T Cell Ig Mucin-3 Promotes Homeostasis of Sepsis by Negatively Regulating the TLR Response

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Sepsis is an excessive inflammatory condition with a high mortality rate and limited prediction and therapeutic options. In this study, for the first time, to our knowledge, we found that downregulation and/or blockade of T cell Ig and mucin domain protein 3 (Tim-3), a negative immune regulator, correlated with severity of sepsis, suggesting that Tim-3 plays important roles in maintaining the homeostasis of sepsis in both humans and a mouse model. Blockade and/or downregulation of Tim-3 led to increased macrophage activation, which contributed to the systemic inflammatory response in sepsis, whereas Tim-3 overexpression in macrophages significantly suppressed TLR-mediated proinflammatory cytokine production, indicating that Tim-3 is a negative regulator of TLR-mediated immune responses. Cross-talk between the Tim-3 and TLR4 pathways makes TLR4 an important contributor to Tim-3–mediated negative regulation of the innate immune response. Tim-3–signaling inhibited LPS–TLR4–mediated NF-κB activation by increasing PI3K–AKT phosphorylation and A20 activity. This negative regulatory role of Tim-3 reflects a new adaptive compensatory and protective mechanism in sepsis victims, a finding of potential importance for modulating innate responses in these patients.

TLRs are pattern recognition receptors that recognize conserved microbial molecules called pathogen-associated molecular pattern molecules. TLR1, 2, 4, and 6 are expressed on the cell surface and have been shown to detect, and initiate a response against, extracellular pathogens, whereas TLR3, 7, 8, and 9 are located in endosomes in specific immune system cells and are able to recognize conserved structures of DNA or RNA from intracellular pathogens (6, 7). Activation of TLR4 represents an important triggering event in the development of infectious and inflammatory diseases (8). Bacterial LPS binds to myeloid differentiation protein 2 (MD2) in the MD2–TLR4 complex and triggers a signaling cascade causing secretion of MyD88 and TRIF-dependent proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (4). However, uncontrolled TLR activation can result both in excessive inflammation, such as septic shock, and in immune suppression. For example, recent data clearly show that TLR4 plays important roles in septic organ failure, such as cardiovascular depression (9), whereas Schwandt et al. (10) recently reported that TLR4 signaling upregulates type I IFN and contributes to immune suppression. As many reports (e.g., Refs. 11, 12) have demonstrated the central role of TLRs in the pathogenesis of sepsis, information on the mechanisms that regulate TLR signaling is of great importance and may provide a new therapeutic strategy for severe sepsis.

T cell Ig and mucin domain protein 3 (Tim-3) was initially identified on terminally differentiated Th1, Th17, and Tc1 cells, and was thought to function directly in regulating T cell responses (13, 14). Indeed, binding of Tim-3 to its natural ligand galectin-9 (Gal-9) induces T cell death and tolerance in vivo (15, 16). However, subsequent data showed that Tim-3 is also constitutively expressed on macrophages and dendritic cells and regulates the innate immune response (17–19). Because of its critical roles in both innate and adaptive immune regulation, Tim-3 dysregulation has been correlated with the pathogenesis of many clinical diseases. For example, downregulation of Tim-3 is associated with enhanced T effector cell function in autoimmune diseases, such as...

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Abbreviations used in this article: CLP, cecal ligation and puncture; CRP, C-reactive protein; EU, endotoxin unit; Gal-9, galectin-9; IRF, IFN regulating factor; KO, knockout; PLF, peripheral lavage fluid; rhGal-9, recombinant human Gal-9; siRNA, small interfering RNA; Tim-3, T cell Ig and mucin domain protein 3.
multiple sclerosis (20) and autoimmune hepatitis (21), whereas its overexpression on T cells correlates with T cell paralysis in patients with tumors or chronic viral infection (22, 23). The above data show that the Tim-3 pathway is actively involved in maintaining immune homeostasis in vivo, but the underlying mechanisms by which it regulates immune responses, especially the innate immune response, remain unclear.

Of great interest is the finding that the TLR system can be negatively regulated by Tim-3. For example, data show that Tim-3 controls the extent of the inflammatory response by suppressing the TLR4 response (24, 25). However, the underlying mechanisms remain largely unclear. Because sepsis is characterized by immune disorders in which an uncontrolled TLR response plays a major pathogenic role (2, 4, 26) and because the Tim-3 pathway is actively involved in homeostatic immune regulation and may play a negative regulatory role in the TLR-mediated response (24, 25), it is important to know whether the Tim-3 pathway is also involved in the regulation of sepsis, and, if so, whether it plays a negative regulatory role.

In this study, we demonstrated, for the first time, to our knowledge, that Tim-3 is actively involved in maintaining the homeostasis of sepsis by negatively regulating the TLR response. Downregulation and/or blockade of the Tim-3 pathway correlates with severity of sepsis, indicating that this pathway might be a new target for intervention in the treatment of sepsis.

Materials and Methods

Study population

The study protocol was approved by the Ethics Committee of the General Hospital of the People’s Liberation Army, Beijing, China. All patients gave their written informed consent for the study. The study population comprised three groups of subjects, from whom PBMCs were obtained by gradient centrifugation at room temperature on Ficoll-Hypaque (Lymphoprep; Axis-Shield PoC, Oslo, Norway). Group 1 consisted of normal healthy subjects (n = 16), group 2 of patients with sepsis, (n = 12), and group 3 of patients with severe sepsis (n = 14). Sepsis and severe sepsis were defined as described previously (27). Sepsis corresponded to a systemic response to infection, manifested by two or more of the following conditions as a result of infection: (1) temperature, >38°C or <36°C; (2) heart rate, >90 beats per minute; (3) respiratory rate, >20 breaths per minute or PaCO2 <32 mm Hg; and WBC count, >12,000/μL or <4000/μL or >10% immature WBCs. Severe sepsis corresponded to sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Perfusion abnormalities could include, but were not limited to, lactic acidosis, oliguria, or an acute alteration in mental status. The mean age of the 26 sepsis and severe sepsis patients was 50 y (range, 21–71) and that of the controls, 45 y (range, 31–66).

Mice

Male wild-type C57BL/6 and TLR4-deficient [knockout (KO)] B6, B10ScN-Tlr4-lps-del/JthJ mice (both 7 to 8 wk old) (The Jackson Laboratory, Bar Harbor, ME) were bred in our facilities under specific pathogen-free conditions. All treatment of mice in this study was in strict compliance with the guidelines for the care and use of laboratory animals set out by the Institute of Basic Medical Sciences.

Reagents and Abs

The antagonist anti-mouse Tim-3 mAb (RMT3-23; rat IgG2a, endotoxin concentration < 1.0 endotoxin units (EU)/mg) and isotype control IgG were purchased from eBioscience (San Diego, CA). The recombinant fusion protein sTim-3-Ig was prepared by fusing cDNA coding for the soluble extracellular domain of mouse Tim-3 to that coding for the single-chain Fc fragment of human IgG1 in the pet28a+ vector and expressing the construct in Escherichia coli BL21, as described previously (28, 29). The presence and purity of sTim-3-Ig were confirmed by SDS-PAGE and Western blot analysis using rabbit anti-mouse Tim-3 Abs (Abcam). The Fc fragment of human IgG1 was prepared and purified from E. coli BL21 in an identical manner and used as the negative control. The endotoxin concentration in sTim-3-Ig or Ig was < 1.0 EU/mg. The TLR agonists Pam3CSK4 (TLR1/2), HKLM (TLR2, a freeze-dried heat-killed preparation of Listeria monocytogenes), polyinosine-polycytidylic acid (TLR3), LPS-EK (TLR4, LPS from E. coli K12), ST-FLA (TLR5, flagellin from Salmonella typhimurium), FSL-1 (TLR6/2, Pam2CGDSPKHPKS), ssRNA40 (TLR7), and ODN1826 (TLR9) were purchased from InvivoGen (San Diego, CA). The Tim-3 ligand, recombinant human Gal-9 (rhGal-9), endotoxin concentration < 1.0 EU/mg, was prepared as described previously (30). Rabbit Abs against mouse phospho-AKT, AKT, or IgG1 were purchased from Cell Signaling Technology (Beverly, MA). The PDK inhibitor wortmannin was purchased from Sigma-Aldrich (St. Louis, MO).

Induction of experimental sepsis by cecal ligation and puncture or by LPS injection, Tim-3 blockade, and sample collection

Specific pathogen-free 7- to 8-wk-old male C57BL/6 mice or B6.B10ScN-Tlr4-lps-del/JthJ mice were used to establish the sepsis model. Briefly, the mice were anesthetized by i.p. injection of ketamine; then approximately two thirds of the cecum was ligated through a 2-cm abdominal midline incision and punctured extensively with a 21-gauge needle. After the bowel was repositioned, the abdomen was closed in layers, using a 4.0 surgical suture and metallic clips. Sham-operated mice were handled in the same manner, except the cecum was not ligated and punctured. This cecal ligation and puncture (CLP) model of sepsis is believed to closely simulate sepsis in humans because of the polymicrobially driven inflammatory process. In addition, an LPS-induced endotoxemia model was induced in mice by i.p. injection of 0.5 mg/kg LPS (E. coli 055:B5; Sigma-Aldrich), as described previously (31).

Tim-3 blockade was performed as explained earlier (28, 32). In a first approach to investigating the role of Tim-3 signaling, we used anti-mouse Tim-3 mAb (RMT3-23; rat IgG2a, eBioscience). To assess the effect on survival, 200 μg mAb or control IgG (eBioscience) was injected i.p. on day 1 before CLP (day 0) and on days 1, 3, 5, and 7, and survival was monitored. To test the effects of Tim-3 blockade on the inflammatory response in vivo, mice received a single injection of 200 μg mAb or control IgG on day 1 before CLP or LPS injection (day 0); then splenocytes were collected 24 h later for real-time PCR analysis. In a second approach, Tim-3 blockade was carried out using 200-μg doses of sTim-3-Ig or Fc (control), as described previously (29), using the same protocols as above to test the effect on survival over 7 d and on inflammatory responses at 23 h after CLP.

Cell culture

The mouse macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA). Mouse peritoneal macrophages were elicited as described previously (33). Briefly, C57BL/6 mice were injected i.p. with 2 ml 4% thioglycollate medium (Sigma-Aldrich); then, 3 d later, peritoneal macrophages were collected by peritoneal lavage with cold PBS, incubated for 2 h at 37°C in DMEM supplemented with 10% heat-inactivated FBS (Life Technologies), and washed with PBS to eliminate nonadherent cells (33). Both RAW264.7 cells and peritoneal macrophages were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 U/ml streptomycin in a humidified 5% CO2 atmosphere at 37°C.

Generation of stable cell lines

The cDNA encoding full-length Tim-3 was cloned from mouse splenocytes, and the pCMV-AC/GFP.neo vector was used to express mouse Tim-3 in the RAW264.7 macrophage cell line. Clones were verified by DNA sequencing. Recombinant plasmid or vector control was transfected into RAW264.7 cells using Lipfectamine 2000 (Invitrogen) according to the manufacturer’s protocol; then resistant cells were selected using G418, and transfected clones were selected and expanded for further studies. GFP expression was evaluated by fluorescent microscopy and flow cytometry to estimate transfection efficiency. Tim-3 small interfering RNA (siRNA)–transfected or control siRNA (negative control)–transfected RAW264.7 cell lines were established previously (34).

ELISA

The concentration of TNF-α and IL-6 in cell-free supernatants or in mouse serum was measured using a sandwich ELISA (eBioscience) according to the manufacturer’s protocol.

Quantitative real-time RT-PCR

Gene expression was analyzed by two-step quantitative RT-PCR. Total RNA was extracted from human PBMCs, mouse splenocytes, or mouse peritoneal lavage fluid (PLF)–derived cells using TRI Reagent (Invitrogen), according to the manufacturer’s instructions. RNA (0.2–1 μg) was reverse tran-
scribed in a 20-μl reaction volume (42˚C, 30 min; 95˚C, 5 min), using a QuantiTect Reverse Transcription Kit (Qiagen); then cDNA (2 μl) was amplified using a SYBR Green I Master Mix (Roche, Basel, Switzerland) and a LightCycler 480 PCR System (Roche). All tests were carried out on duplicate 20-μl reaction mixtures in 96-well plates, and a negative control with no cDNA template was included in each run. The specificity of the products was confirmed by visual inspection of the melting curves, and the products were run on a 1.2% agarose gel. The relative expression of a gene was determined using the 2^(-ΔΔCt) method, with GAPDH as the internal control. The primer sequences for targeted cDNAs are listed in Table I.

Western blots
Western blotting was performed to evaluate levels of IκB-α, AKT, phospho-AKT, A20, and GAPDH. Briefly, 50 μg protein was electrophoretically separated on a 12% NaDodSO4-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, which was then blocked by incubation for 1 h at room temperature in 5% fat-free dry milk in TBST. The blots were then incubated overnight at 4˚C with rabbit Abs against IκB-α (1:1000), AKT (1:1000), phospho-AKT (1:500), A20 (1: 500), or GAPDH (1:1000) (Cell Signaling Technology) diluted in TBST containing 5% BSA, washed for 25 min with TBST, and incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG Abs (KPL, Gaithersburg, MD) (1:20,000 in TBST containing 5% BSA); then bound Ab was visualized using ECL kits (Amersham Biosciences).

Plasmids, transfection, and luciferase assay
Lipofectamine 2000 (Invitrogen) was used for transient transfection. RAW264.7 cells were transiently transfected in 12-well plates with 10 ng pNF-kB-Luc plasmid and 1 ng Renilla luciferase plasmid; then, 4 h later, cells were treated with LPS and/or rhGal-9 for an additional 6 h. Luciferase reporter activity was next determined using a dual-specific luciferase assay kit (Promega) as described by the manufacturer, and the relative firefly luciferase activity normalized to the Renilla luciferase activity was calculated as a fold induction relative to the value for vector-transfected cells.

FACS analysis and macrophage sorting
RAW264.7 cells or cells harvested from the PLF were collected and stained with allophycocyanin-conjugated rat anti-mouse CD11b mAb (macrophages and neutrophils, clone M1/70), FITC-conjugated rat anti-mouse F4/80 mAb (clone BM8), FITC-conjugated rat anti-mouse Ly-6G mAb (clone 1A8), and/or PE-conjugated rat anti-mouse Tim-3 mAb (clone GL3) (all from eBioscience) diluted in 2% FBS in PBS; isotype control Abs (clone BM8, FITC-conjugated rat anti-mouse F4/80 and CD11b (eBioscience) were used to isolate macrophages from splenocytes, using FACS cell-sorting methods.

Microarray analysis
The mRNA sample was isolated from Tim-3 siRNA– or control siRNA–transfected RAW264.7 cells. Three biological replicates were used in this experiment, each from separate cultures. Microarray analysis was carried out using a Nimblegen 12k platform, and differentially expressed genes were identified by comparing the expression profiles of the two sets of cells. The candidate gene or genes were further identified by quantitative real-time PCR analysis.

Statistical analysis
Data are expressed as the mean ± SD. Differences between groups were analyzed using the Kruskal–Wallis test and ANOVA, whereas differences between survival curves were assessed using the Wilcoxon test. A p value < 0.05 was considered significant. SPSS software (version 20.0) was used for all statistical procedures.

Results

**Tim-3 downregulation correlates with severity of sepsis**

Recent data have shown that Tim-3 contributes to homeostasis in vivo. To investigate whether the Tim-3 pathway was also involved in the physiopathology of sepsis, we first examined the dynamic expression of Tim-3 and whether a correlation existed between Tim-3 expression and the disease activity of sepsis.

We first examined Tim-3 expression in PBMCs from sepsis patients at different stages (sepsis or severe sepsis). As shown in Fig. 1A, Tim-3 mRNA levels were significantly increased in sepsis patients compared with healthy controls, but, unexpectedly, were significantly lower in severe sepsis patients than in sepsis patients. When we used CRP as a biomarker to track the inflammatory status of patients with sepsis, CRP expression, in contrast to Tim-3 mRNA levels, was markedly increased in severe sepsis patients compared with sepsis patients, and values in both sepsis groups were significantly higher than in controls (Fig. 1B). These data show that downregulation of Tim-3 expression occurs in the late stage of sepsis.

To confirm the correlation between downregulated Tim-3 expression and disease progression, a mouse sepsis model was established by CLP, as described previously (35), with sham-operated mice as controls. When the kinetic expression of Tim-3 mRNA in PLF-derived cells was examined, a sharp increase in levels was seen in both CLP and sham-operated mice at 2 h after operation, but, unexpectedly, at subsequent time points, Tim-3 expression was markedly lower in CLP mice than in sham-operated mice (Fig. 1C). The primer sequences for human and mouse Tim-3 are listed in Table I. We also examined kinetic changes in levels of TNF-α (Fig. 1D) and IL-6 (Fig. 1E) in serum from mice with sepsis and sham-operated mice and found that levels of both remained low in sham-operated mice, but increased sharply at 4 h after CLP and continued to increase for at least 24 h (Fig. 1D) or 12 h (Fig. 1E). These data show that a negative relationship exists between Tim-3 downregulation and increased IL-6 and TNF-α expression in the late stage of sepsis in mice. To address this further, we collected mouse PLF cells at 4 h after CLP and examined Tim-3 expression by flow cytometry. Consistent with the mRNA data, Tim-3 protein expression on PLF cells was significantly downregulated in CLP mice compared with sham-operated mice (Fig. 1F). These data clearly indicate a link between Tim-3 expression and disease activity in sepsis in both humans and mice, with downregulation of Tim-3 expression being seen in severe sepsis. The reason for, and the biological significance of, dysregulated Tim-3 expression in the progression of sepsis were thus of great interest.

**Tim-3 signaling negatively regulates sepsis in mice**

To further confirm the involvement of Tim-3 in the progression of sepsis, we examined the effects of in vivo Tim-3 blockade on sepsis progression, using either an antagonistic mAb against mouse Tim-3 or a fusion protein consisting of the soluble extracellular domain of mouse Tim-3 fused to the Fc region of human IgG1 (sTim-3-IgG1), as described by other authors (28, 32).

As shown in Fig. 2, administration of anti-Tim-3 mAb decreased the survival rate (Fig. 2A) and also increased levels of mRNAs coding for the proinflammatory cytokines IL-6, IL-1β, and HMGB1 (Fig. 2B). As TLR4 and TLR2 are two important mediators of excessive inflammatory responses during polymicrobial sepsis (36), we also measured levels of TLR4 and TLR2 mRNAs after Tim-3 blockade and found that both were increased (Fig. 2B). However, we unexpectedly discovered that mRNA levels for the anti-inflammatory cytokine IL-10 were increased after Tim-3 blockade (Fig. 2B). The primer sequences for targeted cDNAs are listed in Table I. It is well known that the bacteria-induced excessive inflammatory response accounts for the major pathogenesis of sepsis. Tim-3 blockade may further exacerbate the uncontrolled inflammation and lead to a decreased survival rate. The fact that Tim-3 blockade led to exacerbated sepsis suggests that the Tim-3 pathway is actively involved in the progression of sepsis and may play a regulatory role.

Consistent with this idea, administration of sTim-3-IgG also significantly exacerbated sepsis, as shown by a decreased survival...
rate (Supplemental Fig. 1A), as well as increased levels of mRNAs for proinflammatory cytokines and TLR4 (Supplemental Fig. 1B).

Tim-3 blockade and/or downregulation leads to enhanced macrophage responsiveness

The above data showed that blockade and/or downregulation of Tim-3 correlated with severity of sepsis. However, the underlying mechanisms remained unclear. Macrophages are a key component of innate immunity and are required for the innate immune response to bacterial infection. However, uncontrolled macrophage activation might lead to an excessive inflammatory response and a decreased survival rate in sepsis. To explore whether Tim-3 blockade affected progression of sepsis and thus contributed to the exacerbated sepsis, we isolated macrophages at 24 h after CLP from mice that had been injected i.p. with anti–Tim-3 mAb or control IgG at 6 h before CLP. As shown in Fig. 3A, Tim-3 blockade significantly altered macrophage responsiveness, as levels of mRNAs for proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10, and HMGB1) and TLRs (TLR4 and TLR2) were all significantly upregulated in macrophages from mice that had received anti–Tim-3 mAb. The primer sequences for targeted cDNAs are listed in Table I.

In addition, we examined whether dysregulated downregulation of Tim-3 also occurred on macrophages and, if so, whether this downregulation correlated with enhanced macrophage responsiveness. As shown in Fig. 3B, Tim-3 expression on CD11b+ F4/80+ macrophages from PLF cells of CLP mice was significantly lower than on macrophages from sham-operated mice. To test whether Tim-3 downregulation affected macrophage responsiveness, a RAW264.7 macrophage cell line in which Tim-3 expression was measured by real-time PCR (A) and plasma CRP levels by immunonephelometry (B). (C) Splenocytes were isolated from sham-operated and CLP mice at different time points, and Tim-3 mRNA levels were measured by real-time PCR. (D and E) Serum was collected from sham-operated and CLP mice at different time points and tested for TNF-α (D) or IL-6 (E) by ELISA. In (C)–(E), the data are expressed as the mean ± SD and are representative of those obtained in three independent experiments, each with 8–12 mice per group. **p < 0.01. (F) Cells were isolated from the PLF of untreated, sham-operated, and CLP mice at 12 h after the operation, and Tim-3 expression was examined by FACS.

Table I. Primers used in this study

<table>
<thead>
<tr>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tbody>
<tr>
<td>hTim-3</td>
<td>5'-CTTGCGCTGGTTTGATGAC-3'</td>
</tr>
<tr>
<td>mTim-3</td>
<td>5'-GACCCCTCATCTGGAATGAC-3'</td>
</tr>
<tr>
<td>mGal-9</td>
<td>5'-GGCATGACTGAGCTGCTTCC-3'</td>
</tr>
<tr>
<td>mIL-6</td>
<td>5'-GCCCACTCTTCTGTGACACTCTAC-3'</td>
</tr>
<tr>
<td>mIL-1β</td>
<td>5'-ATCTCGAGTACAGAAGCTGACCCA-3'</td>
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<tr>
<td>mTLR4</td>
<td>5'-ACTGTTGCTCTCTTCCGATGAC-3'</td>
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<tr>
<td>mTLR2</td>
<td>5'-CTGGCGCTTAAACTTGGACCTAC-3'</td>
</tr>
<tr>
<td>mHMGB1</td>
<td>5'-GCCACAGAAGCTGAGCTTCC-3'</td>
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<td>mIRF-7</td>
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</tr>
<tr>
<td>mGAPDH</td>
<td>5'-CTGGCGCTTAAACTTGGACCTAC-3'</td>
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h, Human; m, mouse.
tion was stably knocked down (Fig. 3C) was used. As shown in Fig. 3D and 3E, Tim-3 knockdown led to significantly increased production of IL-6 (Fig. 3D) and TNF-α (Fig. 3E) in response to stimulation of TLR1 (Pam3CSK4), TLR2 (HKLM), TLR4 (LPS), TLR6 (FSL-1), or TLR9 (ODN1826). The observation that downregulation of Tim-3 leads to enhanced sensitivity of macrophages to TLR stimulation and to the production of much higher levels of proinflammatory cytokines, such as TNF-α and IL-6,
partially explains why downregulation or blockade of Tim-3 correlates with exacerbation of the inflammatory response and severity of sepsis in vivo and shows that Tim-3 promotes immune homeostasis of sepsis by maintaining the innate immune cells in a steady state, thus preventing an excessive inflammatory response.

**Tim-3 overexpression on macrophages suppresses proinflammatory cytokine production induced by TLR signaling**

The above data show that Tim-3 negatively regulates the TLR-mediated response in macrophages. To address this idea further, we established a RAW264.7 macrophage cell line stably overexpressing Tim-3 mRNA (Fig. 4A) and protein (Fig. 4B) and found that Tim-3 overexpression led to inhibition of production of IL-6 (Fig. 4C) and TNF-α (Fig. 4D) in response to TLR stimulation, again showing that Tim-3 is a negative regulator of the TLR response.

**Cross-inhibition between LPS/TLR4 and Tim-3 makes TLR4 an important contributor to Tim-3-mediated regulation**

We had demonstrated that Tim-3 is a negative regulator of the TLR response in sepsis. However, we did not know why this negative regulator was downregulated during sepsis progression. To analyze this finding, we first mimicked the in vivo microenvironment by incubating mouse peritoneal macrophages with different doses of LPS, a critical component in the pathogenesis of sepsis. When the macrophages were treated with a fixed concentration of LPS (200 ng/ml) for different times (Fig. 5A) or with different doses of LPS for a fixed time (12 h) (Fig. 5B), we found that LPS could bidirectionally regulate Tim-3 mRNA levels, suggesting that Tim-3 downregulation in sepsis, at least on macrophages, might be caused by long-lasting LPS stimulation. To confirm that Tim-3 expression could be actively downregulated by LPS, RAW264.7 cells, which constitutively express high levels of Tim-3, were treated with different doses of LPS for 12 h, and LPS was found to dose-dependently downregulate levels of Tim-3 protein (Fig. 5C) and mRNA (Fig. 5D). These data suggest that a long-lasting high dose of LPS might cause downregulation of Tim-3 in sepsis in vivo. Of interest, we also found that LPS stimulation caused a dose-dependent increase in the expression of Gal-9 (a Tim-3 ligand) in RAW264.7 cells (data not shown), suggesting that, when Tim-3 levels are low, Gal-9 can act in an autocrine fashion to maintain the function of the Tim-3/Gal-9 pathway. To our knowledge, this report is the first to show that LPS can regulate Tim-3 expression. However, the biological significance of this phenomenon remains to be determined.

As shown above (Fig. 5A–D), LPS/TLR4 mediates Tim-3 downregulation, at least on macrophages. In contrast, Tim-3 signaling negatively regulates the TLR4 response to LPS stimulation, as shown in RAW264.7 cells stably overexpressing Tim-3 (Fig. 4C, 4D). To explore this finding further, we activated the Tim-3 pathway using rhGal-9 and examined the effects of Tim-3 signaling on LPS-induced macrophage activation. As shown in Fig. 5, ligation of Tim-3 with Gal-9 inhibited the LPS-induced production of TNF-α (Fig. 5E) and IL-6 (Fig. 5F) by macrophages. The cross-talk between the Tim-3 and TLR4 pathways suggests that TLR4 might play an important role in Tim-3–mediated immune regulation in vivo.

**Tim-3 blockade in TLR4 KO mice does not significantly affect progression of sepsis**

The above data showing that the LPS/TLR4 pathway is involved in the regulation of Tim-3 and that Tim-3 maintains the homeostasis of innate immune cells by suppressing the TLR4 response indicated that the TLR4 pathway is an important mediator of Tim-3–related homeostatic mechanisms. To test the hypothesis that Tim-3–mediated immune regulation occurs through TLRs, particularly TLR4, we injected TLR4 KO mice i.p. with anti–Tim-3 mAb or control IgG and found that Tim-3 blockade did not significantly affect the survival rate for sepsis (Fig. 6A), showing that Tim-3 exerts its negative function by downregulating the LPS/TLR4 pathway. We also examined the expression of proinflammatory cytokines in these mice after Tim-3 blockade and found that levels of mRNAs for IL-1β, IL-10, IL-6, and HMGB1 in splenocytes were only slightly upregulated, compared with controls (Fig. 6B). The primer sequences for targeted cDNAs are listed in Table I. These data clearly show that Tim-3 is actively involved in maintaining the homeostasis of sepsis by inhibiting the TLR4 response.

To further confirm that Tim-3 can actively suppress the TLR4 response, thus contributing to immune homeostasis in vivo, an LPS-induced endotoxinemia model was established in mice and the effects of Tim-3 blockade on its progression were studied. Tim-3 blockade with anti–Tim-3 mAb again significantly enhanced expression of the proinflammatory cytokines IL-6 and IL-1β, the anti-inflammatory cytokine IL-10, and TLR4 (Supplemental Fig. 2). These data further demonstrate a cross-talk between the TLR4 and Tim-3 pathways in the progression of inflammation and indicate that TLR4 is a critical target of Tim-3–mediated negative regulation.
Tim-3 signaling inhibits LPS/TLR4-mediated NF-κB activation by enhancing PI3K-AKT phosphorylation

The molecular mechanism by which Tim-3 signaling negatively regulates the TLR4 response was then examined. As the NF-κB pathway plays critical roles in LPS/TLR4-induced proinflammatory cytokine production (37), we examined whether Tim-3 signaling suppressed LPS/TLR4-induced NF-κB activation. In addition, because the PI3K-AKT pathway is another critical pathway for LPS/TLR4-mediated macrophage activation (38), we analyzed whether Tim-3 signaling affected LPS-induced PI3K-AKT activation. To test these two points, RAW264.7 cells were stimulated with rhGal-9 and/or LPS for different times; then IkBα levels and AKT phosphorylation were assessed by Western blotting. As shown in Fig. 7A, rhGal-9 alone did not significantly affect NF-κB activation but, when combined with LPS, led to increased levels of IkBα, showing suppression of NF-κB activation.

In addition, rhGal-9 alone did not activate AKT, whereas LPS alone did, and the two together resulted in significantly enhanced AKT phosphorylation. To confirm the involvement of NF-κB and the PI3K-AKT pathway in Tim-3 signaling, we treated control RAW264.7 cells or RAW264.7 cells stably knocked down for Tim-3 with different doses of LPS for 10 min, and then examined cell lysates on Western blots. As shown in Fig. 7B and 7C, Tim-3 knockdown RAW264.7 cells, compared with control cells, showed decreased AKT phosphorylation and decreased IkBα expression after stimulation by increasing doses of LPS, indicating that Tim-3 signaling promotes AKT activation and decreases NF-κB activation.

In addition, an NF-κB luciferase assay was performed to further examine whether NF-κB activation was attenuated after coligation of Tim-3 with Gal-9 and TLR4 with LPS. As shown in Fig. 7D, coligation of Tim-3 significantly inhibited LPS-induced NF-κB activation in RAW264.7 cells. Although the underlying mechanism is yet to be determined, these data show that Tim-3 inhibits the LPS-induced response by inhibiting NF-κB activation.

The biological significance of Tim-3 signaling–enhanced PI3K-AKT activation was then assessed. It is known that NF-κB activation can be inhibited by the PI3K-AKT pathway (39, 40). To examine whether Tim-3–enhanced PI3K-AKT activation contributed to the inhibition of NF-κB activation, the PI3K-AKT inhibitor wortmannin was used in the TNF-α production assay after Tim-3 and TLR4 coligation. As shown in Fig. 7E, inhibition of PI3K-AKT prevented the Tim-3 ligation–induced decrease in TNF-α production in LPS-treated RAW264.7 cells. These data show that Tim-3 ligation–enhanced PI3K-AKT activation can inhibit LPS-induced NF-κB activation.

Tim-3 signaling increases A20 expression, which contributes to attenuation of the LPS response in macrophages

As TLR4 activation is an important triggering event in the development of inflammatory diseases, strict regulation of these signals is important for host protection against excessive inflammation. To avoid an excessive LPS response, cells have developed a mechanism called “endotoxin tolerance,” in which many mol-
ecules, such as zinc finger protein A20, FLN29, and SOCS1, are involved (41–43). To determine whether the Tim-3–induced attenuation of the LPS response was mediated by these molecules, we treated Tim-3 knockdown RAW264.7 cells and control cells with different doses of LPS for 15 min. Of interest, as shown in Fig. 7F, Tim-3 knockdown RAW264.7 cells, compared with control cells, showed inhibited A20 expression in response to LPS stimulation, revealing that Tim-3 attenuates the LPS response in macrophages by increasing A20 expression. In contrast, no significant difference in SOCS1 and FLN29 expression was seen between LPS-treated Tim-3 knockdown RAW264.7 cells and LPS-treated control cells (data not shown).

**Tim-3 signaling suppresses LPS-induced IFN regulating factor 7/IFN-αβ expression**

The above data showed that Tim-3 signaling inhibits LPS-induced activation of NF-κB in macrophages and suggest that increased PI3K-AKT and A20 activity may contribute to this process. However, we still did not know how Tim-3 regulation of these effects was accomplished, as Tim-3 does not contain any obvious inhibitory signaling motifs (44). To investigate the Tim-3 signaling cascade further, a gene chip was used to examine changes in gene expression after Tim-3 knockdown in RAW264.7 cells (series entry: GSE42729, www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42729). Approximately 300 differentially expressed genes were present in the knockdown line compared with controls. Of these, TLRs, such as TLR1, and cytokines, such as TNF-α, were found to be at least twice as abundant in Tim-3 knockdown RAW264.7 cells as in control cells (Fig. 8A). These genes covered a wide range of cellular functions, such as binding and response to bacterial products (CD36) (45) and T cell recruitment (CXCL16, CD48) (46). However, we were intrigued by the unexpected finding that expression of IFN regulating factor (IRF)-7 was 2-fold higher in Tim-3 knockdown RAW264.7 cells (Fig. 8A), as IRF-7 has been reported to be a possible inducer of type I IFN.
downstream of the TLR (47). We therefore selected IRF-7 for further study to investigate whether it was related to Tim-3–mediated inhibition of the TLR4 response. Tim-3 knockdown and Tim-3–overexpressing RAW264.7 cells, showing, respectively, increased and decreased IRF-7 mRNA levels (Fig. 8B, 8C, left panels), were assessed for production of type I IFN after LPS stimulation, and the results showed that Tim-3 signaling actively decreased levels of mRNAs for the type I IFNs IFN-β and IFN-α4 (Fig. 8B, 8C, center and right panels). We then examined whether Tim-3 blockade affected the expression of type I IFNs in vivo in mice with sepsis and found increased IRF-7 mRNA levels in both CLP mice (Fig. 8D) and LPS-injected mice (Fig. 8E) injected with anti–Tim-3 mAb, compared with controls injected with IgG, but only a moderate and nonsignificant increase in IFN-α4 and IFN-β1 mRNA levels (data not shown). The primer sequences for targeted cDNAs are listed in Table I. As there are data showing that TLR4-mediated upregulation of type I IFN contributes to the immune suppression in sepsis (10), our data provide another possible mechanism by which Tim-3 inhibits IRF-7/IFN-α expression and promotes homeostasis of sepsis, thus preventing subsequent immune suppression.

The mechanisms by which Tim-3 negatively regulates the TLR response and promotes homeostasis of sepsis are summarized in Fig. 9.

Discussion
In this study, we first examined whether the Tim-3 pathway was involved in the regulation of sepsis, a condition characterized by immune disorders. Our findings that downregulation and/or blockade of Tim-3 correlated with severity of sepsis demonstrate that Tim-3 is an important negative regulator of sepsis. We also showed that Tim-3 negatively regulated the TLR response by suppressing NF-κB activation, thus helping prevent the excessive inflammatory response in sepsis and attenuating sepsis. This negative regulatory role of Tim-3 indicates that this pathway might be a new target for intervention in the treatment of sepsis.

The Tim family of genes was first identified in 2001. In mice, there are eight Tim genes, Tim1–8, and three of these—Tim1, Tim3, and Tim4—are conserved in humans (48, 49). Like other Tim members, Tim-3 is one of the type I membrane proteins, which share a characteristic IgV, mucin, transmembrane, and cytoplasmic domain structure. Tim-3 has received much attention because...
its dysregulation has been linked to many clinical diseases (20–23). However, it is not known whether Tim-3 plays a role in sepsis, a fatal condition that is the leading cause of death in intensive care units (5, 26). We first examined whether this is the case, and, if so, how Tim-3 is involved in the regulation of sepsis. The finding that Tim-3 expression was dysregulated during the progression of sepsis both in humans and in a sepsis mouse model suggested that Tim-3 is involved in the progression of sepsis. To test this further, both an antagonist mAb against Tim-3 and soluble Tim-3 protein were used to examine Tim-3 involvement in the progression of sepsis. Intervention in the Tim-3 pathway using either method exacerbated sepsis, as shown by a decreased survival rate and increased production of proinflammatory cytokines (Fig. 1, Supplemental Fig. 1). These data demonstrate that Tim-3 promotes the homeostasis of sepsis by negatively regulating the inflammatory response. The expression of genes involved in the inflammatory process (i.e., cytokines) was measured at the mRNA level rather than as secreted protein in the blood, as real-time-PCR is considerably more sensitive than methods used to measure protein, such as ELISA. IL-1α, IL-6, and HMGB1 are the major proinflammatory cytokines that contribute to the excessive inflammatory response in sepsis (50). Unexpectedly, Tim-3 blockade in vivo did not significantly affect TNF-α expression, but increased IL-10 expression. Our in vivo findings are supported by recent reports that blockade of Tim-3 signaling in monococytes results in little change in TNF-α production, but increased IL-10 production (51, 52). The increased IL-10 expression after Tim-3 blockade in vivo might explain the nonsignificant upregulation of TNF-α in vivo, as IL-10 is known to downregulate TNF-α expression (53). IL-10 is known to be an anti-inflammatory cytokine, but has also been linked to impaired bacterial clearance in sepsis (54), and elevated IL-10 expression in critically ill patients has been correlated with a poor outcome from sepsis (55). Thus, blockade of Tim-3 upregulation of IL-10 expression may not be beneficial but, rather, detrimental. In addition, we also found increased TLR2 and TLR4 expression in splenocytes from anti–Tim-3 mAb-injected CLP mice, indicating that Tim-3 might maintain the homeostasis of sepsis by directly suppressing expression of TLR2 and TLR4. However, the underlying mechanisms by which Tim-3 regulates TLR2 and TLR4 expression remain to be investigated.

After demonstrating involvement of the Tim-3 pathway in the regulation of sepsis in vivo, two questions arose. One was how Tim-3 works in vivo to maintain the homeostasis of sepsis, and the other was why Tim-3 is downregulated in the acute or severe stage of sepsis. To answer these questions, we examined macrophage activity in CLP mice following Tim-3 blockade, as macrophage activation represents an important triggering event in the development of infectious and inflammatory diseases. Tim-3 blockade significantly enhanced the responsiveness of macrophages in vivo (Fig. 3), suggesting that the macrophage is an important target for Tim-3–mediated negative regulation. We also examined whether Tim-3 was expressed on neutrophils and found a population of Tim-3+ CD11b+Ly-6G+ cells (neutrophils) in PLF from septic mice (data not shown), explaining why two populations of Tim-3–expressing cells were found in PLF (Fig. 1F). However, the role for Tim-3–mediated signals in neutrophils in the pathogenesis of the sepsis remains to be determined. In addition, as Tim-3 silencing in RAW264.7 cells in vitro led to enhanced macrophage activation in response to TLR stimulation, we demonstrated that Tim-3 signaling negatively regulated macrophage activation by inhibiting the TLR response. To our knowledge, this is the first report demonstrating that Tim-3 can negatively regulate the macrophage response by downregulating the TLR response.

Another question we addressed is why Tim-3 is downregulated in the progression of sepsis. Although many papers have reported a correlation between dysregulated Tim-3 expression and clinical disease, the answer to why Tim-3 is dysregulated is largely unclear. In this study, for the first time to our knowledge, we found that LPS can bidirectionally regulate Tim-3 expression on macrophages (Fig. 5). Although these data partially explain the downregulation of Tim-3 during the progression of sepsis, the biological significance of LPS-regulated Tim-3 expression still needs to be determined. In the progression of sepsis, low-dose endotoxin, such as low-dose LPS, would promote macrophage activation and increase Tim-3 expression. Normally, Tim-3 acts as a homeostasis maintainer to prevent an excessive inflammatory response and protect the host from autoimmune damage. However, high-dose or long-lasting LPS sends a signal to the host to amplify the inflammatory response against infection. Thus, by some unknown mechanism, LPS causes downregulation of Tim-3, thus inhibiting its ability to maintain homeostasis and leading to an enhanced inflammatory response to fight a long-lasting infection, but, at the same time, causing host damage. Our data demonstrated that the LPS/TLR4 pathway is involved in the regulation of Tim-3 expression and its regulatory activity. Although we found that Tim-3 signaling also inhibited other TLR-mediated responses, such as those mediated by TLR1, TLR2, TLR6, and TLR9, the data showing that Tim-3 blockade in TLR4KO mice did not significantly affect the progression of sepsis compared with that in wild-type mice (Fig. 6) indicate that TLR4 plays important roles in Tim-3–mediated negative regulation.

Furthermore, we examined the intracellular cascade by which Tim-3 signaling inhibits the TLR response. Unlike other negative regulators of T cell function (e.g., PD-1), Tim-3 does not contain any obvious inhibitory signaling motifs (42), and little is known about Tim-3 signaling in T cells and other immune cells. In this article, we demonstrated that Tim-3 negatively regulates macrophage activation by inhibiting LPS/TLR4–induced NF-κB activation, in which Tim-3 signaling–enhanced PI3K-AKT activation and A20 activity might contribute to this process. Several reports have shown that P13K is involved in suppression of NF-κB activation. For example, Fukao (39) suggested that an excessively activated P13K pathway causes Th1 suppression by inhibiting NF-κB activation, and Guha (40) reported that activation of the PI3K pathway in monocytes suppresses both the MAPK and NF-κB cascade responses to LPS, resulting in decreased TNF-α production. Although our data demonstrated that enhanced P13K-AKT activation accounts for Tim-3–inhibited macrophage activation, the precise mechanism by which it inhibits TLR4 signaling remains obscure. A report has shown that AKT overexpression can decrease both the IRAK-mediated increase in NF-κB activation and the binding of IRAKs to MyD88. In such a scenario, Tim-3 signaling, by a still unknown mechanism, would enhance activation of P13K-AKT, which then inhibits LPS–TLR4–induced NF-κB activation by inhibiting IRAK activity or the binding of IRAKs to MyD88.

A20 (TNF-α–induced protein 3) has been investigated as a negative regulator of the LPS/TLR4 response. A20 is required for the termination of TLR-induced NF-κB activation and proinflammatory gene expression in macrophages by suppressing TRAF6 activity, thus protecting mice from endotoxic shock (56). Our data shown in Fig. 7 suggest that A20 might be differently regulated by Tim-3 under nonstimulated and stimulated conditions. Initially, Tim-3 inhibits expression of A20, a negative regulator of the TLR response, and this may help maintain the cells in a “ready to respond” state. However, following stimulation, Tim-3 switches from inhibiting A20 expression to promoting
of potential importance in modulating the innate responses in these of sepsis. This negative regulatory role represents a novel adaptive
ulator of TLR-mediated immune responses in the pathophysiology
utes to immune suppression must still be determined.

3 signaling did suppress expression of INF-α and INF-β in macrophages in vitro. The roles of type 1 IFN in the progression of sepsis remain unclear. A recent report showing that TLR4 signaling upregulates type 1 IFN and contributes to immune suppression suggests a pathogenic role for type I IFN in the progression of sepsis (10). In addition, type I IFN has been found to have deleterious effects during the early inflammatory phase of sepsis (57, 58). Whether blockade and/or downregulation of Tim-3 also exacerbates sepsis by enhancing type 1 IFN activity and contributes to immune suppression must still be determined.

In summary, our study, to our knowledge, has revealed a previously unrecognized role for Tim-3 as a physiologic negative regulator of TLR-mediated immune responses in the pathophysiology of sepsis. This negative regulatory role represents a novel adaptive compensatory and protective mechanism in sepsis victims, a finding of potential importance in modulating the innate responses in these patients.

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Disclosures

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