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*J Immunol* 2010; 185:3677-3684; Prepublished online 9 August 2010; doi: 10.4049/jimmunol.0904020

http://www.jimmunol.org/content/185/6/3677

Supplementary Material  http://www.jimmunol.org/content/suppl/2010/08/09/jimmunol.0904020.DC1

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
B7-H3 Augments the Inflammatory Response and Is Associated with Human Sepsis

Guangbo Zhang,*†,1 Jian Wang,‡,1 Justin Kelly,§ Guohao Gu,† Jianquan Hou,* Yinghui Zhou,‡ H. Paul Redmond,§ Jiang Huai Wang,§ and Xueguang Zhang*,†

B7-H3, a new member of the B7 superfamily, acts as both a T cell costimulator and coinhibitor, and thus plays a key role in the regulation of T cell-mediated immune responses. However, it is unclear whether B7-H3 is involved in the innate immune monocyte/macrophage-mediated inflammatory response. In this paper, we show that, although B7-H3 alone failed to stimulate proinflammatory cytokine release from murine macrophages, it strongly augmented both LPS- and bacterial lipoprotein-induced NF-κB activation and inflammatory response. This occurred in both a TLR4- and TLR2-dependent manner. Blockage of B7-H3 in vivo attenuated LPS-induced proinflammatory cytokine release and endotoxic shock-related lethality. Furthermore, we found that patients diagnosed with sepsis, in contrast to healthy individuals, exhibited significant levels of raised plasma soluble B7-H3 (sB7-H3) and that this level correlated with the clinical outcome and levels of plasma TNF-α and IL-6. In addition, a putative receptor for B7-H3 was detected on monocytes and peritoneal macrophages from septic patients but not on monocytes from healthy donors. Stimulation of human monocytes with LPS and inflammatory cytokines led to a substantial release of sB7-H3. Taken together, our data indicate that significantly elevated plasma sB7-H3 in septic patients may predict a poor outcome. Furthermore, we demonstrate that B7-H3 functions as a costimulator of innate immunity by augmenting proinflammatory cytokine release from bacterial cell wall product-stimulated monocytes/macrophages and may contribute positively to the development of sepsis. The Journal of Immunology, 2010, 185: 3677–3684.

A common and serious consequence of overwhelming bacterial infection is the development of sepsis, septic shock, and their sequelae. Despite significant advances in our understanding of the molecular and genetic basis of sepsis and its associated immune response, sepsis remains a major cause of death in intensive care units worldwide (1, 2). The innate immune system, via TLRs in sensing pathogen-associated molecular pattern and initiating the inflammatory response, plays a key role in host defense against microbial infection and sepsis (3, 4). However, a persistent or uncontrolled activation of the TLR-mediated intracellular signal transduction pathway, characterized by the excessive release of proinflammatory cytokines, including TNF-α and IL-6, may lead to septic shock syndrome (5).

The B7 superfamily of costimulatory proteins plays an important role in the regulation of Ag-specific T cell-mediated immune responses (6, 7). B7-H3, a newly discovered member of the B7 superfamily, has been identified in both humans and mice by sharing ~88% amino acid sequence identity (8, 9). B7-H3 is not expressed in significant amounts on freshly isolated human lymphocytes but is induced in human monocytes/macrophages and dendritic cells upon inflammatory cytokine stimulation (9–12). Accumulated evidence supports the notion that B7-H3 has a contrasting role in regulating T cell-mediated immune responses by functioning as both a T cell costimulator and coinhibitor. Human B7-H3 was originally described to augment Ag-specific TCR-mediated T cell proliferation, enhance the induction of cytotoxic T cells, and selectively stimulate IFN-γ production (9). Additional studies further support a costimulatory function for this molecule (13–16). In contrast, other groups have reported a negative regulatory function for both human and mouse B7-H3 in T cell-mediated immune responses, including an inhibitory effect of B7-H3 on anti-CD3 mAb-induced both T cell proliferation and cytokine production (11, 12, 17). Furthermore, B7-H3−deficient mice exhibited an enhanced Th1-mediated hypersensitivity and developed an early onset of experimental autoimmune encephalomyelitis (11, 12). Although the definitive counterreceptor(s) for B7-H3 has not yet been identified, previous work revealed that B7-H3 binds to a putative receptor expressed on PHA- or anti-CD3 mAb-activated T cells (8, 9). A recent study further demonstrated that the triggering receptor expressed on myeloid cells-like transcript 2 is a costimulatory receptor for B7-H3 (18).

Despite the extensive research work on the immunologic function of B7-H3 during the past decade, the impact of this molecule on innate immune response remains largely unknown. Our recent work has shown that a soluble form of B7-H3, released from monocytes, dendritic cells, and activated T cells, is detectable in the circulation of healthy humans (19). In this paper, we report that significantly elevated levels of soluble B7-H3 (sB7-H3) in the circulation are observed in patients diagnosed with sepsis, and levels of sB7-H3 in septic patients correlate with their clinical outcomes and circulating
proinflammatory cytokines TNF-α and IL-6. Consistent with this finding, we further show an inflammation-based costimulatory action of B7-H3 by demonstrating that B7-H3 amplifies endotoxin/LPS- and bacterial lipoprotein (BLP)-stimulated NF-κB activation and proinflammatory cytokine production in monocytes/macrophages, which occurs via both TLR4- and TLR2-dependent mechanisms. Furthermore, B7-H3 may function as a positive contributor during the development of sepsis, as administration of anti-B7-H3 neutralizing mAb attenuates proinflammatory cytokine release and improves survival in mice challenged with lethal LPS.

Materials and Methods

Reagents and Abs

Human B7-H3-hIg (hB7-H3Ig), hB7-H3, murine B7-H3 (muB7-H3), recombinant human TNF-α (rhTNF-α), and INF-γ were obtained from R&D Systems ( Minneapolis, MN). LPS (Escherichia coli O55:B5) and BLP (a synthetic bacterial lipopeptide Pam3-Cys-Ser-Lys4-OH derived from the immunologically active NH2 terminus of BLPs) were purchased from Sigma-Aldrich (St. Louis, MO) and EMC Microcollections (Tübingen, Germany), respectively. Both BLP and muB7-H3 were endotoxin-free as confirmed by the Limulus amebocyte lysate assay (Charles River Endosafe, Charleston, SC). FITC-conjugated anti-CD14 mAb and streptavidin were purchased from Immunotech (Marseilles, France) and Vector Laboratories (Burlingame, CA), respectively. The anti–hB7-H3 mAbs 2E6, 4H7, and 21D4 were identified and developed in our laboratory (19). The anti–B7-H3 neutralizing mAb (MH35) was a gift from Dr. M. Azuma (Tokyo Medical and Dental University, Tokyo, Japan) and the control rat IgG was from MP Biomedicals (Solon, OH). The expression vectors pcDNA3-FLAG–tagged TLR2 and pcDNA3-YEP-tagged TLR4 were provided by Dr. A. Bowie (Trinity College Dublin, Dublin, Ireland). All plasmid DNA was isolated and purified with Qiagen Endo-free Plasmid Maxi Kit (Chatsworth, CA). All culture medium and reagents used for cell cultures were purchased from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals, unless indicated, were from Sigma-Aldrich.

Patients

This study was approved by the ethics committee of Soochow University (Suzhou, China) for clinical investigation and written informed consent was obtained from patients or their relatives before enrollment. All experiments and procedures involving human subjects were conducted according to the principles expressed in the Declaration of Helsinki. A total of 27 patients who were admitted into the intensive care unit (First Affiliated Hospital, Soochow University) and diagnosed with sepsis between January 2006 and December 2008 were enrolled into this study. Sepsis was judged according to the diagnostic criteria of the American College of Chest Physicians/Society of Critical Care Medicine (20). Patient demographic characteristics are provided in Table 1. After 7 d of culture, the adherent cells were identified as the bone marrow–derived macrophages (BMMs), based on their positive F4/80 staining by FACScan analysis with an anti-F4/80 Ab (Serotech, Oxford, U.K.). The purity of BMMs was >95% as identified by anti-F4/80 staining.

Bone marrow cells were isolated from the femurs of C3H/HeN mice (6–8 wk old) and cultured in DMEM containing 20% heat-inactivated FCS, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and supplemented with 10 ng/ml recombinant mouse M-CSF (R&D Systems) as described previously (20). After 7 d of culture, the adherent cells were identified as the bone marrow–derived macrophages (BMMs), based on their positive F4/80 staining by FACScan analysis with an anti-F4/80 Ab (Serotech, Oxford, U.K.). The purity of BMMs was >95%. BMMs were then harvested and used for in vitro experiments.

HEK-TrlR4-M2D and HEK-trlR2 cells, stably transfected with a human TLR4-M2D or TLR2 cDNA construct, were a gift from Dr. E.A. Kurt-Jones (University of Massachusetts Medical School, Worcester, MA) and maintained in DMEM supplemented with 10% FCS and G418 (0.5 mg/ml). HEK293 and human THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM or RPMI 1640, supplemented with 10% FCS, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml) and gluconate (2 mM).

Cytokine and sB7-H3 measurement

Purified human monocytes plated in 24-well plates (BD Falcon, Lincoln Park, NJ) (1 × 105 cells/well) were stimulated with LPS (1 μg/ml), rhTNF-α (25 ng/ml), or IFN-γ (1000 U/ml) for 48 h. Cell-free supernatants were collected and stored at −80°C until analysis for sB7-H3. An ELISA kit for sB7-H3 detection was used with anti–B7-H3 mAbs 4H7 as a capture Ab and biotinylated 21D4 as a detecting Ab, which was previously developed in our laboratory (19). In the current study, we modified the kit by using biotinylated 2E6 instead of biotinylated 21D4 as the detecting Ab and improved the sensitivity of B7-H3 detecting limit from a previous 27 to 3.3 pg/ml. Concentrations of sB7-H3 either in the cell culture supernatants or in plasma from septic patients and healthy donors were assessed by the ELISA kit as described above.

Murine BMMs plated in 96-well plates (BD Falcon) (2 × 105 cells/well) were stimulated with LPS (100 ng/ml), BLP (100 ng/ml), and muB7-H3 (1–5 μg/ml) alone or a combination of either LPS or BLP with muB7-H3 for 24 h. Cell-free supernatants were collected and stored at −80°C until analysis. Concentrations of murine TNF-α, IL-1β, IL-6, and IL-12/70 in the supernatants were assessed by MSD 96-well Cytokine Multiarray (Meso Scale Discovery, Gaithersburg, MD). Human TNF-α, IL-6, IL-17, and IFN-γ in plasma from septic patients were assessed by the ELISA kits (R&D Systems).

FACScan analysis of a putative receptor for sB7-H3

PBMCs were isolated from septic patients (n = 5) or healthy donors (n = 5), and peritoneal cells were collected from intraperitoneal peritoneal lavage of septic patients with acute peritonitis (n = 3) and nonseptic patients undergoing elective laparotomy (n = 3). After incubation with human AB serum (10 μI/105 cells) at 4°C for 30 min to block the FcR, cells were stained with biotinylated hB7-H3Ig (100 ng/ml) or biotinylated human IgG (100 ng/ml) as the control, followed by dual-staining with PE-conjugated streptavidin and FITC-conjugated anti-CD14 mAb. FACScan analysis was performed from at least 10,000 events to detect surface expression of a putative B7-H3 receptor by measuring the mean fluorescence intensity (MFI) of biotinylated hB7-H3Ig staining on human peripheral monocytes (CD14-positive cells) and peritoneal macrophages (CD14-positive cells) using a FACScanto flow cytometer and Diva software (BD Biosciences, San Jose, CA).

Transfection transience

HEK-trlR4-M2D, HEK-trlR2, and HEK293 cells plated in 24-well plates (BD Falcon) (1 × 105 cells/well) were transfected with 400 ng NF-κB-driven firefly luciferase plasmid (pNF-κB-Luc) (BD Clontech, Mountain View, CA) and 10 ng CMV promoter-driven Renilla luciferase plasmid (pRL-CMV) (Promega, Madison, WI), along with the plasmid encoding either YFP-tagged TLR4 (40–400 ng) or Flag-tagged TLR2 (20–200 ng) for 16 h. In all cases, the amount of DNA transfected was kept constant by the addition of various amounts of the appropriate empty vector plasmid. After transfection, cells were left untreated or stimulated with hB7-H3 (2.5 μg/ml) for 6 h. HEK-trlR4-M2D and HEK-trlR2 cells were transfected with the NF-κB promoter luciferase plasmid for 16 h and further stimulated with either LPS (100 ng/ml) and BLP (100 ng/ml) alone or in combination with hB7-H3 (1–5 μg/ml) for 6 h. Luciferase activity was determined using the dual-luciferase reporter assay system (Promega). Transfection efficiency was normalized in all experiments with simultaneously measured Renilla luciferase activities.

LPS-induced septic model

Pyrogen-free, 8- to 10-wk-old male C3HHeN mice purchased from Harlan (Oxon, U.K.) were maintained in the University Biological Services Unit, University College Cork (Cork, Ireland). Mice were housed in barrier cages.

Table 1. Clinical characteristics of the sepsis population

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>47 ± 19 (25–82)</th>
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</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
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<tr>
<td>Primary site of infection (n)</td>
<td>Lung 13</td>
</tr>
<tr>
<td></td>
<td>Abdomen 5</td>
</tr>
<tr>
<td></td>
<td>Urinary tract 4</td>
</tr>
<tr>
<td></td>
<td>Other 2</td>
</tr>
<tr>
<td></td>
<td>Unknown 3</td>
</tr>
<tr>
<td>Positive blood cultures (n)</td>
<td>Gram-negative 12</td>
</tr>
<tr>
<td></td>
<td>Gram-positive 15</td>
</tr>
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</table>

Data are mean ± SD. n = 27.
under controlled environmental conditions (12/12-h light/dark cycle, 55% humidity, 23˚C) and had free access to standard laboratory chow and water. Animals were fasted 12 h before experiments and allowed water ad libitum. All animal procedures were conducted in the University Biological Services Unit under a license from the Department of Health (Republic of Ireland). Sepsis was induced in mice by i.p. injection of 35 mg/kg LPS (Escherichia coli O55:B5). Mice received i.p. injection of 250 mg per mouse of anti–B7-H3 mAb (MIH35) or control rat IgG 2 h before LPS injection. Blood samples were collected at 90 min and 2 h postseptic challenge, and serum TNF-α and IL-6 were assessed by cytometric bead array (BD Biosciences). Survival was monitored for at least 10 d.

Statistical analysis
All data are expressed as mean ± SD. Statistical analysis was performed by using the Student t test and ANOVA. Nonnormally distributed values, as assessed by the Kolmogorov-Smirnov test, were analyzed by using the Mann-Whitney U test. Correlations between plasma sB7H3 and plasma TNF-α, IL-6, IL-17, or IFN-γ were assessed by using the Spearman test. The p values <0.05 were considered statistically significant.

Results
Elevated plasma sB7-H3 in septic patients and its correlation with survival and inflammatory cytokines
Given our previous observation of sB7-H3 in both serum and plasma samples from healthy donors (19), we measured sB7-H3 levels in the plasma from 27 patients diagnosed with sepsis. The clinical characteristics of these 27 patients are summarized in Table I. The levels of plasma sB7-H3 in the septic patients (8.37 ± 4.49 ng/ml) were significantly higher than those in healthy controls (2.99 ± 0.98 ng/ml) (p < 0.001) (Fig. 1A). To investigate the potential clinical significance of sB7-H3 in sepsis, we compared plasma sB7-H3 levels between the survivors and nonsurvivors of the 27 patients with sepsis. As shown in Fig. 1B, the levels of sB7-H3 in 8 nonsurvivors of septic patients (12 ± 3.6 ng/ml) were apparently higher than those in 19 survivors (6.85 ± 3.98 ng/ml) (p = 0.004), indicating that elevated plasma sB7-H3 in patients with sepsis may predict a poor outcome.

Having considered the potential contribution of inflammatory cytokines in the development of sepsis, we sought to clarify whether there were any correlations between plasma sB7-H3 and the levels of plasma TNF-α, IL-6, IL-17, or IFN-γ in patients with sepsis. We found that plasma sB7-H3 strongly correlated with the levels of plasma TNF-α (R² = 0.64; p < 0.001) (Fig. 2A) and IL-6 (R² = 0.45; p = 0.02) (Fig. 2B). However, no correlation was found between plasma sB7-H3 and IL-17 (R² = 0.27; p = 0.18) (Fig. 2C) or between plasma sB7-H3 and IFN-γ (R² = −0.15; p = 0.44) (Fig. 2D). Taken together, these data suggest that sB7-H3 is strongly associated with sepsis and could serve as an important prognostic indicator for sepsis.

Endotoxin and inflammatory cytokine stimulated sB7-H3 release from human monocytes
Our previous work has shown that sB7-H3 is released from membrane B7-H3 on human monocytes, dendritic cells, and activated T cells via matrix metalloproteinases (MMPs) (19). To further elucidate the underlying mechanisms responsible for the signifi-

![FIGURE 1.](http://www.jimmunol.org/)

Elevated levels of sB7-H3 in patients diagnosed with sepsis and its correlation with outcome in sepsis. Concentrations of sB7-H3 in plasma isolated from patients with sepsis or healthy individuals were assessed by ELISA. A, Plasma sB7-H3 levels were compared in patients with sepsis (n = 27) and healthy controls (n = 50); p < 0.001. B, Plasma sB7-H3 levels in nonsurviving patients (n = 8) compared with surviving patients (n = 19); p = 0.004. Dots represent individual patients or healthy donors. Horizontal lines represent the mean.

![FIGURE 2.](http://www.jimmunol.org/)

Correlations between plasma sB7-H3 and TNF-α (A), IL-6 (B), IL-17 (C), and IFN-γ (D) in patients with sepsis. Concentrations of plasma sB7-H3, TNF-α, IL-6, IL-17, and IFN-γ in patients with sepsis (n = 27) were assessed by ELISA. Dots represent individual patients. R² values were calculated using the Spearman test.

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cantly elevated plasma sB7-H3 observed in septic patients, we attempted to examine the effect of bacterial endotoxin/LPS and inflammatory cytokines TNF-α and IFN-γ on sB7-H3 release from freshly isolated human monocytes. As shown in Fig. 3A, stimulation of human monocytes with LPS, TNF-α, or IFN-γ resulted in a significant increase in sB7-H3 release compared with naive cells ($p < 0.05$), whereas TNF-α stimulation led to a more profound sB7-H3 release among these three stimulants. A time-response curve of TNF-α–stimulated sB7-H3 release from human monocytes revealed that the released sB7-H3 in the media could be detectable after 8 h of stimulation and reached a maximal level at 48 h after stimulation (Fig. 3B).

A putative receptor for B7-H3 is detectable on septic human monocytes/macrophages and is inducible by LPS stimulation

Although a definite counterreceptor for B7-H3 has not yet been clarified, most data published so far indicate that B7-H3 binds to a putative receptor expressed on PHA- or anti-CD3 mAb-activated T cells (8, 9). To assess whether such a putative B7-H3 receptor is expressed on human monocytes and/or macrophages, we examined MFI of staining for biotinylated hB7-H3Ig on these cells. As shown in Fig. 4A, a putative receptor for B7-H3 was detectable on CD14+ peripheral blood monocytes but not on CD14− PBLs in patients with sepsis. In contrast, neither CD14+ monocytes nor CD14+ lymphocytes from healthy donors showed the binding to biotinylated hB7-H3Ig. Notably, peritoneal macrophages from three septic patients all expressed high levels of this putative B7-H3 receptor compared with nonseptic patients (Fig. 4B). To further elucidate whether the expression of this putative B7-H3 receptor is inducible, we incubated purified human monocytes from healthy donors with LPS ($1 \mu$g/ml) for up to 48 h. There was no binding of biotinylated hB7-H3Ig detectable on naive monocytes; however, LPS stimulation resulted in a time-dependent upregulation of this putative B7-H3 receptor expression (Fig. 4C). Furthermore, the MFI of staining for biotinylated hB7-H3Ig on human mononuclear THP-1 cells was markedly reduced by preincubation with anti–B7-H3 mAbs 4H7, 2E6, or 21D4 (Supplemental Fig. 1), indicating a direct and specific B7-H3 binding.

**FIGURE 3.** B7-H3 is actively released from human monocytes upon activation. Purified human monocytes from healthy donors ($n = 5$) were stimulated with LPS ($1.0 \mu$g/ml), rhTNF-α ($25 \text{ ng/ml}$), and IFN-γ ($1000 \text{ U/ml}$) for 48 h (A) or rhTNF-α ($25 \text{ ng/ml}$) for different time periods (B). PBS was used as the control. The amounts of released sB7-H3 in the media were measured. *$p < 0.05$ versus PBS.

**FIGURE 4.** Surface expression of a putative receptor for B7-H3 on human monocytes/macrophages is upregulated in patients with sepsis. PBMCs (A) were isolated from septic patients ($n = 5$) and healthy donors ($n = 5$), and peritoneal cells (B) were collected from septic patients ($n = 3$) and nonseptic patients ($n = 3$). Cells were stained with biotinylated hB7-H3Ig (100 ng) (open) or biotinylated human IgG (100 ng) as the control (gray filled), followed by dual-staining with PE-conjugated streptavidin and FITC-conjugated anti-CD14 mAb. The rectangles in the dot plots indicate the monocytic (CD14+ PBMCs) and lymphocytes (CD14− PBMCs) (A) or the peritoneal macrophages (CD14+) (B). A, Data shown are one representative of five independent experiments. C, Purified human monocytes from healthy donors ($n = 5$) were incubated with LPS (1 $\mu$g/ml) for up to 48 h. Surface expression of a putative receptor for B7-H3 was assessed at the indicated time points. Data are presented as histograms with biotinylated hB7-H3Ig (100 ng) staining (open) or biotinylated human IgG (100 ng) staining as the control (gray filled) and representative of five independent experiments.

**B7-H3 amplifies inflammatory response to LPS and BLP via a TLR-dependent mechanism**

Our clinical data of elevated plasma sB7-H3 in septic patients and its correlations with outcome of sepsis and plasma TNF-α and IL-6 suggest that B7-H3 may exert an inflammation-based stimulatory action. Therefore, we stimulated murine BMMs with various doses of muB7-H3 or a combination of muB7-H3 with either LPS or BLP.
B7-H3 alone was unable to induce proinflammatory cytokine TNF-α, IL-6, IL-1β, and IL-12p70 release (Fig. 5A, Supplemental Fig. 2A); however, B7-H3, in a dose-dependent manner, significantly augmented LPS- and BLP-stimulated TNF-α, IL-6, IL-1β, and IL-12p70 release (p < 0.05) (Fig. 5B, Supplemental Fig. 2B). This was mirrored by a costimulatory effect of B7-H3 on NF-κB activation. As shown in Fig. 5C, B7-H3 significantly enhanced NF-κB–induced NF-κB activation in HEKTLR4-MD2 cells (p < 0.05) and BLP-induced NF-κB activation in HEKTLR2 cells (p < 0.05). We further used polymyxin B, a specific LPS inhibitor, to ascertain that the observed costimulatory effect of B7-H3 on proinflammatory cytokine release was not due to the contamination of LPS. Polymyxin B at 25 μg/ml efficiently blocked the LPS-stimulated inflammatory response (Fig. 6A) but had no effect on B7-H3–enhanced TNF-α release from BLP-stimulated BMMs (Fig. 6B). By contrast, heat-inactivated B7-H3 lost its costimulatory activity in both LPS- and BLP-treated BMMs (Fig. 6A, 6B). These results clearly rule out any effects from a potential LPS contamination of the muB7-H3 preparation.

To elucidate whether TLR4 and/or TLR2 are involved in the B7-H3–amplified inflammatory response to LPS and BLP, we transfected HEKTLR4-MD2, HEKTLR2, and wild-type HEK293 cells with the plasmids encoding either YFP-tagged TLR4 or FLAG-tagged TLR2, followed by stimulation with hB7-H3. Transfection of the YFP-tagged TLR4 plasmid led to a dose-dependent NF-κB activation in both HEKTLR4-MD2 and HEK293 cells (Fig. 7A). A further increase in NF-κB activation was noted in B7-H3–stimulated HEKTLR4-MD2 cells; however, this effect was not observed in B7-H3–stimulated HEK293 cells (Fig. 7A). Similarly, B7-H3 significantly augmented FLAG-tagged TLR2-induced NF-κB activation in HEKTLR2 cells but failed to enhance NF-κB activation in HEK293 cells (Fig. 7B). These results indicate that B7-H3 amplifies Gram-negative and Gram-positive bacteria-initiated inflammatory responses via both TLR4- and TLR2-dependent mechanisms.

Blockage of B7-H3 attenuates LPS-associated lethality and proinflammatory cytokine release in vivo

Because sB7-H3 augmented proinflammatory cytokine release from LPS or BLP-stimulated monocytes/macrophages in vitro, we further investigated whether B7-H3 had a contributory effect in vivo during LPS-induced shock by using a specific B7-H3 neutralizing mAb, MIH35 (18). Mice receiving anti–B7-H3 mAb showed a significant reduction in their serum TNF-α and IL-6 levels in response to LPS challenge compared with mice receiving control Ig (p < 0.001) (Fig. 8A). Furthermore, blockage of B7-H3 protected mice from LPS-induced lethality, with an increase in survival from 20% in control Ig-treated mice to 47% in anti–B7-H3 mAb-treated mice (p = 0.0347) (Fig. 8B). These results indicate that B7-H3 contributes positively to the development of sepsis.

Discussion

Sepsis results from the inability of the host immune system to limit bacterial spread during an ongoing infection. Although normally helping to eradicate pathogens from a local infection, inflammation initiated by the innate immune response during sepsis develops into a systemic syndrome with abnormal coagulation, increased vascular permeability, ultimately septic shock, and multiple organ failure (3–5, 18).
Deciphering novel host factors that modulate the innate immune response during sepsis not only improves our insight into this complex disease but also provides avenues for designing novel therapies that could minimize mortality. Our previous work has shown that sB7-H3, released from monocytes, dendritic cells, and activated T cells, is detectable in the circulation of healthy humans (19). We also found that markedly increased levels of sB7-H3 were present in the serum samples from patients with bacterial meningitis when compared with healthy controls or patients with aseptic meningitis (23). In this study, we further demonstrated that patients diagnosed with sepsis, in contrast to healthy individuals, exhibited significantly elevated levels of sB7-H3 in their plasma, and more importantly, that plasma levels of sB7-H3 observed in the nonsurvivors of septic patients were substantially higher than those observed in the survivors. In addition, sB7-H3 levels in septic patients closely correlated with their plasma levels of inflammatory cytokines TNF-α and IL-6 but not IL-17 and IFN-γ. Although there were only 27 septic patients recruited into the current study, our data strongly suggest that sB7-H3 is associated with sepsis and could serve as an important prognostic indicator. Nevertheless, measurement of circulating sB7-H3 levels in a subgroup of patients with systemic inflammatory response syndrome (SIRS) may provide additional insights into the pathogenesis of sepsis.

![Figure 6](image.png)

**FIGURE 6.** Inactivation of B7-H3 by heating, but not polymyxin B, prevents B7-H3–augmented TNF-α release from LPS- and BLP-stimulated BMMs. BMMs isolated from C3H/HeN mice were preincubated with polymyxin B (25 μg/ml) and further stimulated with LPS (A), BLR (B), and muB7-H3 alone or their combinations for 24 h. In addition, LPS and muB7-H3 were heated at 80°C for 30 min before stimulation. TNF-α concentrations in the supernatants were measured, and data are expressed as mean ± SD from four to six independent experiments. *p < 0.01, compared with cells preincubated with polymyxin B; **p < 0.01, compared with cells costimulated with LPS and heated muB7-H3; ***p < 0.01, compared with cells costimulated with LPS and heated muB7-H3.

![Figure 7](image.png)

**FIGURE 7.** B7-H3–amplified NF-κB activation depends on the presence of TLR4 and TLR2. HEK293 cells were cotransfected with the NF-κB promoter luciferase plasmid and various amounts of plasmid encoding either YFP-tagged TLR4 (A) or FLAG-tagged TLR2 (B) for 16 h. EV was used as the control. Transfected cells were left untreated (naïve) or stimulated with hB7-H3 (2.5 μg/ml) for 6 h. NF-κB activation was expressed as fold increase of luciferase activity relative to the empty vector-transfected naïve cells. Data are expressed as mean ± SD of triplicate samples and representative of at least three independent experiments. *p < 0.05, compared with the TLR4- or TLR2-transfected naïve cells. EV, empty vector.

![Figure 8](image.png)

**FIGURE 8.** Blockage of B7-H3 attenuates LPS-associated lethality in vivo. Mice received 250 μg per mouse of anti–B7-H3 mAb (MIH35) or control Ig 2 h before a lethal LPS challenge (35 mg/kg) as described in Materials and Methods. A, Serum TNF-α and IL-6 levels were assessed by cytometric bead array. Data shown are the results of peak serum levels of TNF-α at 90 min and IL-6 at 2 h postseptic challenge (mean ± SD for each group of three mice). *p < 0.001, compared with control Ig-treated mice. B, Kaplan-Meier survival curve shows a significantly improved survival in anti–B7-H3 mAb-treated mice (n = 15) compared with control Ig-treated mice (n = 10) following a lethal LPS challenge. p = 0.0347.
dramatic because of noninfectious etiologies will further identify whether B7-H3 could act as a specific predictor for sepsis.

We have previously reported that sB7-H3 in the circulation is shed from membrane B7-H3 expressed on the cell surface of human monocytes, dendritic cells, and activated T cells, possibly through MMPs, as an MMP inhibitor attenuates sB7-H3 release from both B7-H3–transfected L929 cells and A549 cells (19). Circulating sB7-H3 is detectable but usually at very low level in normal humans; however, significantly increased levels of sB7-H3 were present in the circulation in patients with sepsis. To explain this phenomenon, we stimulated freshly isolated human monocytes with LPS, TNF-α, and IFN-γ. Substantial amounts of sB7-H3 were released from monocytes upon stimulation with LPS, TNF-α, or IFN-γ when compared with naïve cells. Patients with sepsis are usually accompanied by an uncontrolled bacterial infection and/or overactivation of the innate immune system with excessive release of inflammatory cytokines, such as TNF-α, which, in turn, may lead to an elevated level of sB7-H3 in the circulation. Although the precise mechanism by which LPS and inflammatory cytokines promote sB7-H3 release from human monocytes is still unclear, enhanced B7-H3 expression (19, 24) and activation of MMPs (25, 26) by these stimulants may be two possible explanations for the elevated sB7-H3 observed in patients with sepsis.

Previous work has indicated that B7-H3 binds to a putative receptor expressed on PHA- or anti-CD3 mAb-activated T cells that is distinct from CD28, CTLA-4, ICOS, and programmed death-1 (8, 9). Research on the immunologic function of B7-H3 clarified that B7-H3 acts not only as a costimulator but also as a coinhibitor in regulating T cell–mediated immune responses. Therefore, it is proposed that there may be both costimulatory and coinhibitory receptors expressed on T cells for B7-H3 (18, 27). A recent study identified triggering receptor expressed on myeloid cells–like transcript 2 as a B7-H3 costimulatory receptor (18), but the coinhibitory receptor(s) for B7-H3 have not yet been discovered. It is possible that multiple counterreceptors for B7-H3 may exist on different types of immune cells (18, 27). In the current study, we provide the first evidence that a putative receptor for B7-H3 was expressed on peripheral monocytes and peritoneal macrophages, but not on lymphocytes, from septic patients. In contrast, this receptor couldn’t be detected on naïve monocytes from healthy individuals but was inducible by LPS stimulation. However, the immunologic and clinical significance of such a putative B7-H3 receptor detected on monocytes/macrophages in septic patients requires further investigation.

Accumulated data have demonstrated that B7-H3, being both a T cell costimulator and coinhibitor, plays a contrasting role in modulating T cell–mediated immune responses (9, 11–17). However, it is unclear whether this molecule is also involved in the innate immunity–associated inflammatory response. Our finding that elevated levels of plasma sB7-H3 in septic patients correlated not only with patients’ clinical outcome but also with the levels of plasma TNF-α and IL-6 implicates a possible link between B7-H3 and the inflammatory response. To test this hypothesis, we stimulated murine BMMs with B7-H3 or in a combination with LPS and BLP. We couldn’t prove a direct stimulatory effect of B7-H3 on proinflammatory cytokine release from murine BMMs; however, B7-H3 dramatically augmented LPS– and BLP-stimulated TNF-α, IL-1β, IL-6, and IL-12p70 release. Consistent with this finding, we further demonstrated that B7-H3 also enhanced LPS– and BLP-induced NF-κB activation, which occurs via both TLR4– and TLR2–dependent mechanisms. The development of septic shock from an uncontrolled bacterial infection is characterized by the excessive release of proinflammatory cytokines from innate immune cells, such as monocytes/macrophages. Although appropriate amounts of these cytokines are essential for innate immune response to bacterial infection, exaggerated production can lead to an uncontrolled systemic inflammatory response, tissue injury, and multiple organ failure (3–5, 21, 22). Therefore, amplification of monocyte/macrophage–mediated inflammatory response by B7-H3 may be detrimental during sepsis. Our in vivo results further support this notion, where substantially reduced serum TNF-α and IL-6 levels, and significantly improved survival were observed in lethal LPS-challenged mice when their B7-H3 was blocked by a specific neutralizing mAb, MH35, suggesting a contributory effect of B7-H3 during sepsis. However, it is necessary to further investigate whether neutralization of B7-H3 also affords the protection against microbial sepsis–related lethality, because the mortality from endotoxic shock is predominantly associated with LPS stimulation exaggerated inflammatory responses, whereas the microbial sepsis–related lethality results not only from an excessive release of proinflammatory cytokines but also an impaired antimicrobial activity of innate immunity.

Taken together, our clinical data suggest that circulating sB7-H3 may serve as a prognostic indicator for sepsis as the significantly elevated sB7-H3 in septic patients is associated with a poor outcome and correlates with the proinflammatory cytokines TNF-α and IL-6. In addition to its regulatory function on T cell–mediated immune response, we further demonstrate, for the first time, that B7-H3 participates in monocyte/macrophage–mediated inflammatory responses by augmenting LPS– and BLP-stimulated proinflammatory cytokine release and thus potentiates the development of sepsis. Targeting this molecule may provide a new therapeutic strategy for microbial sepsis.

Disclosures
The authors have no financial conflicts of interest.

References
17. Ling, V., P. P. Wu, V. Spaulding, J. Kieczczawa, D. Luxenberg, B. M. Carreno, and M. Collins. 2003. Duplication of primate and rodent B7-H3 immunoglobulin V-