key Words

p53 • CD4⁺ T lymphocytes • Cell proliferation • Apoptosis • Immunosuppression • Sepsis

Abstract

Background/Aims: Immunosuppression frequently occurs during the development of sepsis and is closely associated with poor outcome. Characteristics of immunosuppressive CD4⁺ T lymphocytes in sepsis have been reported to include dramatic cell loss and inactivation. p53 acts as a pivotal transcription factor in regulating cell proliferation and apoptosis, which control tumorigenesis. However, few studies have investigated the universal role of p53 in immune cells, especially in the development of sepsis. **Methods:** A mouse model of sepsis was produced by cecal ligation and puncture (CLP), and isolated splenic CD4⁺ T cells or Jurkat cells were exposed to lipopolysaccharide (LPS) stimulation *in vitro*. We used genetic knockout (p53^{-/-}) mice or the specific inhibitor pifithrin- α (PFT) to investigate the regulatory mechanisms of p53. Cell proliferation ability was assessed using a Cell Counting Kit-8 assay, and apoptotic cells were stained with annexin V/propidium iodide and then analyzed using a FACScan flow cytometer. Protein and mRNA expression levels were measured by western blotting and real-time PCR, and cytokine levels in culture supernatants were determined by enzyme-linked immunosorbent assay. **Results:** Splenic CD4⁺ T lymphocytes from CLP mice expressed gradually elevated p53 mRNA and protein levels, which resulted in extracellular regulated protein kinase 1/2 inactivation and expression of apoptotic molecules. Specific inhibition of p53 by PFT or genetic knockout (p53^{-/-}) maintained CD4⁺ T lymphocyte homeostasis, as indicated by protection from cell loss and restoration of immune function. A medium dose of PFT improved the survival rate of mice, while mortality rate showed only a slight improvement in p53^{-/-} mice compared with wild-type mice. The *in vitro* responses to LPS were consistent with these results, and upregulation of p53 clearly affected the proliferation, apoptosis, and immune dysfunction of CD4⁺ T lymphocytes. In addition, we confirmed the regulatory effect of p53 in Jurkat cells, and inhibition of p53 by either inhibition or short hairpin RNA transduction

markedly protected cells from LPS stimulation. **Conclusion:** Elevation of p53 in T lymphocytes during sepsis or endotoxin challenge might be responsible for inhibiting cell proliferation and enhancing both apoptosis and immune dysfunction of T cells.

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Introduction

Sepsis is a life-threatening condition that has become the major cause of mortality in intensive care units (ICUs) and is responsible for over 8 million deaths across the world each year [1]. Traditionally, sepsis is considered an infection-induced systemic inflammatory response syndrome and may lead to organ dysfunction and even death. However, numerous anti-inflammatory therapy trials did not achieve satisfactory results. Recently, sepsis has been redefined as a life-threatening organ dysfunction caused by a dysregulated host response to infection [2]. The imbalance between pro- and anti-inflammatory responses is critically involved in the immune dysregulation and rapidly leads to an immunosuppressive state [3]. Along with a better understanding of the potential mechanism, immunotherapies to improve host immunity have acquired remarkable success, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death-1 (PD-1)-specific antibodies [4]. These successes have further encouraged us to illustrate the mechanism underlying immunosuppression in the development of sepsis, especially the involvement of dysfunction of T lymphocytes. The alterations of T lymphocytes in critical illness are a dramatic loss and inactivation [5, 6]. Similar phenomena are evident in human immunodeficiency virus (HIV) infection, as inactivation and elimination of CD4⁺ T lymphocytes are significantly induced, and the mechanism might be related to p53, which is activated by dsDNA damage [7]. Patients with HIV infection gradually approached to an immunosuppressive state called AIDS, and their progression is correlated with CD4⁺ T lymphocyte counts [8, 9].

p53 is a transcription factor responsible for target gene expression that is involved in multiple pathways and affects cell fate. The most well documented functions of p53 are cell cycle regulation and apoptosis [10]. Approximately half of human cancers express mutant p53. As a result, tumor cells proliferate due to a loss of biological cell arrest regulation. By contrast, senescent cells express a higher level of p53, which induces cell cycle arrest. p53 induces cell cycle arrest through both transcriptional activation of p21 and inactivation of extracellular signal-regulated kinase (ERK)1/2, which lead to inhibition of cyclin/cyclin dependent kinase (CDK) expression and combination [11-13]. In addition, p53 targets apoptosis-related genes, such as Bax and p53 upregulated modulator of apoptosis (PUMA), which evoke cytochrome C leakage from mitochondria and following apoptosis [14]. p53 is a universally expressed protein in eukaryotic cells, including immune cells. As reported in HIV infection, an elevation of p53 in immune cells might contribute to senescence and apoptosis [7, 15]. The loss and dysfunction of T lymphocytes are involved in the development of an immunosuppressive state in critical illness. Nevertheless, the potential role of p53 in immune dysfunction of T lymphocytes remains to be further elucidated in the setting of septic response.

Materials and Methods

Reagents

RPMI 1640 (containing glutamine, penicillin, streptomycin and HEPES) and fetal bovine serum (FBS) were purchased from TianRunShanda Biotech Co. Ltd, Beijing, China. Mouse CD4⁺ T Cell Isolation Kit was purchased from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany. Anti-mouse CD3 antibody and anti-mouse CD28 antibody were purchased from eBioscience, San Diego, CA, USA. Lipopolysaccharide (LPS) and pifithrin- α (PFT) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Nutlin-3 was purchased from Selleck, Houston, TX, USA. Phycoerythrin (PE) Annexin V Apoptosis Detection Kit I was purchased from BD Pharmingen, San Diego, CA, USA. Caspase-3 and Caspase-9 Colorimetric Assay Kits were purchased from

R&D Systems, Minneapolis, MN, USA. Mouse interleukin (IL)-2, IL-4 and interferon (IFN)- γ , and human IL-2, IL-4 and IFN- γ enzyme-linked immunosorbent assay (ELISA) kits were purchased from Exell Inc., Shanghai, China. The nuclear factor of activated T cell (NF-AT)c1 ELISA kit was purchased from Active Mortif, Carlsbad, CA, USA. The Reverse Transcription System was purchased from Promega, Madison, WI, USA. SYBR Green Polymerase Chain Reaction (PCR) Master Mix was purchased from Exell Inc., Shanghai, China. Anti-phospho-p53 (Ser15) antibody, anti-phospho-mitogen-activated protein kinase (MAPK) (42/44) antibody, anti-Bcl-2 antibody, anti-Bax antibody and anti-cleaved caspase-3 antibody were purchased from Cell Signaling Technology Inc., Danvers, MA, USA. Anti-p53 antibody, anti-p21 antibody and anti-histone 3 antibody were purchased from Abcam, Cambridge, MA, USA. Anti- β -actin antibody, HRP labeled mouse anti-rabbits antibody and goat anti-mouse antibody were purchased from Applygen Technologies Inc., Beijing, China. The Amersham ECL™ advance Western blotting detection kit was purchased from Applygen Technologies Inc., Beijing, China.

Experimental animals and study design

Male C57BL/6 mice (age range 7 $\pm$ 1 weeks, weight range 20 $\pm$ 2 g) were purchased from the Laboratory Animal Institute (Chinese Academy of Medical Science, Beijing, China). p53^{-/-} mice on the C57BL/6 background were purchased from Beijing Biocytogen Co. Ltd, Beijing, China. Mice were housed 5 per cage in static microisolation caging with softwood bedding and food ad libitum in an SPF barrier facility. The animal housing room was maintained on a 12:12-h light:dark cycle with constant temperature (24 $\pm$ 1 °C). For each independent experiment, C57BL/6 mice (n=60) were randomly allocated into three groups including the sham, the cecal ligation and puncture (CLP), and the CLP+PFT groups (n=20 per group). p53^{-/-} and C57BL/6-background mice (n=60) were randomly allocated into the sham and the CLP groups (n=20 per group). Prior to CLP operation mice were subject to diet restriction for 12 hours. The CLP procedure is described in the following section. The mortality was recorded at 12, 24, and 48 hours, and the dead mice were removed from the cage immediately. This CLP model led to approximately 40% to 50% mortality within 2 days. Survival mice were sacrificed randomly at 24 or 48 hours after CLP (n=5 $\pm$ 1), and subjected to spleen isolation. The experimental reproducibility was assured in at least three independent trials, whose results were combined for this study.

Cecal ligation and puncture

For induction of polymicrobial sepsis, mice were subjected to CLP as described previously [43]. In brief, a laparotomy was performed after anesthesia with 5% chloral hydrate (0.1 ml/kg). The cecum was isolated, ligated below the ileocecal valve, and punctured through with a 21-gauge needle and a droplet of feces was extruded. The peritoneum and skin were closed by simple interrupted sutures. Sham operation was performed by isolating the cecum without ligation and puncture. Animals were resuscitated by subcutaneously injection of 1 ml prewarmed normal saline. Animals that underwent CLP were given a single intraperitoneally injection of PFT (2 mg/kg) at 30 min prior to surgery. In the control group, animals received an identical volume (200 μ l) of PBS intraperitoneally at the corresponding time point. Mice were resuscitated by postcervical subcutaneous injection of 1 ml prewarmed 0.9% normal saline (NS). Antibiotics were not provided after CLP or sham operation.

Isolation of splenic CD4⁺ T lymphocytes

Spleens were removed from mice and teased apart in cold PBS. Cells were dispersed through a 300s stainless steel mesh and were resuspended in PBS. Mononuclear cells were then obtained using Ficoll-Paque density-gradient centrifugation. CD4⁺ T cells were isolated from splenocytes by positive selection using the Mouse CD4⁺ T Cell Isolation Kit as directed by the manufacturer. In brief, CD4⁺ T cells were separated by passing the cell suspension over a magnetic-activated cell sorter LS column held in a MACS magnetic separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The CD4⁺ T cells passing through the column were harvested for further use.

Cell culture and treatment

Isolated splenic CD4⁺ T cells were resuspended in RPMI 1640 medium supplemented with 10% FBS and seeded into 96 or 6 well plates with precoated anti-mouse CD3 (1 µg/ml) as well as soluble anti-mouse CD28 antibody (0.5 µg/ml). After 12-hour incubation at 37°C in a humidified incubator with 5% CO₂, cells were subjected to LPS (1 µg/ml) stimulation with or without PFT (20 µm) or to nutlin-3 (10 µm) for the indicated times.

Jurkat E6-1 cells (purchased from the Cell Resource Center, Chinese Academy of Medical Sciences, Beijing, China) were cultured in RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, and 2 mM L-glutamine at 37°C in a humidified incubator with 5% CO₂. For experiments, cells were stimulated with LPS (1 µg/ml) in the absence or presence of PFT (20 µm) or nutlin-3 (10 µm) for the indicated times.

Lentivirus transfection

A full-length human p53 cDNA was obtained from GenScript Co. Ltd., Piscataway, NJ, USA. Lentivirus vectors expressing the DNA fragments encoding GFP-tagged full-length human p53 (Lv-p53 mRNA) and p53 mRNA-targeted shRNA were designed, constructed, packed, and purified by Obio Technology Co. Ltd., Shanghai, China. Lentiviral transduction of Jurkat cells was performed according to the protocol provided by the manufacturer. In brief, cells were grown to logarithmic phase, harvested, and washed twice with PBS. Cells were resuspended to 2.5×10⁵ per well in 0.5 ml of complete medium (RPMI 1640 with 10% FBS) in 24-well plates. Lentivirus was added to a final multiplicity of infection (MOI) of 50 colony-forming units per cell. The cells were then incubated for a further 8 hours before washing and replating in fresh medium. Cells were transferred to 12-well plates, 6-well plates and 10 cm² bottles in turns. Afterwards cells were selected by puromycin (5 µg/ml). Transduced cells were resuspended for experiments.

Measurement of cell proliferative ability

Cell proliferative ability was measured by the Cell Counting kit-8 (CCK-8) from Dojindo Laboratories, Kumamoto, Japan. Cells (2×10⁵/well) were transplanted into a 96-well plate with treatment and then cultured in a humidified incubator containing 5% CO₂. After the indicated time, 10 µl CCK-8 was added into each well. After 2-hour incubation, the optical density value (absorbance) was recorded at 450 nm using an ELISA plate reader (Spectra MR, Dynex, Richfield, MN, USA). The assays were performed in quadruplicate.

Determination of cell apoptosis

Apoptotic cells were identified and quantified using the Annexin V-PE kit according to instructions of the manufacturer. Briefly, cells (1×10⁵) were stained with Annexin V and 7-AAD. Cells were collected and washed twice with cold PBS, then resuspended in 100 µl binding buffer, to which were added 2.5 µl Annexin V-PE and 2.5 µl 7-AAD. After incubation for 15 min in darkness at room temperature, the cells were diluted with 200 µl binding buffer and analyzed within 1 hour using a FACScan flow cytometer (BD Biosciences, Mountain View, CA, USA).

Western blot analysis

The whole-cell proteins were isolated in Radio Immune Precipitation Assay (RIPA) buffer, and nuclear protein was isolated using a Nuclear-Cytosol Extraction kit (Applygen Technologies Inc., Beijing, China) following the manufacture's protocols. Cell lysate concentration was determined with a Bradford protein assay kit (Applygen Technologies Inc., Beijing, China). Cell lysate that contained 50 µg of protein in SDS-Laemmli loading buffer per lane was separated by 10%-12% SDS-PAGE and transferred onto nitrocellulose membranes. After transfer, membranes were blocked in 0.5% TBST containing 5% nonfat milk for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies, followed by incubation with a secondary antibody for 1 hour at room temperature. The blots were detected with an ECL system. The protein bands were quantified by densitometry using National Institutes of Health ImageJ software.

SYBR green real-time reverse transcription PCR

Total RNA was extracted from cultured cells using Trizol reagent according to the manufacturer's instructions. The mRNAs for p53, p21 and β-actin were quantified in duplicate by SYBR Green 2-step real-time reverse transcription-PCR. Total RNA from each sample was used for reverse transcription using the Reverse Transcription System following the manufacturer's protocol. PCR mixture were prepared using SYBR Green

PCR Master Mix with the following primers: mouse p53, 5'-GCGTAAACGCTTCGAGATGTT-3' (forward) and 5'-TTTTTATGGCGGGAAGTAGACTG-3' (reverse); mouse p21, 5'-CGAGAACGGTGGAACCTTTGAC-3' (forward) and 5'-CCAGGGCTCAGGTAGACCTT-3' (reverse); mouse β -actin, 5'-GGCTGTATCCCCTCCATCG-3' (forward) and 5'-CCAGTTGGTAACAATGCCATGT-3' (reverse); human p53, 5'-CAGCACATGACGGAGGTTG-3' (forward) and 5'-TCATCCAAATACTCCACACGC-3' (reverse); human p21, 5'-TGTCCGTCAGAACCCATGC-3' (forward) and 5'-AAAGTCGAAGTTCCATCGCTC-3' (reverse), human β -actin, 5'-CCTCGCCTTTGCCGATCC-3' (forward) and 5'-CTCGTCGCCCACATAGGAAT-3' (reverse). Thermal cycling conditions were 20 seconds at 95°C followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds on a sequence detection system. p53 and p21 gene expression were normalized to β -actin mRNA content.

ELISA assay

IL-2, IL-4, and IFN- γ levels in culture supernatants were quantitated by ELISA kits for mice or humans following the protocols provided by the manufacturer. Plates were read in a microplate reader at 450 nm.

Measurement of caspase-3 and caspase-9 activities

Caspase activities were measured using caspase-3 and caspase-9 assay kits from R&D Systems, Minneapolis, MN, USA. Cells were lysed according to the manufacturer's instructions, and protein concentration was quantified using the Bradford method. Cell lysates (from 10^6 cells) were incubated with 5 mM dithiothreitol, and caspase-3 and caspase-9 colorimetric substrates were conjugated to color reporter molecule p-nitroanilide (DEVD-pNA, IETD-pNA, LEHD-pNA) in reaction buffer for 2 hours at 37°C. Cleavage of substrate was quantified using a microplate reader to determine absorbance at 405 nm.

Assay of NF-ATc1 activity

Nuclear protein was extracted from cultured cells using a nuclear extraction kit. In brief, cells were washed and collected in ice-cold PBS in the presence of phosphatase inhibitors to limit further protein modifications. Then, cells were resuspended in a hypotonic buffer and treated with detergent to allow leakage of the cytoplasmic proteins into the supernatant. After collection of the cytoplasmic fraction, the nuclei were lysed, and nuclear proteins were solubilized in lysis buffer containing protease inhibition cocktail. Protein concentrations were determined by the Bradford protein assay. The ELISA-based electrophoretic mobility shift assay was used to quantify the NF-ATc1 in nuclei (6 mg). Briefly, the NF-ATc1 activity was purified from a nuclear lysate upon binding an immobilized oligonucleotide containing a 5-AGGAAA-3 motif and was determined by ELISA.

Statistical analysis

Data are expressed as the means $\pm$ SD or the means $\pm$ SEM of three independent experiments conducted in triplicate. Data were analyzed with one-way analysis of variance (one-way ANOVA). Fisher's least significant difference was used to evaluate significant differences between groups. *P* values <0.05 were considered statistically significant.

Results

Loss and dysfunction of CD4⁺ T lymphocytes in septic mice

In the current study, almost half of septic mice died within 48 hours (Fig. 1A). Splenic CD4⁺ T lymphocytes isolated from septic mice lacked effective proliferative abilities even in the absence of stimulation with anti-CD3 and anti-CD28 antibodies (Fig. 1B). Meanwhile, CD4⁺ T lymphocytes gradually underwent apoptosis after CLP (Fig. 1C). Both declined proliferation and increased apoptosis might have been attributable to the loss of T cells, and the remaining alive cells were inactivated with a lower autocrine of IL-2 production (Fig. 1D). In addition, the ratio of IL-4 to IFN- γ production altered significantly in mice after CLP (Fig. 1D).

Fig. 1. Loss and dysfunction of CD4⁺ T lymphocytes in septic mice. (A) C57BL/6 mice (n=20) were subjected to CLP, and survival rates were observed at 24, 48, and 72 hours. (B, D) CLP or sham mice were sacrificed after indicated time points (n=5). Splenic CD4⁺ T cells were isolated and cultured for further 3 days in the absence of anti-CD3 and anti-CD28 antibody. Afterwards cell proliferative rates were measured by CCK-8 kit. The values of sham group (0) were taken as 1, and were means $\pm$ SEM of at least three independent experiments. *Significant difference when compared with 0 group ($P < 0.05$) (B). The supernatants were subjected to ELISA analysis. The original values of IL-2 (left panel) and the ratio of IL-4 to IFN- γ (right panel) were means $\pm$ SD (D). *Significant difference when compared with sham group ($P < 0.05$). (C) Splenic CD4⁺ T cells were isolated after CLP at indicated time points and stained with Annexin-V/7-AAD, and then cells were analyzed by flow cytometry. Annexin⁺/7-AAD⁻ indicated early apoptosis.

Fig. 2. Enhanced p53 expression in CD4⁺ T lymphocytes after sepsis. (A, B) Splenic CD4⁺ T cells were isolated from the sham (n=5) or CLP mice at indicated time points (n=5), and p53 mRNA levels were analyzed by PCR. The values of sham group (0) were taken as 1, and were means ± SEM of at least three independent experiments. *Significant difference when compared with 0 group (P<0.05) (A). Phosphorylated p53 (p-p53) and p53 protein levels were determined by Western blot (B). β-actin was used as loading control. (C) C57BL/6 mice (n=20 for each group) were subjected to CLP with or without a 30 min prior intraperitoneal injection of pifithrin-α (PFT, 2 mg/kg). (D) Wild type (WT, n=20) or p53^{-/-} C57BL/6 mice (n=20) were subjected to CLP, and the data were presented as Kaplan-Meier survival curve. *Significant difference when compared with WT CLP group (P<0.05)

Enhanced p53 expression in CD4⁺ T lymphocytes after sepsis

In splenic CD4⁺ T lymphocytes of mice, p53 mRNA was upregulated after CLP from 24 hours on (Fig. 2A). p53 protein was rapidly phosphorylated, which was followed by stabilization and accumulation (Fig. 2B). The tendency of gradually rising p53 level was consistent with the mortality of mice that underwent CLP. To investigate the potential role of p53, we blocked its activity by intraperitoneal injection of a specific inhibitor, PFT, prior to CLP. As shown in Fig. 2C, a medium dose of PFT improved the survival rate of mice, while mortality rate only showed a slight improvement in p53^{-/-} mice compared with that of WT mice (Fig. 2D).

p53 appears to be responsible for inhibiting cell proliferation

In septic mice, nuclear p53 was gradually increased in CD4⁺ T lymphocytes (Fig. 3A) and exerted its transcriptional function, indicated by an upregulated p21 mRNA level, a major downstream target gene of p53 (Supplemental Fig. 1B - for all supplemental material see www.karger.com/10.1159/000495241/). ERK1/2, which regulates T cell proliferation, is also an identified downstream target of p53 via MAPK phosphatase-3 (MKP-3) [4]. After septic challenge, ERK1/2 activation was suppressed in CD4⁺ T lymphocytes, along with

Fig. 3. p53 was responsible for limited cell proliferation. (A, B) Splenic CD4⁺ T cells were isolated from the sham or CLP WT mice at indicated time points (n=5). Cytosolic and nuclear proteins were separately extracted and subjected to Western blot analysis of p53. Histone-3 was used as nuclear protein loading control. The quantification analysis was shown in the bottom. The values of sham group (0) were taken as 1, and were means $\pm$ SD. *Significant difference when compared with 0 group (P<0.05) (A). Total protein was subjected to analysis of phosphorylated ERK1/2 (p-ERK1/2) (B). (C) Splenic CD4⁺ T cells were isolated from WT mice (n=10) and subjected to LPS (1 μ g/ml) stimulation, and cells were collected and subjected to analysis of p-p53 as well as p53 after indicated time points. (D) Cells were stimulated with LPS in the presence or absence of PFT (20 μ M) for indicated time points. Cell proteins were subjected to analysis of p21 and p-ERK1/2. β -actin was used as loading control. (E) Splenic CD4⁺ T cells were isolated and subjected to LPS stimulation with or without PFT, after 24 hours incubation, cell proliferative rate was measured by CCK-8. The values of untreated cells were normalized to 1, and were means $\pm$ SEM of at least three independent experiments. (F, G) WT mice (n=20 for each group) were subjected to CLP with or without a 30 min prior intraperitoneal injection of PFT (F). WT (n=20) or p53^{-/-} mice (n=20) were subjected to CLP (G). After indicated time points, mice were sacrificed and the splenic CD4⁺ T cells were isolated. Cells were cultured for further 3 days with growth stimuli, and then cells proliferative rate was measured. The values of sham group were taken as 1, and were means $\pm$ SEM of at least three independent experiments. *Significant difference when compared with sham group (P<0.05). #Significant difference when compared with CLP group or WT (P<0.05).

Fig. 4. p53 mediated the apoptosis of CD4⁺ T lymphocytes during sepsis. (A–C) Splenic CD4⁺ T cells were isolated from WT mice (n=20) and subjected to LPS (1 μg/ml) stimulation with or without PFT. After indicated time points, cell proteins were extracted and subjected to analysis of Bcl-2, Bax and cleaved caspase-3. β-actin was used as loading control (A). Cell proteins were subjected to analysis of caspase-3 activities. The original values were shown as means ± SD. *Significant difference when compared with 0 group (P<0.05). #Significant difference when compared with the PFT group (P<0.05) (B). Cells were stained with Annexin-V/7-AAD and then analyzed by flow cytometry (C). (D, E) WT mice (n=20 for each group) were subjected to CLP with or without a 30 min prior intraperitoneal injection of PFT (D). WT (n=10) or p53^{-/-} mice (n=10) were subjected to CLP (E). Mice were sacrificed at 24 hours and the splenic CD4⁺ T cells were isolated. Cells were stained with Annexin-V/7-AAD and then analyzed by flow cytometry. Annexin⁺/7-AAD⁺ indicated early apoptosis.

p53 accumulation (Fig. 3B). We further isolated splenic CD4⁺ T lymphocytes and activated cells with anti-CD3 and anti-CD28 to evaluate cell proliferation. Additional LPS stimulation *in vitro* was used to mimic a septic environment, which is reported to affect T lymphocyte proliferation depending on interactions with CD28 or CD3 [16, 17]. Consistent with those data, LPS stimulation gradually activated p53 phosphorylation and led to its accumulation (Fig. 3C). Effective blockade of p53 activity with PFT reversed LPS induced ERK1/2 deactivation (Supplemental Fig. 1B and Fig. 3D), and as a result cell proliferation ability was promoted, although LPS did not markedly impair the cell proliferation *in vitro* (Fig. 3E). *In vivo*, the CLP-induced inhibition of the cell-proliferative response was obviously diminished by PFT injection (Fig. 3F), and CD4⁺ T cells isolated from p53^{-/-} mice presented a much better proliferative ability (Fig. 3G).

p53 mediates apoptosis of CD4⁺ T lymphocytes during sepsis

LPS-treated CD4⁺ T lymphocytes had a dramatically elevated Bax and a slight decrease in Bcl-2 *in vitro*, and LPS initiated caspase-3 cleavage, indicating the activation of apoptosis cascade, which was blocked by p53 inhibition (Fig. 4A and 4B). Flow-cytometric analysis revealed a larger amount of Annexin⁺/7-AAD⁻ stained early apoptotic cells after LPS stimulation, but this was reduced by the treatment with PFT (45.74% vs. 38.04%) (Fig. 4C). Some 16.48% of the freshly isolated CD4⁺ T lymphocytes from CLP mice underwent apoptosis, as shown in Fig. 4D, while injection of PFT protected cells from apoptosis (10.94%). Consistently, cells from p53^{-/-} mice also showed slightly higher apoptosis compared to that from WT mice (13.68% vs. 8.67%) (Fig. 4E).

Inactivation of CD4⁺ T lymphocytes involved p53

After 24 hours' exposure of LPS, CD4⁺ T lymphocytes showed a significantly depressed NF-ATc1 activation, which was reversed by p53 inhibition (Fig. 5A). *In vivo*, CLP resulted in suppression of NF-ATc1 activation in CD4⁺ T lymphocytes. However, injection of PFT reserved the relative normal activity (Fig. 5B), and consistent results were acquired from p53^{-/-} mice (Fig. 5C). Inactivated T cells presented an altered pattern of cytokine productions during sepsis both *in vitro* and *in vivo*, as indicated by decreased IL-2 production and increased IL-4/IFN- γ ratio (Fig. 1D). These cytokine phenotypes almost recovered to normal range following treatment with PFT, suggesting a restoration of cell activation and normal Th1 differentiation (Fig. 5D and Supplemental Fig. 2A). Injection of PFT also markedly reversed cytokine secretion *in vivo* (Fig. 5E and Supplemental Fig. 2B). In p53^{-/-} mice, sepsis did not suppress IL-2 production. In contrast, IL-2 was gradually elevated after CLP. Although the IL-4/IFN- γ ratio increased after septic challenge in p53^{-/-} mice, it was still lower than that in WT mice (Fig. 5F and Supplemental Fig. 2C).

Role of p53 in dysfunction of Jurkat cells treated by LPS

To further investigate whether p53 plays a potential role as in CD4⁺ T lymphocytes of mice, Jurkat cells were exposed to LPS stimulation. As shown in Fig. 6A, p53 was phosphorylated and obviously accumulated within 24 hours. Along with p53 activation, ERK1/2 phosphorylation was suppressed, and caspase-3 was cleaved together with an increased activity. Then, we treated cells with p53 inhibitor PFT, or with a well-used p53 activator, Nutlin-3 as a positive control [18]. Blockade of p53 reversed LPS-induced ERK1/2 dephosphorylation, caspase-3 cleavage and activation, while induction of p53 by Nutlin-3 showed the same effects as LPS stimulation (Fig. 6B and 6C). Consequently, LPS exposure resulted in the dysfunction of cell proliferation and apoptosis, which were blocked by treatment with PFT (Fig. 6D and 6E). In addition, LPS inhibited NF-AT activity and IL-2 production, and the ratio of IFN- γ /IL-4 was shifted to a Th2 type (Fig. 6F, 6G and Supplemental Fig. 3).

Fig. 5. Inactivation of CD4⁺ T lymphocytes involved p53. (A, B) Splenic CD4⁺ T cells were isolated from WT mice (n=20) and subjected to LPS stimulation with or without PFT. (C, D) WT mice were subjected to CLP with or without a 30 min prior intraperitoneal injection of PFT. (E, F) WT or p53^{-/-} mice were subjected to CLP. After indicated time points, mice were sacrificed and the splenic CD4⁺ T cells were isolated. Nuclear proteins were extracted from cells and subjected to assessment of NF-ATc1 activity. The original values were means ± SD (A-C). Cells were cultured for further 3 days with growth stimuli and the supernatants were subjected to ELISA analysis. The original values of IL-2 (left panel, D-F) and the ratio of IL-4 to IFN-γ (right panel, D-F) were means ± SD of at least three independent experiments. *Significant difference when compared with control or sham (P<0.05). #Significant difference when compared with the CLP or WT group (P<0.05).

Interference with p53 gene expression in LPS-exposed Jurkat cells

To further investigate the regulatory mechanism underlying p53 action in T cell immune response, we transfected Jurkat cells with lentivirus containing either p53 shRNA or mRNA (Supplemental Fig. 4A). In p53-knock-down cells, ERK1/2 had a normal phosphorylation level, without obvious caspase-3 cleavage. In p53-overexpressing cells, ERK1/2 phosphorylation and caspase-3 cleavage were inhibited, even in the absence of LPS (Fig. 7A). Consistent with those data, cell proliferation blunted by p53 was absent in knock-down cells and was further inhibited in overexpression cells (Fig. 7B). LPS-promoted apoptosis was mitigated in knock-down cells (11.02% vs. 14.06%). By contrast, p53 overexpression itself markedly enhanced apoptosis (10.87% vs. 5.76%), and apoptosis was further induced after LPS exposure (20.27%) (Fig. 7C). Meanwhile, NF-ATc1 activity was maintained in p53-knock-down cells but showed a decrease in overexpression cells (Fig. 7D). IL-2 production showed the same tendency. Although IL-4 and IFN-γ levels did not change obviously, their ratios revealed a slight decrease in p53-knock-down cells, especially after LPS stimulation. In overexpression cells, the basal ratio of IL-4 and IFN-γ was higher but strongly decreased after LPS treatment (Fig. 7E and Supplemental Fig. 4B).

Fig. 6. Effect of chemical p53 regulators on LPS-treated Jurkat cells. (A) Jurkat cells were treated with LPS (1 μ g/ml) at various time points, and then cells were subjected to Western blot analysis of p-p53, p53, p-ERK1/2, and cleaved caspase-3. β -actin was used as loading control. (B-G) Cells were treated with LPS in the presence or absence of PFT (20 μ M), or treated with nutlin-3 (10 μ M) for 24 hours. Afterwards cells were subjected to Western blot analysis of p-ERK1/2 and cleaved caspase-3 (B), assessment of caspase-3 activity (C) or cell proliferation (D). Treated cells were stained with Annexin-V/7-ADD and then analyzed by flow cytometry (E). The nuclear proteins were extracted from treated cells and subjected to assessment of NF-ATc1 activity (F). The supernatants were subjected to ELISA analysis. The original values of IL-2 (left panel, G) and the ratio of IL-4 to IFN- γ (right panel, G) were means $\pm$ SD of at least three independent experiments. *Significant difference when compared with control ($P < 0.05$). **Significant difference when compared with the LPS group ($P < 0.05$).

Fig. 7. Effect of p53 mRNA interference on LPS-treated Jurkat cells. Jurkat cells were transfected with p53 shRNA or mRNA expressing lentivirus (Lv-p53 shRNA or Lv-p53 mRNA). After stable expression, cells were treated with LPS for 24 hours. GFP expressing lentivirus (Lv-GFP) was used as a cell control. (A) Treated cells were subjected to analysis of p-ERK and cleaved caspase-3. β-actin was used as loading control. (B) Treated cells were subjected to cell proliferation analysis by CCK-8, and the values of each untreated cells were taken as 1, and were means ± SD of at least three independent experiments. (C) Cells were stained with Annexin-V/7-AAD after stimulation and then analyzed by flow cytometry. (D) The nuclear proteins were extracted from treated cells and subjected to assessment of NF-ATc1 activity. (E) The supernatants were subjected to ELISA analysis of IL-2, IL-4 and IFN-γ. The original values NF-ATc1 activity and IL-2 (left panel, E) and the ratio of IL-4 to IFN-γ (right panel, E) were means ± SD. *Significant difference when compared with Lv-GFP control (P<0.05). #Significant difference when compared with Lv-GFP LPS group (P<0.05).

Discussion

Tumor-suppressor protein p53 was discovered for over 30 years by its suppression effect on tumors, and it is inactivated or mutant in more than half of human tumors [19]. Tumor therapy strategies based on p53 activation are focused on tumor cell apoptosis and cell cycle arrest to protect from tumorigenesis or metastasis [20]. Recent compelling evidence indicates that p53 plays a crucial role in tumor suppression through regulating immune surveillance in the tumor microenvironment (TME) [21]. Meanwhile, elevated p53 is detected in contexts of chronic inflammation, autoimmune diseases and virus infections, including inflammatory bowel disease, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and HIV infection [22]. Increasing evidence suggests the potential role of p53 in inflammation-induced immune responses. Herein, we found that p53 is critical in activation of T lymphocytes not only by regulating proliferation and apoptosis but also because of its effective function in the pathogenesis of septic complications.

Previous reports have suggested p53 elevation in the septic response, but it has only been evaluated in target organs and is closely related to death because of a p53-dependent apoptosis and cell cycle arrest, consequently leading to organ failure [23-25]. Organ cells, endothelial cells and neutrophils are susceptible to ischemia/reperfusion injury and the resultant cellular oxidative stress, which activate p53 [26]. However, the activation of p53 in adaptive immune cells involves other, more complicated mechanisms that are still unclear. According to reports on HIV-1 infection, DNA double-strand breaks could induce genotoxic stress and lead to p53 activation in CD4⁺ T lymphocytes [7]. In chronic infection of viruses such as hepatitis C virus, p53 induction also depends on the type 1 IFN pathway [27]. The IFN α/β pathway might activate the transcription of p53 through an ISRE site in its promoter, as well as inducing phosphorylation of p53 at Ser15, thus stabilizing its protein level [28, 29]. A major intracellular signal transducer of the type 1 IFN pathway, signal transducer and activator of transcription (STAT)-1, is suggested to inhibit mouse double minute 2 homolog (MDM2) transcription, which directly binds with p53 and leads to continuous degradation of p53 [30]. In our current reports, however, the elevation of p53 was independent of MDM2 expression. In both *in vivo* and *in vitro* experiments, MDM2 transcription gradually inclined and showed the same expression pattern as p53. MDM2 itself is a transcriptional target of p53, which forms a negative-feedback loop to regulate p53 signaling [31, 32]. In our context, MDM2 expression did not take charge in regulating p53 protein stabilization, indicating an effective p53 transcriptional activity (data not shown).

We observed an induction of p53 from mRNA transcription to phosphorylation (Ser15)-induced protein stabilization. The N-terminal transcriptional activation domain contains numerous phosphorylation sites that are considered the primary targets for stress signals sent through protein kinase transduction [33]. The MAPK family (JNK1/2, p38 and ERK1/2) respond to several types of stress and transduce signals to p53 phosphorylation on various sites [34]. However, ERK1/2 is different from the other two pathways, as its activation is regulated by p53-mediated MKP-3 transcription, and MKP-3 works as a specific ERK1/2 diphosphatase [11]. Consistent with these pathways, we showed that accumulated p53 decreased ERK1/2. On the other hand, ERK1/2 signaling is an essential pathway for regulating cell proliferation that might be partially responsible for the limited proliferation of CD4⁺ T lymphocytes [35, 36]. Collectively, our results might suggest an enhanced expression of p53 in sepsis, which was mediated by both transcriptional activation and phosphorylation-induced protein stabilization. The precise mechanism may involve multiple pathways and is under further investigation.

In the current study, the development of sepsis was attributed to p53, not only through leading CD4⁺ T lymphocytes to growth arrest and apoptosis but also through participating in the activation and cytokine production of CD4⁺ T lymphocytes. IL-2 plays an essential role in directing T lymphocyte proliferation and differentiation to effector T cells, and its autocrine response is regulated by the transcription factor NF-AT. Our data from mouse CD4⁺ T lymphocytes and endotoxin-exposed Jurkat cells showed dramatically depressed

NF-ATc1 activity, which was closely related to p53 levels, as indicated by either gene knock-out/knock-down or chemical inhibitor-aroused recovery. In X-ray irradiation-induced cell injury, p53 combines with NF-AT and facilitates its target gene expression [37]. Accordingly, p53 and NF-AT might have some shared sites or domains through which they facilitate or compete with each other's activation of target genes.

Our data imply that the dysfunction of CD4⁺ T lymphocytes can be represented as a shift to Th2 phenotype, supported by an alteration of IL-4/IFN- γ production. The involvement of p53 may be to directly regulate cytokine production in CD4⁺ T lymphocytes. In senescing cells with an activated p53 pathway, the cells present an altered cytokine production pattern, which is called the senescence-associated secretory phenotype (SASP), including cytokines as well as chemokines [38]. Interestingly, a majority of the SASP cytokine release appears to depend on the nuclear factor (NF)- κ B pathway, which evidences a cross-talk between p53 and NF- κ B. The Th2 differentiation can also be due to hyperactivation of regulatory T cells (Tregs). Existing reports in autoimmune disease indicate that a high level of p53 in T cells promotes inducible Treg growth, thus regulating T cell differentiation, polarizing T helper cell differentiation towards a Th2 immunosuppressive type [22].

In addition to T lymphocytes, p53 plays indispensable roles in other immune cells. For instance, nutlin-3-triggered p53 activity promotes the production of pro-inflammatory cytokines in human macrophages, including TNF- α , IL-6, and IL-8, via cooperation with the NF- κ B pathway [39]. Other reports indicated a contrary phenomenon in which macrophages from p53^{-/-} mice produced increased pro-inflammatory cytokines in response to LPS [40]. Furthermore, p53 plays an essential role in promoting M2 macrophage polarization, which expresses anti-inflammatory cytokines [41]. In dendritic cells (DCs), p53 mediates surface molecule expression and maturation, in turn affecting T lymphocyte proliferation [42]. The transcription factor p53 indeed has expansive functions through its targets and is indispensable in regulating immune responses. According to a brief review of existing reports, the effects of p53 on immune response are disputable and context-dependent. In the setting of sepsis, our results strongly suggest an important role of p53 in regulating proliferation, apoptosis, and dysfunction of CD4⁺ T lymphocytes, all of which are part of immunosuppression.

The current results were obtained from mouse CD4⁺ T lymphocytes and the human Jurkat cell line. Clinical data from septic patients were not acquired and need to be studied. Additionally, in both *in vivo* and *in vitro* experiments, we isolated CD4⁺ T lymphocytes, composed of helper T cells and Tregs, which have reciprocal and contrary effects in immune responses [43]. Although there are only a small number of Tregs among CD4⁺ T lymphocytes, the potential role of p53 in Treg proliferation and activation should not be ignored. Hereafter, we propose to further investigate the possible role and regulatory mechanism of p53 based on various subtypes of CD4⁺ T lymphocytes in the setting of septic challenge.

Nevertheless, the limitations in this study should be overcome in further investigations. In this study, we did not provide analgesic treatment for sepsis model mice. This is an important ethical issue and should be remedied to eliminate severe pain during or after the CLP procedure. Several reports have suggested that low-dose buprenorphine or tramadol is safe and does not cause significant changes in immune response in CLP mice. However, it is still controversial whether buprenorphine might alter septic outcome, especially in male C57BL/6 mice [44, 45]. In the future, we would like to search for a more appropriate analgesics treatment for our animal models.

Another limitation in study design was the lack of treatment after CLP, including fluid resuscitation and antibiotics. In the current experiment, we focused on the potential role of p53 in the development of immunosuppression induced by sepsis, and our findings suggest it as a possible therapeutic target for management of sepsis. Therefore, antibiotics and resuscitation should be administered to closely mimic clinical practice [46]. To get more reliable results from sepsis models, we will consider mimicking clinical treatment and use fluid resuscitation as well as antibiotics, depending on mouse age and weight in future experiments.

Conclusion

In summary, accumulating evidence has demonstrated novel role of p53 in regulating cell cycle arrest and apoptosis in the host immune response. Along with the development of sepsis, inactivation and exhaustion of immune cells might result in marked immune suppression, which is now considered the major cause of poor outcome. Exploring the participation of p53 in immune depression might deepen our understanding the underlying pathophysiological mechanism of immune dysfunction and shed light on novel target of immunomodulation treatment, or at least alert us to the patients who are prone to p53-related immune suppression in septic response.

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Disclosure Statement

The authors declare no conflicts of interest.

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