Sepsis Mortality in CIITA Deficient Mice is Associated with Excessive Release of High-mobility Group Box 1

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Background: Down regulation of major histocompatibility complex class II transactivator (CIITA) has been identified as a major factor of immunosuppression in sepsis and the level of CIITA expression inversely correlates with the degree of severity. However, it has not been fully elucidated whether the lower expression of CIITA is a cause of disease process or a just associated sign. Here we determined whether the CIITA deficiency decreased survival rate using murine sepsis model.

Methods: Major histocompatibility complex class II (MHC-II) deficient, CIITA deficient and wild type B6 mice were subjected to cecal ligation puncture (CLP) surgery. CIITA and recombination activation gene (RAG)-1 double deficient mice were generated to test the role of lymphocytes in CIITA-associated sepsis progression.

Results: Sepsis mortality was enhanced in CIITA deficient mice, not by impaired bacterial clearance resulted from CD4 T cell depletion, but hyper-inflammatory response such as excessive release of a pro-inflammatory cytokine, high-mobility group box 1 (HMGB1).

Conclusion: Our results demonstrate that CIITA deficiency affects the course of sepsis via the unexpected function of CIITA, regulation of cytokine release.


INTRODUCTION

Sepsis is a major cause of death in intensive care units, accounting for over 200,000 deaths per year in the United States alone. Despite the recent advances in intensive care treatment and the discovery of antibiotics, sepsis remains associated with a high mortality rate (1,2). Although commonly initiated by an infection, the pathogenesis of sepsis is characterized by an overwhelming systemic inflammatory response and subsequent immune dys-function, which can lead to lethal multiple organ failure (3). Endotoxin and other pathogen components stimulate macrophages to produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α. Although TNF-α can cause a lethal systemic inflammation and neutralization of TNF-α can prevent the development of septic shock in animal model, TNF-α blocking therapy produced modest clinical effects in critically ill patients (4). Recently, it has been reported that other late pro-inflammatory mediators may contribute to the progression of sepsis (5).

High-mobility group box 1 (HMGB1) was originally described as a nuclear non-histone DNA binding protein that functions as a structural cofactor critical for proper transcriptional regulation. It also facilitates numerous nuclear transactions including replication, recombination, and DNA transposition. Recently, it has been reported that HMGB1 can be secreted into the extracellular milieu by activated macrophages and identified as a late mediator of lethal systemic inflammation in sepsis (6). Several reports suggest that HMGB1 is a sufficient and necessary mediator of sepsis (5-9).

MHC Class II transactivator (CIITA), a non-DNA binding co-activator, is a critical regulator for major histocompatibility complex class II (MHC-II) and other genes related to antigen presentation (10,11). Recently, it has been reported that CIITA can influence the expression of a variety of genes that are involved in distinct functions, such as interleukin (IL)-4 (12), collagen α2 (13,14), Fas ligand (15), and plexin A1 (16). In sepsis patients, the expression of MHC-II molecules is reduced, which is mediated by the down-regulation of its

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transactivator CIITA. Interestingly, these lower expressions of MHC-II and CIITA correlates with disease severity (17,18).

Here we show that the CIITA deficiency enhances sepsis mortality, accompanied with hyper-inflammatory response such as excessive HMGB1 release. The impairment of bacterial clearance or the enhanced cellular apoptosis had no effects on the sepsis mortality. Instead, we demonstrate that cytokine dys-regulation affects sepsis outcome in the CIITA deficiency.

MATERIALS AND METHODS

Mice

MHC-II$^{-/-}$, recombination activation gene (RAG)-1$^{-/-}$ and CIITA$^{-/-}$ mice (all on a C57BL6 background, H-2$^b$ haplotype) were purchased from The Jackson Laboratory (Bar Harbor, ME). For some experiments CIITA$^{-/-}$ mice were bred onto a RAG-1$^{-/-}$ background. All the mice were maintained and crossed under specific pathogen-free conditions at the animal facility of Hallym University College of Medicine. Experiments were performed after receiving approval of the Animal Experimentation Committee at Hallym University.

Sepsis model

Cecal Ligation and Puncture (CLP). For CLP, mice were anesthetized with tribromoethanol (Sigma; St. Louise, MO). A small laparotomy was performed to allow exposure of the cecum. The cecum was mobilized and ligated below the ileocecal valve, punctured through both surface once with a 21-or 23- (for less severe CLP) gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites to ensure patency. The cecum was replaced into the peritoneal cavity and the abdominal incision was closed. Survival was monitored and recorded for 7 days (19).

Determination of colony-forming units (CFUs)

10 $\mu$l of blood and peritoneal lavage fluid were diluted serially in sterile physiological saline. 10 $\mu$l of each dilution was aseptically plated and cultured on tryptose blood agar plates (BD Biosciences; San Jose, CA) at 37°C. After 24 h, the number of bacterial colonies was counted. Quantitative cultures are expressed as CFUs per 10 $\mu$l of blood or peritoneal lavage fluid.

RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen: Carlsbad, CA) and reverse transcribed into complementary DNA using Superscript (Invitrogen). Primer sequences were as follows: b-actin, 5'-CACCAGCTGTTATCTCTCAGTC-3' and 5'-CGGGACGTGGAGCCAGAGGAG-3'; Mip2, 5'-AGTGAACCTGGCCTGCAATG-3' and 5'-TTTGGTTCCTCGGTTGAGG-3'; Cxcl1, 5'-CTGGGATTTCACCTCAAGAACCACCACTGAGG-3' and 5'-TTACTGGGGGACACTTCTTGCAGAACTC-3'; Cxcl5, 5'-CTGCCGCT- TGCAGGCTAGCTCATA-3' and 5'-TGACATTCCGCTTAGCTTTG-3'; Tnf, 5'-GCCAGAGTCCTTGAAGCTTGAAGTAACAG-3' and 5'-CAATTAGGGCTAGGTCCTGCAGAACTC-3'; Il6, 5'-GCCAGAGTCCTGCTAGGCT- TAGACACCACCACTGAGGAG-3'; Il10, 5'-GCCAGAGTCCTGCTAGGCT- TAGACACCACCACTGAGGAG-3'; Il12, 5'-GCCAGAGTCCTGCTAGGCT- TAGACACCACCACTGAGGAG-3'; Ifnb, 5'-CTTCTCCACCCACACCGCTCTCATA-3' and 5'-CCCAACACCTGCTTGCAGCAA-3'; Ifng, 5'-GATGCTCTTCGACCTCGAACATCAGCAT-3' and 5'-GATGCTCTTCGACCTCGAACATCAGCAT-3'. The PCR products were electrophoresed on a 1.5% agarose gel.

Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL)

Tissues from CLP or sham operated mice were rapidly excised and placed in 10% neutral buffered paraformaldehyde. Paraffin-embedded tissue slices were dewaxed, rehydrated and stained apoptotic nuclei with DNA strand breaks according to manufacturer’s instructions using an apoptosis detection kit (Takara: Shiga, Japan).

Measurement of cytokines. Concentrations of TNF-$\alpha$, IFN-$\beta$, IFN-$\gamma$, IL-4, IL-6, IL-10, and IL-12 were measured using ELISA kits (BD pharmingen; San Diego, CA or R&D systems; Minneapolis, MN) and according to the manufacturer’s instructions. The level of HMGB1 was determined by immunoblotting using anti-HMGB1 antibodies (Upstate Biotechnology; Lake Placid, NY or Abcam; Cambridge, MA) and quantified by densitometric scanning of the exposed X-ray film. The data were normalized to 12 hours -stimulated CIITA deficient sample giving a relative densitometric value of 1.

Western blotting

Samples of peritoneal lavage fluid or culture supernatant were concentrated with protein concentration beads (Elips Biotech: Daejon, Korea) or centrifric (Millipore: Billerica, MA) and then separated on 12% SDS-PAGE gel, transferred to nitrocellulose
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Figure 1. CIITA\textsuperscript{−/−} mice are more susceptible to sepsis. Sepsis is induced using the CLP model of polymicrobial infection and survival was recorded. (A) Whereas almost all of CIITA\textsuperscript{−/−} mice succumbed to sepsis in the first 72 hours after CLP, 61% of wild type B6 mice survived. The sepsis mortalities of anti-CD4 antibody treated (B) and MHC-II\textsuperscript{−/−} mice (C) were comparable to control mice. (D) Absence of lymphocyte did not affect the survival rate of CIITA\textsuperscript{−/−} mice. Wild type and mutant mice were backcrossed with RAG-1\textsuperscript{−/−} mice to exclude the effect of lymphocytes and subject to CLP surgery.

Figure 2. CIITA\textsuperscript{−/−} mice are not defective in the capacity of clearing bacterial pathogens or recruiting leukocytes. Peripheral blood (A) and peritoneal lavage fluid (B) are collected indicated time and 10\textsuperscript{μl} of serial dilutions are plated on blood agar plates. Data represent bacterial counts in individual mice. (C) Neutrophil numbers in peritoneal lavage fluid do not show a significant difference between B6 and CIITA\textsuperscript{−/−} mice. Data represent the average of neutrophil numbers in 5 different high power fields (magnification: 200 ×; *, p=0.0220). (D) The levels of chemokine expression were also similar between B6 and CIITA\textsuperscript{−/−} mice.

RESULTS
CIITA\textsuperscript{−/−} Mice Are More Susceptible to Experimental Sepsis. The association of lowered expression of CIITA with severity score and death in sepsis patients leads us to examine the response of CIITA\textsuperscript{−/−} mice in a murine model of polymicrobial sepsis. To this end, we applied the cecal ligation and puncture (CLP) model that closely mimics human sepsis (20) to CIITA\textsuperscript{−/−} mice and monitored their survival rate. Whereas most of the wild type B6 mice were resistant to sepsis, almost all of CIITA\textsuperscript{−/−} mice succumbed to sepsis within 3 days after CLP surgery (Fig. 1A, B6 vs CIITA\textsuperscript{−/−}, p<0.0001), indicating that the presence of CIITA renders the mice resistant to sepsis. In a less severe sepsis model (23G puncture), CIITA\textsuperscript{−/−} mice were also more susceptible to sepsis (B6 vs CIITA\textsuperscript{−/−}, p=0.0097; n=8, 12 for each group). Since the presence of CIITA is an essential part of MHC II expression and subsequent CD4 T cell selection, next we examined sepsis survival in CD4 T cell depleted mice. Mice were intraperitoneally injected twice with anti-CD4 antibody (clone: GK1,5) or rat sera and subjected to CLP surgery (We confirmed CD4 T cell depletion in GK1,5 treated mice with FACS analysis, data not shown). Interestingly, both mice did not show any difference in survival rate (Fig. 1B, rat sera vs GK1,5 treatment, p=0.8595). We also did the same experiment using MHC-II deficient mice and did not find significant differences between two groups (Fig. 1C, B6 vs MHC-II\textsuperscript{−/−}, p=0.0902). Finally, we performed CLP surgery in lymphocyte-deficient RAG-1\textsuperscript{−/−} (RAG-1 KO) and CIITA\textsuperscript{−/−} plus...
RAG-1\(^{-/-}\) (CIITA/RAG-1 DKO) mice, to exclude the difference in lymphocyte profiles. While 34% of Rag-1 KO mice succumbed to sepsis in the first 21.5 hours after surgery, all of DKO mice died during the same period (Fig. 1D, RAG-1 KO vs CIITA/RAG-1 DKO, \(p=0.0006\)). The above data suggest the early mortality shown in CIITA\(^{-/-}\) mice is caused by the absence of CIITA itself, not by absence of MHC-II molecule or the subsequent immunodeficiency.

**Bacterial clearance or neutrophil recruitment are not impaired in CIITA\(^{-/-}\) mice**

To explore the mechanisms responsible for the survival disadvantage of CIITA\(^{-/-}\) mice, we measured blood and peritoneal bacterial contents. Both wild type B6 and CIITA\(^{-/-}\) mice showed comparable numbers of bacterial colony-forming units. In addition, we did not find any marked differences in neutrophil numbers in peritoneal fluid (Fig. 2A-C). To confirm the competence of neutrophil recruitment, we compared the levels of various chemokines using RT-PCR assay and could not find significant differences between B6 and CIITA\(^{-/-}\) mice (Fig. 2D). The above findings suggest that the higher mortality in CIITA\(^{-/-}\) mice is not due to the impairment of bacterial clearance (immunodeficiency), consistent with CLP survival data (Fig. 1D).

**Apoptosis levels between B6 and CIITA\(^{-/-}\) mice treated by CLP**

Previously it was reported that the Fas ligand expression was greatly increased in CIITA\(^{-/-}\) mice and forced expression of CIITA reduced Fas ligand expression and subsequent apoptotic cell death (15,21). In addition, since sepsis induces apoptosis and inhibition of apoptosis improves sepsis survival (22), we tested whether more cells undergo apoptosis in CLP-treated CIITA\(^{-/-}\) mice. At 12 hours after CLP surgery, mice were sacrificed and the level of apoptosis was examined with Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL) staining method (Fig. 3). In sepsis, the TUNEL method demonstrated brown-colored nuclei positively stained for apoptosis in many organs from both mice. Thymi from both mice were the most extensively stained tissues and greater than half of the cells were apoptotic in many fields. The degree of apoptosis in thymus from CIITA\(^{-/-}\) mice was not more than that in septic B6 mice. In other organs, we could not observe any significant differences between B6 and CIITA\(^{-/-}\) mice (spleen, colon and liver). No evidence of apoptosis was seen in lungs from both mice.

**Early and excessive release of HMGB1 in CIITA\(^{-/-}\) mice**

Finally, we sought to probe cytokine responses that might be affected by CIITA. Peritoneal fluid was aspirated 3, 6, and 12 hours after CLP surgery and analyzed for cytokine productions. At early time points, small and transient differences in the levels of certain cytokines were detected. In CIITA\(^{-/-}\) mice, IL-12 were increased, whereas anti-in-
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Figure 4. Cytokine patterns in peritoneal lavage fluids (A∼F) or macrophage culture supernatants (G and H) of wild type and CIITA−/− mice. Mice are treated with CLP and peritoneal fluids are collected for the measurement of various cytokines. (A) IL-10; (B) IL-12; (C) IL-6; (D) TNF-α; (E) HMGB-1; (F) representative data from HMGB-1 western blotting. Macrophages from CIITA−/− mice do not release larger amount of HMGB1 in the presence of LPS or E. coli stimulation. Data are expressed as mean±SEM. *, p<0.05; **, p<0.01.

Flamatory cytokine IL-10 were decreased (Fig. 4A and B). The levels of IL-6 and TNF-α were indistinguishable between two groups (Fig. 4C and D) and IFN-γ and IL-4 were below the detection ranges (data not shown). In contrast to these transient changes, the levels of HMGB1 were consistently elevated in CIITA−/− mice after the stimulation of LPS, live bacteria (E. coli) or IFN-γ. Unexpectedly, macrophages isolated from CIITA−/− mice did not produce significantly larger amount of HMGB1 compared to wild type cells (Fig. 4G and H). Although we also examined endotoxin or E. coli-induced HMGB1 translocation, an essential step for HMGB1 release, nuclear HMGB1 were translocated to the cytoplasm in both B6 and CIITA−/− mice in the same degree (data not shown).

DISCUSSION

Leukocytes from many critically ill patients show a low level of MHC-II, partly through the suppression of CIITA expression (18,23). However, it remains to be elusive whether the expression levels of CIITA or MHC-II contribute to sepsis progression. In the present study, CIITA−/− mice showed more accelerated sepsis progression and increased mortalities, which is independent on MHC-II molecules or CD4 T cells. Interestingly, while bacterial clearance was not changed significantly, the levels of HMGB1 were consistently higher in CIITA−/− mice. These data suggest that increased HMGB1 might contribute to higher mortalities in CIITA−/− mice. To find the cellular source of HMGB1 in CIITA−/− mice, we generated CIITA and RAG-1 double mutant mice (to exclude the role of lymphocytes) and isolated MHC-II expressing cells such as macrophages and dendritic cells (data not shown) and then tested HMGB1 release. However, macrophages from B6 control or CIITA−/− mice released HMGB1 in the same degree (Fig. 4G and H), which suggests that CIITA deficiency does not directly affect LPS or bacteria-induced HMGB1 release. There are two ways in which HMGB1 can release into extracellular space. One is an active process; the other is a passive process in which HMGB1 diffuses out of dying cells (6). Although an active process is dominant in LPS treated macrophages, both active and passive processes could play roles in vivo settings. These findings led us to consider whether CIITA deficiency affects cell death and then contributes to the HMGB1 release indirectly, although apoptosis levels were not changed in CIITA−/− mice (Fig. 3). In addition, it could be also possible that the cells expressing CIITA in IFN-γ-dependent manner contribute to the HMGB1 release. Probably this speculation could be verified using various CIITA promoter deficient mice (11).

It has been reported that IL-10 protects mice from lethality by attenuating the development of a systemic inflammatory response by a mechanism that involves inhibition of TNF re-
lease (24-26). Interestingly, the level of IL-10 was transiently decreased in CIITA−/− mice. In addition, bacterial clearance and neutrophil recruitment to the peritoneal cavity were slightly enhanced in CIITA−/− mice (Fig. 2, although the differences were not statistically significant), which were also shown in IL-10−/− mice. However, the levels of MIP-2 (Fig. 2D) and TNF (Fig. 4D) were indistinguishable between B6 control and CIITA−/− mice, suggesting that the reduced IL-10 in CIITA−/− mice partially contributes to enhanced bacterial clearance and neutrophil recruitment.

Recent papers suggest that CIITA expression is inversely correlated with sepsis severity and the level of HMGB1 is higher in sepsis non-survivors. Based on these findings from human patients and our experimental data, we assumed that in severe sepsis patients the level of CIITA would be lower and HMGB1 concentration would be more elevated, compared to mild sepsis patients.

In summary, our results demonstrate that CIITA deficiency affects the course of sepsis via the unexpected function of CIITA, regulation of cytokine release. We anticipate further studies reveal the cellular sources and molecular mechanisms of CIITA-associated HMGB1 secretion.

REFERENCES

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