The Journal of Infectious Diseases

MAJOR ARTICLE

Overexpression of HO-1 Contributes to Sepsis-Induced Immunosuppression by Modulating the Th1/Th2 Balance and Regulatory T-Cell Function

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Background. Countervailing anti-inflammatory response and immunosuppression can cause death in late sepsis. Depletion and dysfunction of T cells are critical for developing sepsis-induced immunosuppression. Heme oxygenase-1 (HO-1) has a regulatory effect on differentiation and function of T cells and anti-inflammatory properties. We therefore investigated the immunosuppressive role of HO-1 in sepsis with a focus on its effects on helper T-cell (Th) differentiation and regulatory T cells (Treg).

Methods. Sepsis was induced by cecal ligation and puncture (CLP). Mice were intraperitoneally injected with zinc protoporphyrin (ZnPP; 25 mg/kg), an HO-1 inhibitor, or hemin (20 mg/kg), an HO-1 inducer, at 24 and 36 hours post-CLP. Splenocytes were isolated 48 hours post-CLP. Mice were intranasally infected with *Pseudomonas aeruginosa* 4 days post-CLP as a secondary pneumonia infection model.

Results. ZnPP improved survival and bacterial clearance, whereas hemin had the opposite effect in septic mice. CLP induced lymphocyte apoptosis and a proinflammatory Th1 to anti-inflammatory Th2 shift, which was attenuated by ZnPP. ZnPP attenuated the CLP-induced Treg population and protein expression of inhibitory costimulatory molecules. Furthermore, ZnPP improved survival in the secondary pneumonia infection model.

Conclusions. Our findings suggest that HO-1 overexpression contributes to sepsis-induced immunosuppression during late phase sepsis by promoting Th2 polarization and Treg function.

Keywords. apoptosis; heme oxygenase; immunosuppression; regulatory T-cells; sepsis; Th1/Th2 shift.

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to bacterial infection, with an unacceptably high mortality rate, and remains a leading cause of death in intensive care units (ICUs). Septic patients initially suffer from hyperinflammatory response, which results in organ damage. As the disease progresses, patients develop a counterregulated anti-inflammatory state. Numerous attempts have been made to inhibit the initial hyperinflammatory response using anti-inflammatory agents, but unfortunately, overall mortality has not been reduced [1]. Accumulating evidence indicates that >60% of patients with sepsis die during the immunosuppressed phase because of failure to clear the primary infection and/or development of secondary infections [2]. Along with absolute decreases in lymphocyte number [3], the septic response induces considerable alterations in lymphocyte function. T-cell secretion of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-2, is significantly depressed in septic patients, and the degree of IL-2 and TNF-α suppression is directly correlated with patient survival [4]. However, the precise mechanism of immunosuppression during sepsis remains unknown.

Helper T cells (Th) play important roles in immune homeostasis during sepsis [5]. Helper T cells are characterized into Th1 and Th2 subsets according to the type of cytokines they excrete after stimulation. In inflammatory diseases, imbalance between the pro-inflammatory Th1 subtype and anti-inflammatory Th2 subtype are reported. Splenocytes isolated from septic mice produced more Th2-derived IL-4 [6], and patients with sepsis also showed an abnormal $CD4^+$ T-cell subpopulation with increased representation of the Th2 subtype [7].

Regulatory T cells (Tregs), formerly known as suppressor T cells, play an indispensable role in suppressing excessive immune responses and maintaining tolerance to self-antigens. However, excessive Tregs contribute to lymphocyte exhaustion in sepsis. Increased Treg populations have been reported in the spleens of septic mice [8] and peripheral blood of septic patients [9]. Tregs directly induce cell death and dysfunction of immune cells via binding of coinhibitory molecules, including cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1). Septic mice showed markedly increased CTLA-4 expression in splenic Tregs [10], and anti-PD-1 is currently being evaluated in a

Received 16 January 2017; editorial decision 16 March 2017; accepted 17 March 2017; published online March 23, 2017.

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The Journal of Infectious Diseases® 2017;215:1608–18

DOI: 10.1093/infdis/jix142

phase 2 clinical trial of patients with cancer who suffer from immunosuppression.

Heme oxygenase (HO) is the rate-limiting microsomal enzyme that catabolizes heme into carbon monoxide (CO), free iron, and biliverdin. HO-1 is an inducible enzyme and possesses anti-inflammatory properties through CO production. One study reported that HO-1 protected against septic injury during the early phase of sepsis by suppressing IL-1β and nuclear factor-κB [11]. Moreover, HO-1 inhibition with zinc protoporphyrin (ZnPP) pretreatment aggravated proinflammatory cytokine production 6 hours after cecal ligation and puncture (CLP) [12], suggesting that early HO-1 inhibition in disease progression may lead to exacerbation of septic injury. In contrast, overexpression of HO-1 had a detrimental role as reflected by increased bacterial load in septic mice [13]. A recent study demonstrated that HO-1 also possesses immunomodulatory potential [14], and HO-1 was found to inhibit the differentiation of $CD4^+$ T cells to Th1 subsets in patients with sickle cell disease [15]. Overexpressed HO-1 accelerated tumorigenesis by inducing Treg function [16]. Moreover, upregulation of HO-1 with hemin promoted basophil apoptosis in bone marrow cells from mice [17].

The aim of this study was therefore to investigate the role of HO-1 in sepsis-induced immunosuppression during the late phase of sepsis, with a particular focus on the effect of HO-1 on Th-cell differentiation and Treg function.

METHODS

Animals and Polymicrobial Sepsis

Male C57BL/6 mice (25–27 g; Orient Bio) were acclimatized under temperature-controlled conditions with a 12-hour light/dark cycle, and given water and food ad libitum. All animal procedures were approved by the Sungkyunkwan University Animal Care Committee and performed in accordance with the guidelines of the National Institutes of Health (publication 86–23, revised 1985). Polymicrobial sepsis was induced by CLP according to the method described by Chaudry et al [18]. After anesthetization with ketamine (100 mg/kg; Yuhan Corporation) and xylazine (10 mg/kg; Bayer), a 1-cm ventral midline incision was made, and cecum was carefully exposed, ligated, and punctured using a a 23-gauge needle. The punctured cecum was returned to the abdominal cavity, and the abdominal incision was closed in 2 layers. Sham-operated animals were subjected to laparotomy and intestinal manipulation, but neither ligated nor punctured. All animals received 1 mL saline subcutaneously immediately post-CLP. The mice were observed for morbidity each hour after the CLP insult, and any moribund mice were immediately killed with overdose of ketamine and xylazine.

Experimental Design

Hemin (20 mg/kg; Sigma-Aldrich) and ZnPP (25 mg/kg; Sigma-Aldrich) were dissolved in 0.2 M sodium hydroxide in the dark (pH = 7.4). Animals were intraperitoneally administered vehicle, hemin, or ZnPP 24 and 36 hours after CLP. Doses and routes of administration were selected based on our preliminary study [19]. Animals were divided randomly into 6 groups (n = 6–8): (1) vehicle-treated sham; (2) ZnPP-treated sham; (3) hemin-treated sham; (4) vehicle-treated CLP; (5) ZnPP-treated CLP; and (6) hemin-treated CLP. Animals were monitored for survival for 10 days post-CLP (n = 15). Independently, blood was collected from the inferior vena cava, and peritoneal fluid was obtained 24 hours post-CLP under anesthesia. Spleens were collected 0 (immediately), 12, 24, 48, and 72 hours after CLP.

HO Activity

Spleens were homogenized in 1.15% potassium chloride. Microsomal fraction was isolated by centrifugation (105 000*g*, 60 minutes, 4°C) and resuspended in 0.1 M potassium phosphate buffer. HO activity was assayed by detecting bilirubin formation, which was calculated from the difference in absorbance [20]. The HO activity was expressed as pmol bilirubin/minute/mg protein.

Colony-Forming Units

Blood, peritoneal fluid, and spleens were collected 48 hours post-CLP, and spleen tissues were homogenized. Samples were diluted with phosphate-buffered saline, spread on blood agar plates, and incubated at 37°C; 24 hours later, the resultant colonies were counted.

Splenocyte Isolation

Spleens were homogenized with a syringe plunger, and passed through a nylon-mesh strainer to remove debris. Red blood cells (RBCs) were lysed using RBC lysis buffer (eBioscience). Isolated splenocytes were conserved in RPMI medium supplemented with 10% fetal bovine serum and 1% antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin; Gibco).

Flow Cytometry Analysis

Isolated splenocytes were blocked with antimouse CD16/CD32-purified antibodies (eBioscience) for 15 minutes and stained with antimouse CD3 (T cell), CD4 (helper T cell), CD8 (cytotoxic T cell), CD11c (dendritic cell [DC]), CD49b (natural killer [NK] cell), CD45R (B cell), CD25, Foxp3 (Tregs), CTLA4, or PD-1 antibodies (eBioscience). Apoptosis was assessed using the Annexin V kit (eBioscience) with 7-amino-actinomycin D.

Total Protein Extraction

Spleen tissue was homogenized using PRO-PREP Solution (iNtRON Biotechnology). Lysates were centrifuged at 13 000*g* for 5 minutes at 4°C. Protein concentrations were determined using a Bicinchoninic acid Protein Assay kit (Pierce Biotechnology).

Western Blotting

Protein samples (8–20 μg) were loaded on 6.5%–17.0% polyacrylamide gels, separated by polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes using a Semi-Dry Trans-Blot Cell (Bio-Rad). Membranes were blocked with 5% skim milk in 0.1% Tween-20 in Tris-buffered saline and incubated overnight at 4°C with primary antibodies against fas-associated protein with death domain (FADD), Bim (Cell Signaling), or HO-1 (Santa Cruz). Membranes were incubated with the appropriate secondary antibodies and immunoreactive bands were detected using ECL reagents (iNtRON Biotechnology) according to the manufacturer's instructions. The intensity of each band was determined using TotalLab TL120 software (Nonlinear Dynamics Ltd). All signals were standardized to that of β-actin (Sigma-Aldrich).

Caspase Activity

Caspase-3 and -8 activities were measured using colorimetric peptide substrates. Spleen tissue was homogenized in 25 mM-Tris, 5 mM magnesium chloride, and 1 mM ethylene glycol tetraacetic acid. The homogenate was centrifuged (15 minutes, 40 000*g*), and supernatant was collected for determination of caspase activities according to the manufacturer's instructions.

Cytokine Production

Isolated splenocytes were stimulated with anti-CD3e/CD28 antibodies (eBioscience). Culture medium was harvested 24 hours after incubation, and interferon gamma (IFN-γ), IL-2, IL-4, and IL-10 production was measured using a commercially available enzyme-linked immunosorbent assay kit (BD Biosciences) according to the manufacturer's instructions.

Secondary Pneumonia Infection Model

Pseudomonas aeruginosa (American Type Culture Collection) was counted by spectrophotometry (1×10^7 CFUs). Mice were subjected to CLP using 27-gauge needles, and imipenem was administered intraperitoneally 1 hour post-CLP. At 4 days post-CLP, 20 μL *P. aeruginosa* culture was injected intranasally and mice were monitored (n = 10 per group).

Statistical Analysis

Survival data were used to construct Kaplan-Meier curves and analyzed by the log-rank test. All results are reported as mean ± standard error of the mean (SEM). The significance of differences between groups was examined by 1-way analysis of variance; $P < .05$ was considered statistically significant. The Bonferroni correction was performed for multiple comparisons.

RESULTS

HO-1 Expression and Activity Are Increased in Sepsis

Splenic HO-1 protein expression began to increase 12 hours post-CLP and peaked 48 hours post-CLP, followed by a slight decline 72 hours post-CLP (Figure 1A). HO-1 protein expression also significantly increased 12 hours post-CLP in lung, liver, and thymus, all of which are organs vulnerable to sepsis (Supplementary Figure 1). Splenic HO activity increased significantly 24 hours post-CLP and peaked 48 hours post-CLP, followed by a slight decline 72 hours post-CLP (Figure 1B). This increase was attenuated by ZnPP, a potent HO-1 inhibitor, whereas it was augmented by hemin, a naturally occurring HO-1 substrate, 48 hours post-CLP (Figure 1C).

HO-1 Inhibition Improves Survival and Bacterial Clearance

The survival rate in the CLP group was 86.7% on the first day and decreased to 66.7% on the second day. Survival then stabilized at 46.7% on the fifth day. ZnPP significantly improved the survival rate compared with the CLP group ($P = .0497$), while hemin decreased the survival rate ($P = .0468$; Figure 2A). Bacterial load in the blood, peritoneal cavity, and spleen was significantly increased 48 hours post-CLP. These increases were attenuated by ZnPP, whereas hemin further increased the bacterial load (Figure 2B–D).

HO-1 Decreases Immune Cell Populations

Compared with the sham group, CLP caused a significant loss in total splenocytes, which was attenuated by ZnPP (Figure 3A). CLP also significantly decreased DC, NK-cell, B-cell, and T-cell populations in the spleen to 46.1%, 31.1%, 35.8%, and 59.1%, respectively, of those in the sham group. ZnPP attenuated these decreases in DC, B-cell, and T-cell populations, but not that of NK cells (Figure 3B–E). In the sham group, the percentages of splenic $CD3^+CD4^+$ and $CD3^+CD8^+$T cells were 20.0% ± 1.2% and 13.7% ± 0.5%, respectively. CLP significantly decreased these percentages, which was attenuated by ZnPP (Figure 3G and 3H). CORM-2 augmented decreases in the percentages of splenic $CD3^+CD4^+$ T-cells in sepsis (Supplementary Figure 2).

HO-1 Induces T-Cell Apoptosis

CLP significantly increased apoptotic splenic $CD4^+$ and $CD8^+$T cells by 2.2-fold and 2.6-fold, respectively, compared with the sham group, which were attenuated by ZnPP (Figure 4B and 4C). CORM-2 augmented apoptotic splenic $CD4^+$T cells (Supplementary Figure 3). ZnPP attenuated CLP-induced terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)–positive cells (Figure 4D) and increased FADD and Bim protein expression in the spleen of CLP mice (Figure 4E and 4F). The activities of splenic caspase-8 and -3 were also significantly increased (1.5-fold and 1.8-fold, respectively) in the CLP group compared with the sham group, which were attenuated by ZnPP (Figure 4G and 4H).

HO-1 Induces Cytokine Shift From Th1 to Th2

Splenocytes from 48 hours post-CLP secreted fewer Th1-derived cytokines such as IFN-γ and IL-2 (65.2% and 65.1% of those in the sham group, respectively). ZnPP attenuated these decreases (Figure 5A and 5B). CLP significantly increased Th2-derived

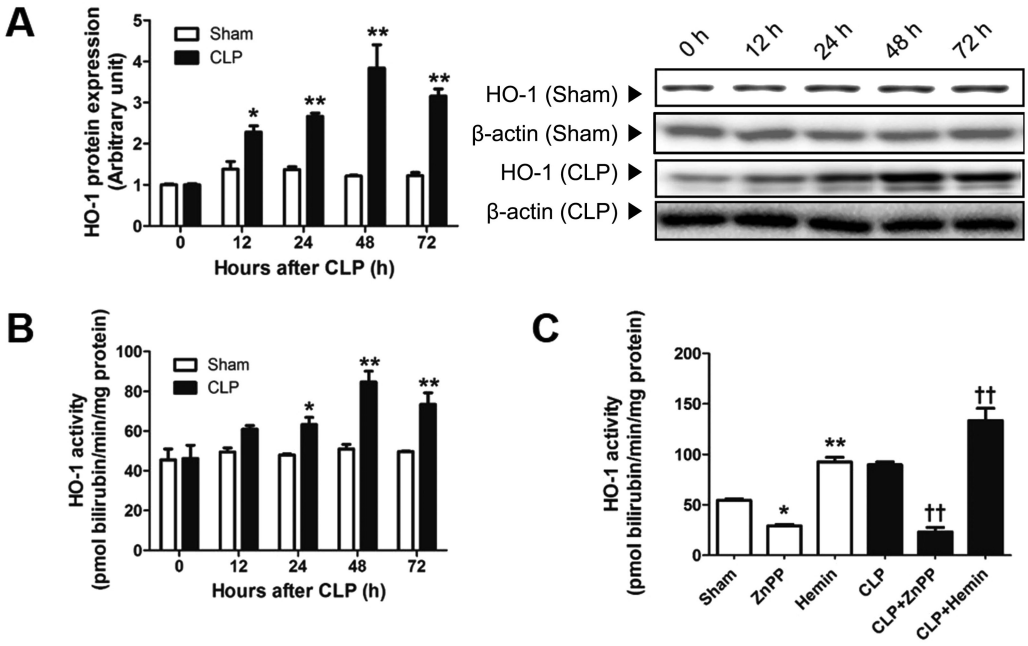

Figure 1. Heme oxygenase 1 (HO-1) protein expression and activity patterns in spleen during sepsis. *A* and *B*, Splenic HO-1 protein expression and activity were measured 0 (immediately), 12, 24, 48, and 72 hours after cecal ligation and puncture (CLP). *C*, Splenic HO activity was measured after administration of zinc protoporphyrin (ZnPP) or hemin at 24 and 36 hours after CLP. Values are presented as mean ± standard error of the mean (n = 6–8 mice per group). $^{*}P < .05$, $^{**}P < .01$ (Bonferroni test; significant differences from sham group). $^{\dagger\dagger}P < .01$ (Bonferroni test; significant difference from CLP group).

cytokines IL-4 and IL-10 production by 1.5- and 2.7-fold relative to the sham group, respectively; these increases were attenuated by ZnPP (Figure 5C and 5D).

HO-1 Increases Treg Population and Function

CLP significantly increased the percentages of splenic $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs by 2.4-fold

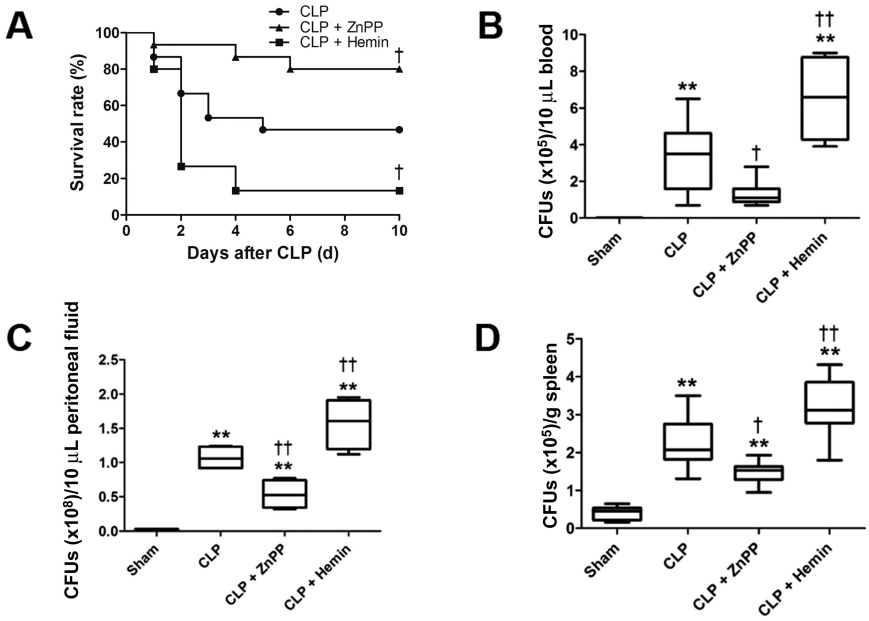

Figure 2. Effect of heme oxygenase 1 on cecal ligation and puncture (CLP)–induced mortality and bacterial clearance. Mice were intraperitoneally administered vehicle, zinc protoporphyrin (ZnPP), or hemin at 24 and 36 hours after CLP (colony-forming units [CFU]). *A*, Animals were monitored 10 days after CLP (n = 15 mice per group). $^{\dagger}P < .05$ (log-rank test; significant difference from CLP). *B–D*, Blood, peritoneal fluid, and spleen tissue were collected 48 hours after CLP and cultured on blood tryptic soy agar plate for 24 hours (n = 6–8 mice per group). Values are presented as mean ± standard error of the mean (n = 6–8 mice per group). $^{**}P < .01$ (Bonferroni test; significant difference from sham group). $^{\dagger}P < .05$, $^{\dagger\dagger}P < .01$ (Bonferroni test; significant differences from CLP group).

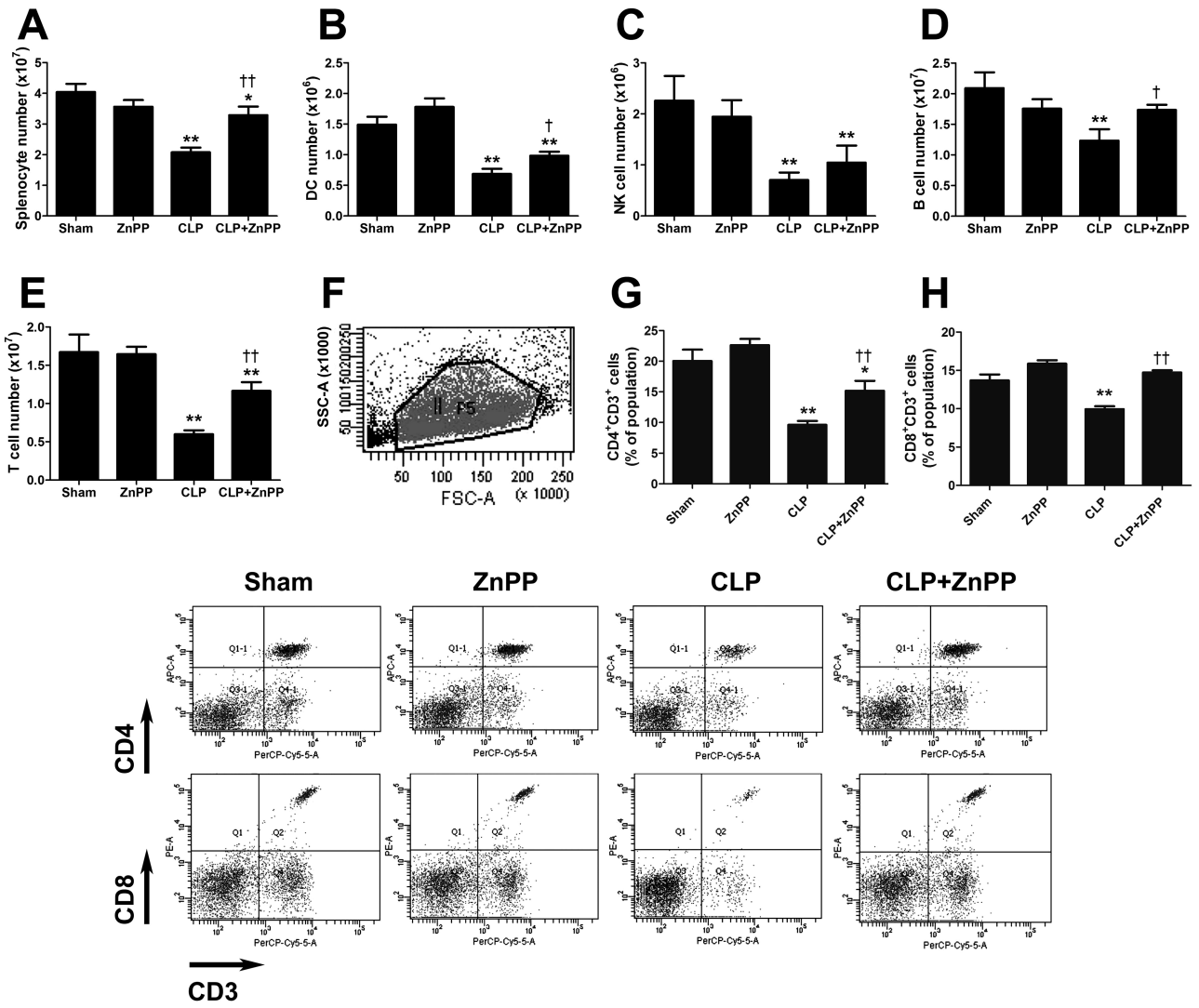

Figure 3. Effect of heme oxygenase 1 on immune cell depletion. Mice were intraperitoneally administered vehicle or zinc protoporphyrin (ZnPP) at 24 and 36 hours after cecal ligation and puncture (CLP). *A*, Splenocytes were isolated and counted 48 hours after CLP. *B–E*, Isolated splenocytes were labeled with fluorescent cell-specific markers for dendritic cells (DC; CD11c), natural killer cells (NK; CD49b), B cells (CD45R), and T cells (CD3) 48 hours after CLP. *F*, Gating strategy of lymphocytes for (*G*) and (*H*) depending on the forward scatter (FSC) and side scatter (SSC). *G* and *H*, The population of $CD4^+$ and $CD8^+$ cells in $CD3^+$ cells were measured using fluorescent cell-specific markers 48 hours after CLP. Values are presented as mean ± standard error of the mean (n = 6–8 mice per group). $^*P < .05$, $^{**}P < .01$ (Bonferroni test; significant differences from sham group). $^{\dagger}P < .05$, $^{\dagger\dagger}P < .01$ (Bonferroni test; significant differences from CLP group).

and 2.3-fold, respectively, compared with the sham group. These increases were attenuated by ZnPP (Figure 6A and 6C). The percentages of splenic $CD4^+CD25^+CTLA4^+$ and $CD4^+PD1^+$ T cells increased significantly by 4.1-fold and 4.3-fold, respectively, compared with the sham group 48 hours post-CLP. ZnPP attenuated these increases (Figure 6D and 6E).

HO-1 Inhibition Improves Survival in Secondary Pneumonia Infection

To increase survival from initial CLP induction, we intraperitoneally injected imipenem to septic mice 1 hour after CLP. The survival rate of *P. aeruginosa*–infected mice was 90% on the first day, 80% on the fifth day, and stabilized at 60% on the sixth day post-CLP. ZnPP improved these survival rates ($P = .0404$; Figure 7).

DISCUSSION

Postmortem studies of patients who died of sepsis have highlighted key immunological defects, including immune cell depletion and dysfunction. Pronounced anti-inflammatory response leads to impairment of host immune function, known as immune paralysis, which perpetuates failure to clear bacterial burden and recover organ function. Of note, patients with sepsis who are treated with immune-enhancing drugs such as granulocyte-macrophage colony-stimulating factor have fewer ventilator days and shorter ICU and hospital stays [21]. However, the immunosuppressive mechanisms of sepsis are still poorly understood. HO-1 has been reported to show cytoprotective effects associated with its

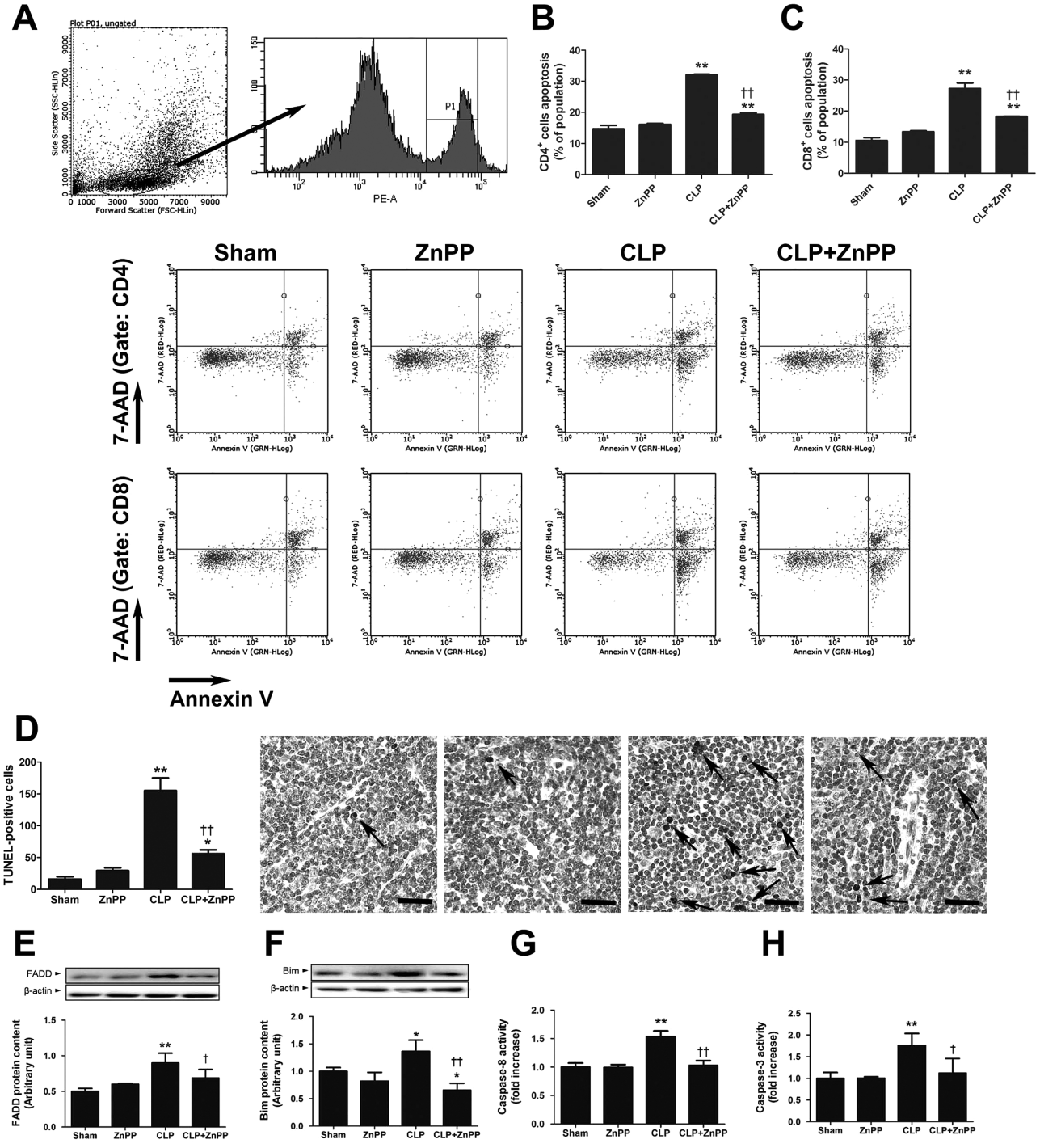

Figure 4. Effect of heme oxygenase 1 on T-lymphocyte apoptosis. Mice were intraperitoneally administered vehicle or zinc protoporphyrin (ZnPP) at 24 and 36 hours after cecal ligation and puncture (CLP). *A*, Gating strategy of $CD4^+$ and $CD8^+$ T cells for (*B*) and (*C*). *B–C*, The percentage of Annexin V–positive and 7-amino-actinomycin D (7-AAD)–negative cells were measured using fluorescent cell-specific markers 48 hours after CLP. *D*, Spleen tissues were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) 48 hours after CLP. Typical images were chosen from each experimental group (original magnification ×200; scale bar = 200 μm) and the arrow indicates TUNEL-positive cell. *E* and *F*, Fas-associated protein with death domain (FADD) and Bim protein expression were measured by Western blot 48 hours after CLP using indicated antibodies. The bands were standardized to those of β-actin. *G* and *H*, The activities of caspase-8 and caspase-3 were measured 48 hours after CLP. Values are presented as mean ± standard error of the mean (n = 6–8 mice per group). $^{*}P < .05$, $^{**}P < .01$ (Bonferroni test; significant differences from sham group). $^{\dagger}P < .05$, $^{\dagger\dagger}P < .01$ (Bonferroni test; significant differences from CLP group).

anti-inflammatory properties in diverse inflammatory diseases including early-phase sepsis [22]. In contrast, HO-1 was recently reported to have a pathogenic effect by propagating chronic inflammation and inducing an immunosuppressive condition. In septic patients, expression of intracellular HO-1 is coincident with the degree of monocyte deactivation

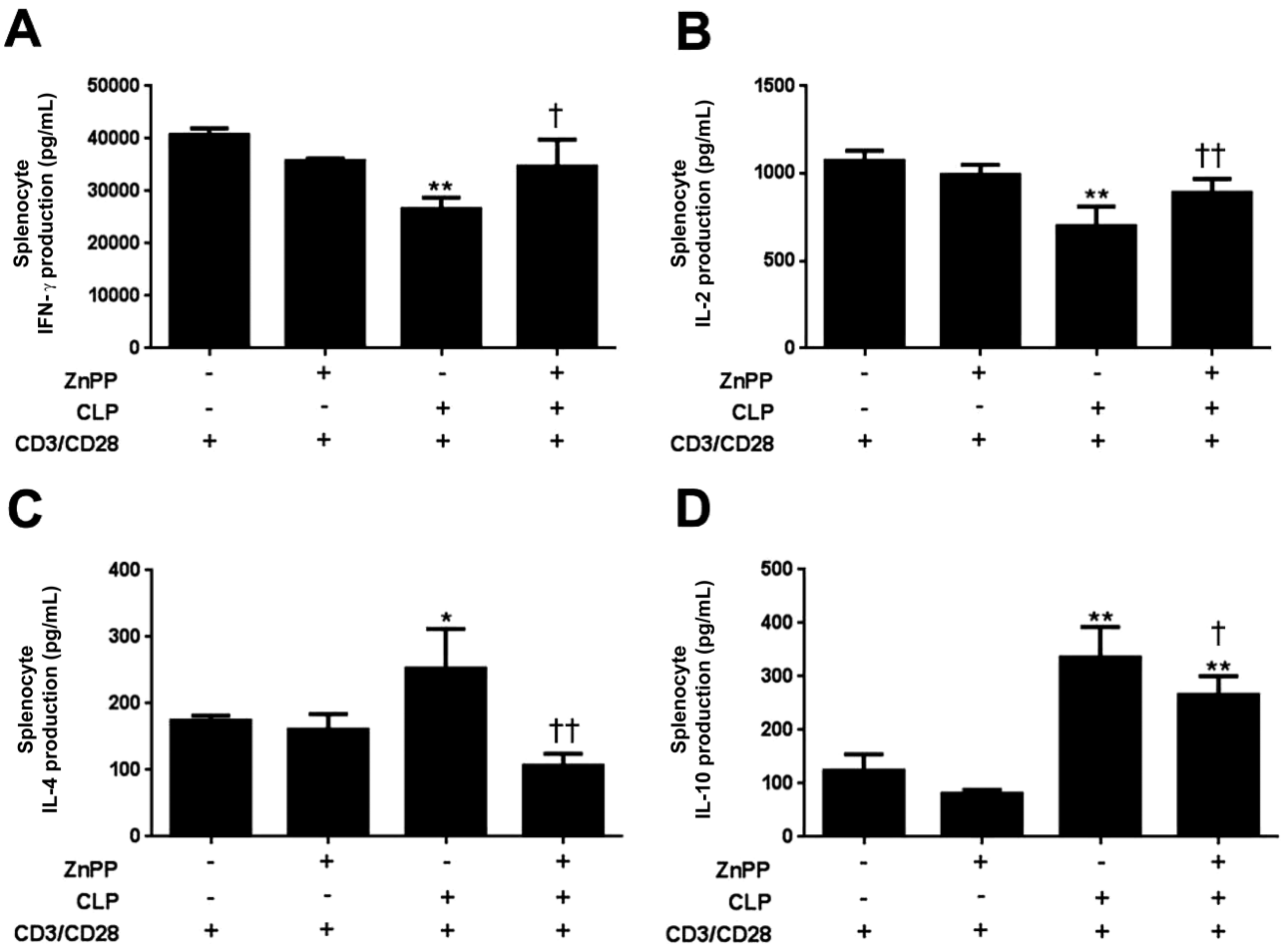

Figure 5. Effect of heme oxygenase 1 on splenic interferon gamma (IFN-γ; *A*), interleukin (IL)-2 (*B*), IL-4 (*C*), and IL-10 (*D*) production. Mice were intraperitoneally administered vehicle or zinc protoporphyrin (ZnPP) at 24 and 36 hours after cecal ligation and puncture (CLP). Splenocytes were isolated 48 hours after CLP and stimulated with CD3/CD28. Cytokine production was measured after 24 hours of incubation with enzyme-linked immunosorbent assay. Values are presented as mean ± standard error of the mean (n = 6–8 mice per group). $^{*}P < .05$, $^{**}P < .01$ (log-rank test; significant difference from sham group). $^{\dagger}P < .05$, $^{\dagger\dagger}P < .01$ (Bonferroni test; significant differences from CLP group).

[23]. Moreover, HO-1 inhibition was shown to increase the migration of neutrophils to inflammatory sites, attenuate lung damage, and therefore reduce mortality rate in *Klebsiella pneumoniae*–induced sepsis [24].

In the present study, we first examined changes in HO-1 protein expression in diverse lymphoid organs of septic mice during late phase of sepsis [25]. In the spleen, a lymphoid organ where lymphocytes mature and transit, HO-1 protein expression increased significantly at 12, 24, 48, and 72 hours post-CLP. In parallel with HO-1 protein expression, HO activity in the spleen markedly increased during the late phase of sepsis (Figure 1). In the liver and lung, which are organs that play vital and dominant roles in immune responses to infection, HO-1 protein expression also increased at 12, 24, and 48 hours post-CLP (Supplementary Figure 1). To investigate the role of overexpressed HO-1 in septic injury, we injected ZnPP, a potent HO-1 inhibitor, or hemin, a naturally occurring HO-1 substrate, into septic mice at 24 and 36 hours post-CLP. Mortality in septic mice was attenuated by delayed administration of ZnPP, but worsened by hemin (Figure 2). This result is similar to that reported by Melley et al [26]; those authors reported that excessive induction of HO-1 was associated with increased mortality of patients in ICUs. Furthermore, sepsis resulted in failure to eradicate bacterial pathogens from the spleen, blood, and peritoneal fluid, while ZnPP administration decreased bacterial loads. Collectively, our findings suggest that overexpression of HO-1 reduces bacterial clearance and contributes to mortality during late-phase sepsis.

Immune cell depletion is an important characteristic of sepsis-induced immunosuppression. Septic patients are characterized by significant losses of T cells, B cells, and DCs in the spleen [27]. Among immune cells, T cells are vulnerable to cell death in sepsis, which is detrimental to patient survival [28]. HO-1 was shown to suppress the G_0/G_1 cell cycle of $CD4^+$T cells under anti-CD3/CD28 stimulation [29], suggesting a

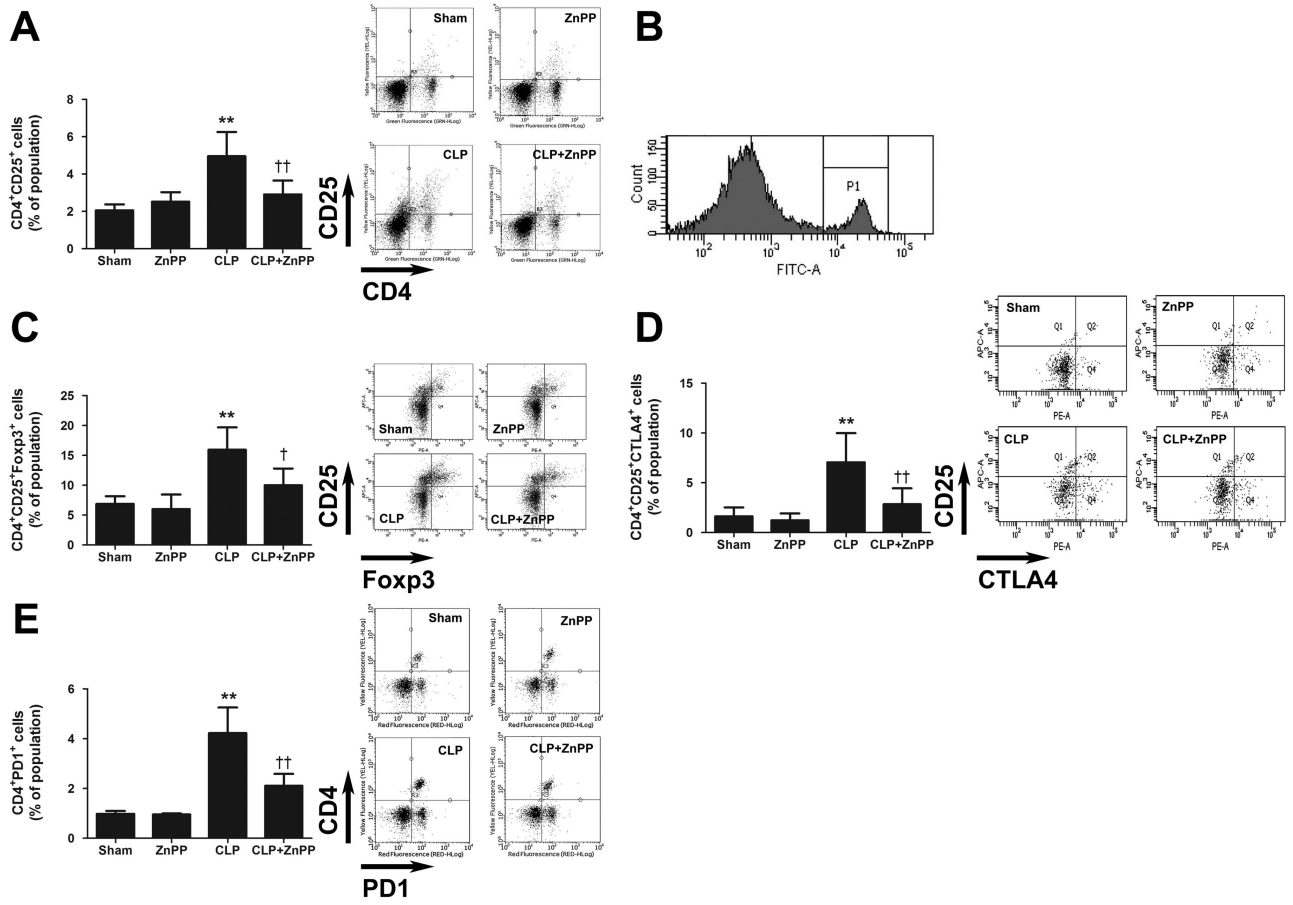

Figure 6. Effect of heme oxygenase 1 on regulatory T-cell (Treg) population and function. Mice were intraperitoneally administered vehicle or zinc protoporphyrin (ZnPP) at 24 and 36 hours after cecal ligation and puncture (CLP). *A,* The population of $CD4^+CD25^+$ cells was measured using fluorescent cell-specific markers 48 hours after CLP. *B,* Gating strategy of $CD4^+$ T cells for (*C–E*). *C–E,* The populations of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+CTLA4^+$, and $CD4^+PD1^+$ cells were measured using fluorescent cell-specific markers 48 hours after CLP. Values are presented as mean ± standard error of the mean (n = 6–8 mice per group). $^{**}P < .01$ (Bonferroni test; significant difference from sham group). $^{\dagger}P < .05$, $^{\dagger\dagger}P < .01$ (Bonferroni test; significant differences from CLP group).

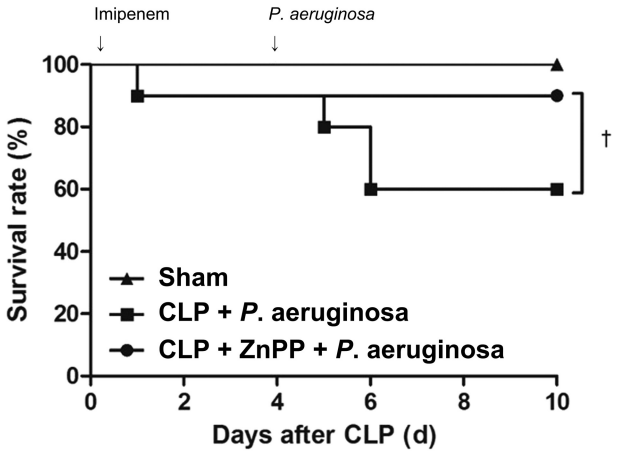

Figure 7. Effect of heme oxygenase 1 on secondary infection with *Pseudomonas aeruginosa.* Mice were intraperitoneally administered vehicle or zinc protoporphyrin (ZnPP) at 24 and 36 hours after cecal ligation and puncture (CLP). Mice were intraperitoneally administered imipenem at 1 hour after CLP and intranasally injected *P. aeruginosa* at 4 days after CLP. Animals were monitored 10 days after CLP (n = 10 per group). $^{\dagger}P < .05$ (log-rank test; significant difference from CLP).

possible linkage between HO-1 and T-cell proliferation. In the present study, CLP markedly decreased $CD4^+$ and $CD8^+$ T-cell populations, whereas ZnPP attenuated these decreases (Figure 3). HO-1 mediates its physiological functions through its byproducts including CO and biliverdin. Among these, CO has been shown to exert an anti-proliferative effect [30]. Under CD3/CD28 stimulation, CO inhibited the Jurkat T-cell population by suppressing production of proliferative factor IL-2 [29]. Furthermore, CO exposure decreased proinflammatory cytokine production and T-cell populations in the kidney of a lupus mouse model [31]. To determine if CO contributes to CLP-induced T-cell depletion, we administered the CO donor CORM-2 to septic mice. CORM-2 augmented the CLP-induced decrease in the $CD4^+$T-cell population, indicating that HO-1–derived CO mediates $CD4^+$T-cell depletion during the late phase of sepsis (Figure 3).

Apoptosis is responsible for depletion of immune cells in sepsis. Extensive apoptotic cell death occurs in the spleen of patients dying of sepsis, and the degree of apoptosis is correlated

with sepsis severity [32]. Apoptosis can proceed by 2 distinct pathways: a death-receptor-initiated extrinsic pathway and a mitochondria-initiated intrinsic pathway. FADD mediates the extrinsic pathway by sequentially activating caspase-8 and caspase-3. Intrinsic apoptosis is activated based on the balance of proapoptotic and antiapoptotic proteins. Specifically, during sepsis, FADD knockout mice showed decreased lymphocyte apoptosis, which is associated with reduced caspase-8 activity [32]. In septic patients, a decrease in Bim, a proapoptotic protein, were observed in circulating T cells [33]. HO-1 inhibited TNF-mediated apoptosis through the activation of p38 MAPK in cardiac-transplanted endothelial cells [34]. In contrast, HO-1 increased basophil apoptosis in allergic airway inflammation via CO production [17]. Moreover, a low concentration of CO promoted FAS-induced apoptosis in Jurkat T cells, which was associated with ERK activation [35]. These discrepant findings may be explained by cell type, the degree of HO-1 expression, and microenvironmental signals. Here, annexin V staining revealed a significant increase in apoptosis of $CD4^+$ and $CD8^+$T cells in septic mice, and this was attenuated by ZnPP. This result was strongly supported by histological TUNEL staining of spleen tissue. Of note, ZnPP suppressed both extrinsic and intrinsic apoptotic pathways, as demonstrated by decreased FADD protein levels and caspase-8 and caspase-3 activities, and decreased Bim protein expression, respectively (Figure 4). Overall, our results indicate that HO-1 induces T-cell apoptosis via extrinsic and intrinsic pathways.

Inappropriate T-cell differentiation induces T-cell anergy and exacerbates immunosuppression in sepsis. T-helper cells play diverse physiological roles through differentiation into proinflammatory Th1 and anti-inflammatory Th2 subsets. An appropriate Th1/Th2 balance is crucial for host defense against microbial infections and resolution of inflammation. Th1 polarization is associated with several inflammatory conditions, including inflammatory bowel disease [36], while Th2 polarization has been reported to play a role in allergic diseases, such as asthma [37]. Interestingly, Boomer et al [38] found little production of the Th1-derived cytokines IFN-γ and TNF-α in spleen tissue isolated from patients with sepsis, which strongly suggests a state of impaired Th1 cell function. This result was reinforced by the findings of another study that expression of T-bet, a transcription factor of Th1, was decreased in patients with sepsis [39]. In contrast, the sepsis-induced Th2 population shows increased production of IL-10 [40]. Recently, adenoviral-mediated HO-1 upregulation induced a Th2 shift and protected fetuses from rejection [41]. However, little is known about the effect of HO-1 on Th1/Th2 balance in sepsis. In the spleen of septic mice, production of the Th1 cytokines IFN-γ and IL-2 was suppressed, and production of the Th2 cytokines IL-4 and IL-10 was increased (Figure 5). ZnPP attenuated these changes, suggesting that HO-1 promotes sepsis-induced Th2 polarization.

$CD4^+CD25^+$ Tregs abrogate the excessive functions of effector T cells through cytokine deprivation, apoptosis, and cytolysis. Foxp3, a master transcriptional regulator for Tregs, is essential for Treg proliferation and function [42]. In septic mice, the number of $CD4^+CD25^+Foxp3^+$ Treg cells significantly increased in both the spleen and blood, which contributed to T-cell anergy [9]. Moreover, Treg inhibition with anti-CD25 antibody improved the proliferative capacity of effector T cells and Th1 cytokine production in splenocytes from septic mice [9]. The coinhibitory molecules CTLA-4 and PD-1 are essential for Treg function. CTLA-4 is expressed only on Tregs, and its binding with ligand on effector T cells strongly inhibits differentiation and function of effector T cells [43]. PD-1, a member of the B7/CD28 family, inhibits TNF-α and IL-2 production, and induces cell death through binding to its ligand, PD-L1. Recently, increased CTLA-4 and PD-1 expression was observed in splenic T cells from patients with sepsis [44, 45]. CTLA-4 has received considerable attention as a biomarker of immunosuppression in sepsis [46]. Blocking CTLA-4 increased IFN-γ production and improved survival rate in *Candida*-infected mice [47]. HO-1 promoted Treg function by enhancing foxp3 expression in allergic airway inflammation [48]. HO-1 expression markedly increased as tumor grade increased, and this increase was directly correlated with the Treg population in brains from glioma patients [16]. However, there is no information about the effect of HO-1 on coinhibitory molecules CTLA4 and PD-1. In the present study, CLP significantly increased the splenic $CD4^+CD25^+$ Treg population and foxp3 expression in Tregs. Moreover, Treg surface expression of CTLA-4 and $CD4^+$ T-cell surface expression of PD-1 were increased in CLP mice. ZnPP attenuated these increases (Figure 6). Collectively, our data indicate that overexpressed HO-1 increases the Treg population and its function in sepsis.

Most patients with sepsis are exposed to nosocomial infections during prolonged periods of hospitalization. Despite advances in antibiotics, secondary infection remains a frequent cause of deaths in sepsis. *Pseudomonas aeruginosa* is a bacterial pathogen, which is frequently found in patients with sepsis [49]. The "2-hit" model of sepsis provides a more protracted host response and has proved useful in examination of therapies targeted at reversing immunosuppression [50]. To investigate the clinical aspects of nosocomial infection during sepsis, we generated a CLP model followed by pneumonia. Mice were injected with imipenem to comply with the mainstay of sepsis. ZnPP administration before *P. aeruginosa* challenge significantly improved survival in septic mice (Figure 7). These results suggest that HO-1 increases susceptibility to secondary infection after septic insult.

In conclusion, we demonstrated that overexpression of HO-1 decreased bacterial clearance and increased mortality in late-phase sepsis. HO-1 depleted the immune cell population by inducing apoptosis, and promoted a Th1 to Th2 cytokine shift.

Moreover, HO-1 enhanced Treg population and function, as indicated by increased expression of inhibitory costimulatory molecules. In conclusion, overexpressed HO-1 contributes to sepsis-induced immunosuppression by inducing a Th1 to Th2 cytokine shift and enhancing Treg function. Thus, regulation of excessive HO-1 expression should be considered in the clinical setting, particularly for the treatment of immunodeficiency disorders.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Financial support. This work was supported by the Mid-Career Researcher Program through an NRF grant funded by the Ministry of Education, Science, and Technology (MEST) in Korea (NRF-2016R1A2B4009880). S.-J. K. received a Global PhD Fellowship (NRF-2013H1A2A1034472) from the NRF (MEST).

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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