



# IL-15 Improves Aging-Induced Persistent T Cell Exhaustion in Mouse Models of Repeated Sepsis

Saito, Masafumi ; Inoue, Shigeaki ; Yamashita, Kimihiro ; Kakeji, Yoshihiro ; Fukumoto, Takumi ; Kotani, Joji

---

(Citation)

Shock, 53(2) :228-235

(Issue Date)

2020-02

(Resource Type)

journal article

(Version)

Accepted Manuscript

(Rights)

© 2019 by the Shock Society

(URL)

<https://hdl.handle.net/20.500.14094/90008020>



**(a) Manuscript title**

IL-15 improves aging-induced persistent T cell exhaustion in mouse models of repeated sepsis

**(b) Author's name and affiliation**

Masafumi Saito<sup>1,2</sup>, Shigeaki Inoue<sup>1,2\*</sup>, Kimihiro Yamashita<sup>3</sup>, Yoshihiro Kakeji<sup>3</sup>, Takumi  
Fukumoto<sup>4</sup>, Joji Kotani<sup>2</sup>

1. Department of Emergency and Critical Care Medicine, Tokai University School of  
Medicine

2. Department of Disaster and Emergency and Critical Care Medicine, Kobe University  
Graduate School of Medicine

3. Kobe University Division of Gastrointestinal Surgery Department of Surgery Graduate  
School of Medicine Kobe Japan

4. Department of Hepato-Biliary-Pancreatic Surgery, Kobe University Graduate School of  
Medicine, Kobe, Japan

**(c) Corresponding author**

\*Shigeaki Inoue

Professor

17 Dept of Disaster and Emergency Medicine Kobe University. Graduate School of Medicine

18 Kusunoki-cho 7-5-2, Chuo-ward, Kobe, Japan

19 Tel: +81-78-382-6521; Fax: +81-78-341-5254; E-mail: inoues@med.kobe-u.ac.jp

20 **(d) Conflict interest**

21 The authors have no conflict of interest.

22 **(e) Running head**

23 IL-15 improves persistent T cell exhaustion

24

## ABSTRACT

Aging is a grave problem in sepsis, and T cell exhaustion is the main cause of sepsis-induced immunosuppression. Sepsis- and aging-induced T cell exhaustion is related to secondary infection with a poor long-term outcome in the elderly. However, the trend, impact, and mechanism of T cell exhaustion are still unclear. Interleukin (IL)-15 improves survival rate of septic mice via its anti-apoptotic effect on T cells, however, it's still unclear how IL-15 reverses prolonged T cell exhaustion in aged septic mice. The purpose of this study was to clarify the trend of sepsis-induced T cell exhaustion and whether IL-15 prevents aging-induced persistent T cell exhaustion in septic mice. Preserved cecal slurry was injected intraperitoneally into young (6-week-old) and aged mice (18- to 24-month-old) four times, to induce clinically-relevant repeated sepsis. IL-15 (1.5  $\mu$ g) or PBS was injected subcutaneously three times, body weight was serially measured, and peripheral blood cells from their cheek were serially collected for 50 days. Sepsis-induced T cell exhaustion was significantly severe in aged mice than in young mice and was accompanied with decreased naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells ( $p < 0.01$ ) and increased expression of program death 1 on T cell ( $p < 0.01$ ) and regulatory T cell population ( $p < 0.01$ ). IL-15 significantly improved sepsis-induced T exhaustion, with significantly increased numbers of NK cells and macrophages,

41 and significantly enhanced phagocytosis activity in aged septic mice ( $p < 0.05$ ). It decreased the  
42 long-term mortality associated with sepsis survivors by improving T cell exhaustion over an  
43 extended duration and also ameliorated aging-induced persistent T cell exhaustion in septic mice.

44

## INTRODUCTION

Sepsis is defined as a life-threatening condition associated with multi-organ dysfunction originating due to the dysregulated innate immune response against pathogen infections (1) and affects more than 19 million people each year (2). Nearly 60% septic patients are  $\geq 65$  years of age (3), and their 3-month survival rate is significantly low than that of adult patients aged 18-64 years (4). Advances in treatment at intensive care unit (ICU) have improved the short-term prognosis of sepsis patients; however, sepsis continues to have a poor prognosis especially in elderly patients.

Several studies revealed that sepsis can cause immunosuppression, which may lead to ICU-acquired infections. Elderly septic patients have secondary infections at 2 to 4 weeks after initial ICU admission (5). In line with this evidence, reduced number of immunocompetent T cells and persistent lymphopenia were observed in elderly septic patients (6). They also displayed increased numbers of circulating regulatory T cells (Treg), which negatively regulate the host immune response, and expression of programmed death-1 (PD-1) on these T cells. This phenomenon of T cell dysfunction induced by sepsis is called “T cell exhaustion” (7), which appears to be more

severe in elderly patients. However, the precise mechanisms of induction of severe and persistent T cell exhaustion in elderly septic patients and recovery from this status are not known and are immunotherapeutic targets in sepsis (8-9).

Interleukin (IL)-15 is an attractive therapeutic target in sepsis because it plays an essential role in the development and homeostasis of naive CD8<sup>+</sup> T cells, memory T cells and natural killer (NK) cells, which have a key role in pathogen elimination (10-13). In addition, our previous study revealed that IL-15 inhibited CD8<sup>+</sup> T cell apoptosis and improved survival rate in septic mice (14).

Although very few studies have been conducted, the above mentioned facts indicate the contributory role of IL-15 to improve T cell exhaustion on a long-term prognosis. Clinically relevant septic model was induced by using cecal slurry (CS) model, which is established by Wynn et al. (15) and modified by Starr et al. (16) in this study. Cecal ligation and puncture (CLP) is regarded as the “gold standard” for establishing an experimental sepsis model in rodents because it closely mimics the clinical course of intra-abdominal sepsis. However, variability in this model has been shown as severity of sepsis is highly dependent on the cecal content (e.g quantity or microflora), wound-healing capability, skill of the researcher (e.g accuracy or rapidity), etc. (15, 17). Unlike CLP model, CS model can induce intra-abdominal sepsis by injection of

suspended cecum contents, therefore, this model may have high reproducible method regardless of the skill of researcher or individuality of mice. This advantage of in CS method enables us to mimic the repeated clinically-relevant infection with minimally invasive technique on mice.

The first purpose of this study was to clarify the trend of sepsis-induced T cell exhaustion among young and aged mice with clinically-relevant repeated sepsis over 50 days. The second purpose was to investigate the effects of IL-15 on sepsis-induced T cell exhaustion in young and aged septic mice over an extended duration.



## MATERIALS AND METHODS

### *Chemicals and recombinant mouse IL-15*

Ammonium chloride (NH<sub>4</sub>Cl), Tris-hydroxymethyl aminomethane (Tris-HCl) and glycerol were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Nacali Tesque (Kyoto, Japan). Recombinant mouse IL-15 was purchased from PeproTech (Rocky Hill, NJ, USA).

### *Antibodies (Abs)*

All Abs were purchased from Biolegend (San Diego, CA, USA). Mouse Abs used for this were as follows: PerCP/Cy5.5-conjugated mouse anti-CD4, PerCP/Cy5.5-conjugated mouse anti-NK1.1, APC/Cy7-conjugated mouse anti-CD8, APC/Cy7-conjugated mouse anti-F4/80, fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD25, FITC-conjugated mouse anti-Ly6C, PE-conjugated mouse anti-CD62L, PE/Cy7-conjugated mouse anti-CD279 (Programed death-1; PD-1), APC-conjugated mouse anti-CD127 (IL-7 Receptor- $\alpha$ ), APC-conjugated mouse anti-CD11b, and Pacific blue-conjugated mouse anti-Ly6G. Mouse Fc-blocker was procured from BD Pharmingen (San Jose, CA, USA).

*Animals and housing*

**Experiment 1.** Young (5-weeks old; n = 19) and aged (18-24 months old; n = 19) female

C57BL6/J mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed in

groups of 4 to 6 per cage and allowed to acclimatize for a week before the initiation of the test.

Mice were maintained under specific pathogen-free conditions with a 12 h light-dark cycle in the

Department of Laboratory Animal Science at Tokai University. The experimental protocol was

approved by the Institutional Animal Care and Use Committee at Tokai University (#181068 and

#181070). All experiments followed the recommendations of the International Expert Consensus

Initiative for Improvement of Animal Modeling in Sepsis as previously described (18).

**Experiment 2.** In another experimental set, young female and male (5-weeks old; 10

females, 10 males) and 18-month old female C57BL6/J mice (n = 10) were obtained from CLEA

Japan, Inc. (Tokyo, Japan). The mice were housed in groups of four per cage and allowed to

acclimatize for a week before the initiation of the test. Mice were maintained under specific

pathogen-free conditions with a 12-h light-dark cycle in the Department of Laboratory Animal

Science at Kobe University. The experimental protocol was approved by the Institutional Animal

Care and Use Committee at Kobe University (#P180806-R1).

117 *Preparation of cecal slurry*

118 Cecal slurry (CS) was prepared as described previously by Starr et al. (16). Briefly, male  
119 ICR mice (6 to 8 week old) were scarified and whole cecums were harvested. Mice cecums were  
120 snicked, transferred to nylon-mesh bag, 1 - 2 mL of sterile water was poured and filtered twice.  
121 The mixture was collected and centrifuged at 11,000 rpm for 1 min. The supernatant was  
122 discarded and the residue was suspended in 30% glycerol with a final concentration 0.5 mg/mL.  
123 CS (400 to 500  $\mu$ L) was transferred to cryotubes and stored at -80 °C till use.

124 *Study design*

125 **Experiment 1.** CS (50  $\mu$ L) was injected four times peritoneally into young (5-weeks old;  
126 n = 19) and aged (18-24 months old; n = 19) female mice on day 0, 4, 7 and 10 to induce sepsis,  
127 and simultaneously 1.5  $\mu$ g of IL-15 or PBS was also injected consecutively three times on day 3,  
128 7 and 10 (Figure 1A). Body weight was serially measured, and peripheral blood cells were  
129 collected from their cheek nine times within 50 days.

130 **Experiment 2.** Young female and male mice and aged female mice were sacrificed at day  
131 12. PBMCs and spleen were harvested to investigate cell population dynamics. Peritoneal lavage  
132 was also collected from each mouse to investigate the phagocytic capacity of peritoneal

macrophages and to estimate bacterial load. This experiment was conducted as described in Figure 1A, except for the timing of sacrifice (Supplemental Figure 1A).

#### ***Phagocytosis assay***

The mice were anesthetized and their peritoneal lavage fluid was collected with 10 mL of phosphate buffered saline containing 0.1% BSA (0.1% BSA /PBS). Peritoneal exudate cells ( $2 \times 10^5$ /100  $\mu$ L) were incubated with 100  $\mu$ L of 0.2 mg/ml pHrodo™ *Escherichia coli* BioParticles™ conjugate for phagocytosis (REF:P35361; Life Technologies, Carlsbad, CA, USA) for 30 min at 37°C. After a 10-min incubation with Fc blocker, the cells were stained to identify F4/80<sup>+</sup> macrophages and the expression of intra-cellular PE-labeled *E. coli* was quantified by flow cytometry using a FACS Verse device (BD Biosciences, San Jose, CA, USA).

#### ***Bacterial colony counting***

The mice were anesthetized and lavage of their peritoneal cavities was carried out using 10 mL of warm saline on day 12. Peritoneal lavage fluids were placed in sterile vials and equal volumes were used for bacterial culture. Ten-fold serial dilutions of each lavage fluid were made and cells were plated; next, colonies were counted after 24 h of incubation.

#### ***Flow cytometric analysis of murine T cell, NK cell and macrophage distribution***

Blood samples were collected from mice cheek, and murine peripheral blood mononuclear cells (PBMC) were isolated by density gradient cell separation using Histopaque1083™ (Sigma-Aldrich, St Louis, MO, USA). Separated PBMC were treated with red blood cell lysis buffer

containing 139.5 mM NH<sub>4</sub>Cl and 1.7 mM Tris-HCl (pH 7.65) at 37°C for 10 min, and then washed with 0.1% BSA /PBS. Murine PBMC were incubated with Abs mixture for 30 min at 4°C after treating with mouse Fc-blocker to block non-specific binding sites. Stained cells were analyzed using FACS Verse. The proportion of the designated cell fraction was determined by recording 10,000 events (Figure 1B), and data files were analyzed using the FlowJo software (Tree Star, OR, USA).

Mice were gently sacrificed by cervical dislocation under anesthesia after peripheral blood samples had been collected. Each spleen was surgically removed and separated by gently pressing the organs through a 70-micron filter. The collected spleen cells were washed using 0.1% BSA/PBS washed and the red blood cells were lysed as well as the blood samples. After treating with mouse Fc-blocker, the spleen cells were incubated with the mixture of Abs for 30 min at 4°C and analyzed using the FACS Verse device.

Collected mouse peritoneal lavage fluids were aseptically collected, treated with red blood cell lysis buffer at 37°C for 10 min, and centrifuged at 300×g for 5 min. The samples were washed with 0.1% BSA /PBS and incubated with the mixture of Abs for 30 min at 4°C after treating with mouse Fc-blocker. Stained cells were analyzed using the FACS Verse device.

169     *Statistical analysis*

170             Statistical analysis was performed using the EZR statistical software (19). Group  
171     differences in body weight and T cell distribution were assessed by repeated-measures ANOVA.  
172     For survival studies, a log rank test was used.  $P < 0.05$  was considered as statistically significant.  
173     Results are presented as mean  $\pm$  SD. Two-way analysis of variance (ANOVA) was performed to  
174     determine the main effects of IL-15 (treated versus non-treated mice) and age (young versus aged),  
175     as well as the interaction between these two factors.

176

## RESULTS

### *Aging induced lower body weight and survival rate in clinically-relevant repeated sepsis model*

To investigate sepsis-induced chronic immunosuppression in Experiment 1, a repeated sepsis model was established by injection of CS into mice (Figure 1A). The reaction produced was characteristic of the induction of mild inflammatory responses. Body weight drastically decreased after the initial injection of CS in both young and aged mice. However, unlike that in aged septic mice, body weight of young mice was recovered until day 10 after the initial injection of CS. Notably, administration of IL-15 prevented the initial reduction of body weight in young septic mice at day 3 (Figure 1B). In the case of aged septic mice, their body weight loss persisted for over 50 days (Figure 1B). Although IL-15 administration attenuated persistent body weight loss in aged septic mice, there were no statistically significant differences with or without IL-15 administration.

Survival rates in young slurry and aged slurry group mice were 91.7% (11/12) and 69.2% (9/13) respectively (Figure 1C). All IL-15-administered mice survived, but the differences in with or without IL-15 administration groups were not statistically significant (young mice:  $p = 0.41$ ; aged mice:  $p = 0.15$ ; Figure 1C).

***IL-15 reversed aging-induced CD4<sup>+</sup> and naïve CD4 T cell reduction in septic mice***

To investigate whether IL-15 administration influenced CD4<sup>+</sup> T cells in septic mice, we monitored CD4<sup>+</sup> T cell and subpopulations in peripheral blood (Figure 2A). As shown in Figure 2B, the distribution of CD4<sup>+</sup> T cell was consistently lower in aged septic mice for at least 50 days than in young septic mice ( $p < 0.01$ ). Although CD4<sup>+</sup> T cells were observed, and the frequencies were not significantly different between two young septic mice groups ( $p = 0.07$ ), the IL-15-treated aged septic mice showed a significant increase in CD4<sup>+</sup> T cells than non-treated mice ( $p = 0.03$ ). In addition, in both IL-15-treated young and aged septic mice, naïve CD4<sup>+</sup> T cell population was significantly higher than that in non-treated mice (young mice:  $p < 0.01$ ; aged mice:  $p < 0.01$ ; Figure 2C).

***IL-15 inhibited aging-induced increase of PD-1 on CD4<sup>+</sup> T cells and Treg in septic mice***

To investigate whether IL-15 improves sepsis-induced T cell exhaustion, we analyzed the population of PD-1<sup>+</sup> CD4<sup>+</sup> T cells and Treg cells, which are well known hallmarks of T cell exhaustion. Flow cytometric analysis revealed that PD-1<sup>+</sup> CD4<sup>+</sup> T cell population drastically increased after primary injection of CS in both aged and young septic mice (Figure 2D). PD-1 expression was sustained in CD4<sup>+</sup> T cells of aged septic mice as compared to young septic mice



(Figure 2D). In addition, Treg population as well as PD-1<sup>+</sup>CD4<sup>+</sup> T cells of aged septic mice were also consistently higher than those in young septic mice ( $p < 0.01$ ; Figure 2E). Meanwhile, IL-15 inhibited the increase in these cell populations especially in aged septic mice. PD-1<sup>+</sup>CD4<sup>+</sup> T cell population was consistently low in IL-15-administered aged septic mice as compared with aged slurry mouse group. Importantly, in this group, PD-1<sup>+</sup>CD4<sup>+</sup> T cells were significantly down-regulated at day 3 after the initial injection of CS ( $p < 0.01$ ; Figure 2D). Furthermore, Treg cell population was also consistently down-regulated in IL-15-administered aged septic mice compared to non-treated aged septic mice ( $p < 0.01$ ; Figure 2E).

#### ***IL-15 reversed aging-induced naïve CD8 T cells reduction in septic mice***

We examined whether IL-15 prevents CD8<sup>+</sup> T cell exhaustion in septic mice over an extended duration. In young septic mice, the frequency of CD8<sup>+</sup> T cell population upon IL-15-administration was consistently higher than that in not-treated young septic mice ( $p = 0.03$ ; Figure 3A). In contrast, IL-15 did not influence the frequency of CD8<sup>+</sup> T cells in the peripheral blood of aged septic mice ( $p = 0.91$ ; Figure 3A). In addition, the frequency of naïve CD8 T cells was significantly higher in IL-15-treated aged septic mice in comparison with non-treated aged septic mice group ( $p < 0.01$ , Figure 3B), as well as in young septic mice ( $p < 0.05$ , Figure 3B).

***IL-15 inhibited aging-induced increase of PD-1<sup>+</sup> CD8<sup>+</sup> T cells population in aged septic mice***

Flow cytometric analysis revealed that PD-1<sup>+</sup>CD8<sup>+</sup> T cell population drastically increased after primary injection of CS in both aged and young septic mice, as well as PD-1<sup>+</sup>CD4<sup>+</sup> T cell (Figure 3C). Although PD-1<sup>+</sup> CD8<sup>+</sup> T cell was not significantly difference in young septic mice, this cell population was down-regulated in IL-15-administered aged septic mice compared to non-treated aged septic mice ( $p < 0.01$ ; Figure 3C). All the results are summarized in Table 1.

***IL-15 up-regulated splenic CD4<sup>+</sup> T cell and NK cells in aged septic mice***

To investigate whether IL-15 effects splenic immune cells in septic mice, we sacrificed young and aged septic mice 12 days after the initial injection of CS in Experiment 2 (Supplemental Figure 1A). The frequency of splenic CD4<sup>+</sup> T cells was significantly higher in IL-15-treated mice in both young and aged mice in comparison with the non-treated group ( $p < 0.05$ , Supplemental Figure 1B). Although CD8<sup>+</sup> T cells tended to increase in the spleens of IL-15-treated mice, no significant difference was evident ( $p = 0.07$ , data not shown).

IL-15 activates and maintains NK cells (10-13). Thus, we next investigated whether IL-15 could increase NK cells in aged septic mice on day 12. As we anticipated, flow cytometry analysis revealed a significant increase in NK cells in spleens of IL-15-treated aged septic mice compared

to non-treated mice ( $2.5 \pm 0.1\%$  vs  $2.0 \pm 0.4\%$ ,  $p = 0.02$ ; Supplemental Figure 1B). In addition, IL-15 tended to increase the frequency of NK cells in PBMCs in aged female mice. However, the increase was not statistically significant ( $6.2 \pm 3.9\%$  vs  $2.4 \pm 0.9\%$ ,  $p = 0.11$ , data not shown). We also investigated the expression of markers of activated NK cells (CD25, CD69, and CD107a). The expressions of these markers were not significantly different when IL-15 was administered or not administered in both young and aged septic mice (data not shown).

#### ***IL-15 increased circulate macrophage and enhanced phagocytosis activity in aged septic mice***

We examined the phagocytic activity and bacterial clearance in IL-15 treated mice. First, we analyzed the distribution of macrophages in PBMCs in septic mice. We observed that the frequency of macrophages was statistically different between young and aged septic mice ( $p < 0.01$ , Supplemental Figure 1C). Moreover, significantly more circulating macrophages were evident in IL-15-treated aged septic mice than in non-treated aged mice ( $p = 0.03$ , Supplemental Figure 1C). We next examined whether IL-15 affected phagocytic activity, which was defined as the percentage of cells with one or more engulfed PE-conjugated *E. coli* beads within the phagocytic cell population in peritoneal lavage. The phagocytic activity of aged mice was significantly reduced compared to young septic mice ( $p < 0.01$ , data not shown). The phagocytic

cell population was significantly enhanced in IL-15-treated aged septic mice in comparison with non-treated aged septic mice ( $p = 0.01$ , Supplemental Figure 1B). Finally, we investigated the effectiveness of IL-15 on bacterial colony formation. No bacterial colonies formed after 24 h storage at 37°C of peritoneal lavage of IL-15-treated aged septic mice. However, there was no statistically significant difference between the treated and non-treated groups (Supplemental Figure 1E).

We sought to determine the levels of IL-6, IL-10, interferon-gamma (IFN- $\gamma$ ), and tumor necrosis factor-alpha (TNF- $\alpha$ ) in the plasma of septic mice using cytometric bead flow cytometry assay. However, these cytokines were under the limit of detection in all mice in this model (data not shown).

## DISCUSSION

Changes in T cell function and the relative proportions with aging are well known, and these age-related immunological changes involve the decline of immune response against new pathogens in elderly people (20-21). Consistent with this evidence, our results revealed that the frequency of CD4<sup>+</sup> T cells and naïve CD4<sup>+</sup> T cells in aged mice significantly decreased in comparison with young mouse at day 0 (Figure 2B and 2C). Additionally, we also showed that the frequency of PD-1 expressing CD4 and Treg (Figure 2D and 2E) and CD8 T cell (Figure 3B and 3C) was significantly higher in aged mouse than in young mouse. These findings imply that aging is associated with increased susceptibility to and severity of infection.

Recent studies reported that sepsis is associated with a greater risk of long-term mortality (22-24). Interestingly, Prescott et al. reported only a 5-year survival rate of 20% for severe sepsis patients (24). One of the main reasons of long-term mortality in sepsis is a persistence of T cell exhaustion. In addition, our previous studies demonstrated that T cell exhaustion is one of the causes in elderly sepsis patients suffering from secondary infections (4-5). Therefore, establishing strategies to rescue T cell exhaustion is important to prevent long-term mortality on sepsis (7-8).

Hence, our data is important to provide evidence that sepsis-induced T cell exhaustion might be more prolonged than previously assumed. In the case of young septic mice, their body weight and CD8<sup>+</sup> T cell populations were recovered for 50 days (Figure 1B and 3A). On the contrary, CD4<sup>+</sup> T cells of young septic mice were only recovered to approximately 81% of the initial frequency on the last day (day0:  $45.6 \pm 2.4\%$  → day50:  $37.0 \pm 2.8\%$ , Figure 2B). These results indicate that host immune function needs time to recover from sepsis induced T cell exhaustion even in young mice. In aged septic mice, we observed consistent reduction of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 2B and 3A), and up-regulation of Treg and PD-1<sup>+</sup> T cells during 50 days (Figure 2D, 2E and 3C). These results suggest that elderly septic mice show severe and prolonged T cell exhaustion than young septic mice, leading to secondary infection after sepsis in elderly survivors. Rossi et al. have published several studies regarding lymphopenia with skewed differentiation of hematopoietic stem cells in the elderly (25-26). This age-dependent skewed distribution of aged HSCs may be related to the reduction of T cells in the elderly.

The term exhaustion has been used to describe the state of functional unresponsiveness, replicative senescence, and ultimate physical deletion of T cells during chronic infection in mice and humans (4,7,27). It is also well known that the population of PD-1<sup>+</sup>CD4<sup>+</sup> T cells increases

300 with aging (28). This T cell anergy retains the capacity to produce low levels of cytokine such as  
301 IL-2 and interferon- $\gamma$  (29), explaining one of the reasons why elderly people have high  
302 susceptibility to infection. However, our study revealed that sepsis increased PD-1 expression for  
303 only a limited period of time during CS injection; the frequency of this cell population increased  
304 after initial injection of CS, and maintained at high level up to day 10, but decreased and returned  
305 to initial level by day 14 (Figure 2D, and 3C). On the contrary, the frequency of circulating Treg  
306 was maintained at a consistently high level in both young and aged mice with CS-induced sepsis  
307 during the period (Figure 2E). Recent studies have shown that Treg is the main contributor to the  
308 induction and maintenance of immunosuppression (30-31), rather than PD-1 expression on T cells  
309 during sepsis. Taken together, these results suggest that the PD-1 expressed T cell population  
310 might have only a limited contribution to sepsis-induced “persistent” T cell exhaustion, whereas  
311 Treg cell is the one of the key players in the persistent T cell exhaustion. Since our data showed  
312 that IL-15 could inhibit a consistently high level of Treg distribution in peripheral blood in both  
313 young and aged septic mice (Figure 2E), IL-15 has a potential to improve sepsis-induced  
314 persistent T cell exhaustion and reinvigorate immune response against pathogens in aged mice.  
315 Nascimento et al. revealed that IL-33 has a major function in the induction of sepsis-induced long-

term immunosuppression via expansion of Treg, type 2 macrophages, and type 2 innate lymphoid cells (31). Therefore, we further examined whether IL-15 was associated with the production of Treg inducible cytokines like IL-10, IL-33, and transforming growth factor- $\beta$ , and how IL-15 can inhibit the expansion of Treg populations during sepsis.

IL-15 is secreted primarily by dendritic cells, monocytes, and epithelial cells during infection. Since it shows IL-2-like activity, IL-15 is not only essential for development and activation on T cells and NK cells (10-13), but also it can prevent sepsis-induced T cell apoptosis by regulating of apoptotic associated molecules (14). Thus, it has attracted attention as a potential therapeutic for patients with chronic infection including sepsis (14). In fact, no deaths occurred in IL-15-treated young and aged septic mice (Figure 1C). In addition, IL-15 significantly inhibited reduction of CD4<sup>+</sup> T cell (Figure 2B), and continuously up-regulated naive CD4<sup>+</sup> and CD8<sup>+</sup> T cell in aged septic mice after injection of CS (Figure 2C and 3B). Furthermore, PD-1 expression was suppressed on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and Treg cell expansion by IL-15 treatment in aged septic mouse group (Figure 2D, 2E and 3C). Taken together, IL-15 could improve sepsis-induced T cell exhaustion on long-term prognosis and reinvigorate immune response against pathogen in aged mice, and it might prevent and reduce the risk of secondary infection.



Recent studies have revealed that PD-1 signaling inhibits phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway, which is involved in T cell proliferation, development and activation, and results in T cell dysfunction (32). On the other hand, IL-15 activates PI3K/Akt pathway via activation of Janus kinase (JAK)-3/Signal transducers and activators of transcription (STAT)-5 pathway in lymphocyte (33). We further examine whether IL-15 up-regulates Akt phosphorylation on exhausted T cells. It is still unclear why administration of IL-15 in acute phase of sepsis improve aging-induced long-term persistence of T cell exhaustion. We did not anticipate that IL-15 would inhibit sepsis-induced immunosuppression over an extended duration because it has a short half-life *in vivo* (34). As clinically important, administration of IL-15 in acute phase of sepsis could prevent the sepsis-induced severe immunosuppression, and results in the possibility of reducing the risk of secondary infection. We further study to elucidate the molecular mechanism by which IL-15 prevents sepsis-induced immunosuppression for a long time.

Our data indicate that aged septic mice display more severe T cell exhaustion than young mice. The function of the acquired immune system decreases with age. This can be problematic since the initial response to sepsis may be important. Data have indicated that increased mortality is associated with a failure of protective immunity, with aged mice reported to display a failure of

innate immune response against sepsis, with no failure seen in young mice (35). Presently, IL-15 increased splenic NK cells, which play a role in initiating the host defense and coordinating innate and adaptive immune response by producing IFN- $\gamma$  and TNF- $\alpha$ , and also enhanced phagocytic activity in aged septic mice (Supplemental Figure 1B and 1D). These results indicate that the inhibition of severe and prolonged T cell exhaustion by IL-15 occurs via activation of innate immune cells, such as NK cells and macrophages in aged mice and results in an increased survival rate of aged septic mice from 70% to 100% (Figure 1C).

In conclusion, sepsis induced T cell exhaustion was more severe and prolonged in aged mice. IL-15 could improve sepsis-induced T exhaustion by increasing the frequency of naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cell distribution and down-regulating the expression of PD-1 on T cell and Treg population, with increasing NK cells and macrophage and phagocytosis activity in aged septic mice. IL-15 may potentially improve T cell exhaustion over an extended period.

## ACKNOWLEDGMENTS

This study was supported by the Teaching and Research Support Center of the School of Medicine of Tokai University. We are thankful to Dr. Yoshiaki Okada and Yumiko Iida for extensive technical support and advice on flow cytometric analysis. We are thankful to Nobuo Watanabe, Yoshiko Shinozaki, Katsuko Naito and Kayoko Iwao for extensive support during mouse-handling and observation of animal health.

We are thankful to Kazumichi Fujioka (Department of Pediatrics, Kobe University Hospital), Takashi Matozaki and Yasuyuki Saito (Division of Molecular and Cellular Signaling, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine) for extensive technical support and advice on flow cytometric analysis.

## REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, et al: The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 315 (8):801-810, 2016.
2. Fleischmann C, Scherag A, Adhikari NK, Hartog CS, Tsaganos T, Schlattmann P, Angus DC, Reinhart K: International Forum of Acute Care Trialists: Assessment of global incidence and mortality of hospital-treated sepsis: current estimates and limitations. *Am J Respir Crit Care Med* 193(3): 259-272, 2016.
3. Martin GS, Mannino DM, Eaton S, Moss M: The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 348:1546–1554, 2003.
4. Inoue S, Suzuki-Utsunomiya K, Okada Y, Taira T, Iida Y, Miura N, Tsuji T, Yamagiwa T, Morita S, Chiba T, Sato T, Inokuchi S: Reduction of immunocompetent T cells followed by prolonged lymphopenia in severe sepsis in in the elderly. *Crit Care Med* 41(3): 810-819, 2013.
5. Hotchkiss RS, Monneret G, Payen D: Sepsis-induced immunosuppression: from cellular

388 dysfunctions to immunotherapy. *Nat Rev Immunol* 13: 862–874, 2013.

389 6. Inoue S, Suzuki-Utsunomiya K, Okada Y, Iida Y, Taira T, Miura N, Tsuji T, Yamagiwa T,  
390 Morita S, Chiba T, et al: Reduction of immunocompetent T cells followed by prolonged  
391 lymphopenia in severe sepsis in the elderly. *Crit Care Med* 41:810–819, 2013.

392 7. Wherry EJ, Kurachi M: Molecular and cellular insights into T cell exhaustion. *Nat Rev*  
393 *Immunol* 15(8): 486-499, 2015.

394 8. Gentile LF, Cuenca AG, Efron PA, Ang D, Bihorac A, McKinley BA, Moldawer LL, Moore  
395 FA: Persistent inflammation and immunosuppression: A common syndrome and new horizon  
396 for surgical intensive care. *J Trauma Acute Care Surg* 72(6): 1491-1501, 2012.

397 9. Chang K, Svabeck C, Vazquez-Guillamet C, Sato B, Rasche D, Wilson S, Robbins P,  
398 Ulbrandt N, Suzich J, Green J, et al: Targeting the programmed cell death 1: programmed  
399 cell death ligand 1 pathway reverses T cell exhaustion in patients with sepsis. *Crit Care* 18:  
400 R3, 2014.

401 10. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, Ma A: IL-15 receptor  
402 maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation.  
403 *Immunity* 9: 669-676, 1998.

- 404 11. Berard M, Brandt K, Paus SB, and Tough DF: IL-15 promotes the survival of naive and  
405 memory phenotype CD8<sup>+</sup> T cells. *J Immunol* 170: 5018-5026, 2003.
- 406 12. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, Matsuki N, Charrier  
407 K, Sedger L, Willis CR, et al: Reversible defects in natural killer and memory CD8<sup>+</sup> T cell  
408 lineages in interleukin 15-deficient mice. *J Exp Med* 191(5):771–780. 2000.
- 409 13. Boyman O, Krieg C, Homann D, Sprent J: Homeostatic maintenance of T cells and natural  
410 killer cells. *Cellu Mol Life Sci* 69(10):1597–1608. 2012.
- 411 14. Inoue S, Unsinger J, Davis CG, Muenzer JT, Ferguson TA, Chang K, Osborne DF, Clark AT,  
412 Coopersmith CM, McDunn JE, et al: IL-15 prevents apoptosis, reverses innate and adaptive  
413 immune dysfunction, and improves survival in sepsis. *J Immunol* 184: 1401-1409, 2010.
- 414 15. Wynn JL, Scumpia PO, Delano MJ, O'Malley KA, Ungaro R, Abouhamze A, Moldawer LL:  
415 Increased mortality and altered immunity in neonatal sepsis produced by generalized  
416 peritonitis. *Shock* 28: 675-683, 2007.
- 417 16. Starr ME, Steele AM, Saito M, Hacker BJ, B. Evers BM, Saito H: A new cecal slurry  
418 Preparation protocol with improved long-term reproducibility for animal models of sepsis.  
419 *PLoS ONE* 9 (12): e115705, 2014.

- 420 17. Gentile LF, Nacionales DC, Lopez MC, Vazant E, Cuenca A, Cuenca AG, Ungaro R, Szpila  
421 BE, Larson S, Joseph A, et al: Protective immunity and defects in the neonatal and elderly  
422 immune response to sepsis. *J Immunol* 192:3156-3165, 2014.
- 423 18. Osuchowski MF, Ayala A, Bahrami S, Bauer M, Boros M, Cavaillon JM, Chaudry IH,  
424 Coopersmith CM, Deutschman C, Drechsler S, et al: Minimum quality threshold in pre-  
425 clinical sepsis studies (MQTiPSS): an international expert consensus initiative for  
426 improvement of animal modeling in sepsis. *Int Care Med Exp* 6:26, 2018.
- 427 19. Kanda Y: Investigation of the freely available easy-to-use software ‘EZR’ for medical  
428 statistics. *Bone Marr Transpla* 48: 452-458, 2013.
- 429 20. Weng NP: Aging of the immune system: how much can the adaptive immune system adapt?  
430 Immunity 24: 495-499, 2006.
- 431 21. Hakim FT Gress RE: Immunosenescence: deficits in adaptive immunity in the elderly. *Tissue*  
432 *Antigens* 70: 179-189, 2007.
- 433 22. Prescott HC, Langa KM, Iwashyna TJ: Readmission diagnoses after hospitalization for sever  
434 sepsis and other acute medical conditions. *JAMA* 313 (10):1055-1057, 2015.
- 435 23. Prescott HC, Osterholzer JJ, Langa KM, Angus DC, Iwashyna TJ: Late mortality after sepsis:

436 propensity matched cohort study. *BMJ* 353: i2375, 2016.

437 24. Iwashyna TJ, Ely EW, Smith DM, Langa KM: Long-term cognitive impairment and  
 438 functional disability among survivors of severe sepsis. *JAMA* 304 (16):1787-1794, 2010.

439 25. Rossi DJ, Seita J, Czechowicz A, Bhattacharya D, Bryder D, Weissman IL: Hematopoietic  
 440 stem cell quiescence attenuates DNA damage response and permits DNA damage  
 441 accumulation during aging. *Cell cycle* 6 (19) :2371-2376, 2007.

442 26. Rossi DJ, Bryder D, Weissman IL: Hematopoietic stem cell aging: Mechanism and  
 443 consequence. *Exp Gerontol* 42 (5): 385-390, 2007.

444 27. Gallimore A, Glithero A, Godkin A, Tissot AC, Pluckthun A, Elliott T, Hengartner H,  
 445 Zinkernagel R: Induction and exhaustion of lymphocytic choriomeningitis virus-specific  
 446 cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility  
 447 complex class I-peptide complexes. *J Exp Med* 187:1383–1393, 1998.

448 28. Shimada Y, Hayashi M, Nagasaka Y, Ohno-Iwashita Y, Inomata M: Age-associated up-  
 449 regulation of a negative co-stimulatory receptor PD-1 in mouse CD4<sup>+</sup> T cells. *Exp Gerontol*  
 450 44:517-522, 2009.

451 29. Shimatani K, Nakashima Y, Hattori M, Hamazaki Y, Minato N: PD-1<sup>+</sup> memory phenotype



- 452 CD4<sup>+</sup> T cells expressing C/EBP $\alpha$  underlie T cell immunodepression in senescence and  
453 leukemia. *Proc Natl Acad Sci USA* 106:15807-15812, 2009.
- 454 30. Venet F, Chung CH, Kherouf H, Geeraert A, Malcus C, Poitevin F, Bohe' J, Lepape A, Ayala  
455 A, Monneret G: Increased circulating regulatory T cells (CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup>) contribute to  
456 lymphocyte anergy in septic shock patients. *Intensive Care Med* 35: 678-686, 2009.
- 457 31. Nascimento DC, Melo PH, Pineros AR, Ferreira RG, Colon DF, Donate PB, Castanheira FV,  
458 Gozzi A, Czaikoski PG, Niedbala W, et al: IL-33 contributes to sepsis-induced long-term  
459 immunosuppression by expanding the regulatory T cell population. *Nat Commun* 8: 14919,  
460 2017.
- 461 32. Patsoukis N, Brown J, Petkova V, Liu F, Li L, Boussiotis VA: Selective effects of PD-1 on  
462 Akt and Ras pathways regulate molecular components of the cell cycle and inhibit T cell  
463 proliferation. *Sci Signal* 5: ra46, 2012.
- 464 33. Johnston JA, Bacon CM, Finbloom DS, Rees RC, Kaplan D, Shibuya K, Ortaldo JR, Gupta  
465 S, Chen YQ, Giri JD: Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus  
466 kinases by interleukins 2 and 15. *Proc Natl Acad Sci U S A* 92: 8705–8709, 1995.
- 467 34. Wong HC, Jeng EK, Rhode PR: The IL-15-based superagonist ALT-803 promotes the

468 antigen-independent conversion of memory CD8<sup>+</sup> T cells into innate-like effector cells with  
469 antitumor activity. *Oncoimmunol* 2 (11): e26442, 2013.

470 35. Nacionales DC, Gentile LF, Vanzant E, Lopez MC, Cuenca A, Cuenca AG, Ungaro R, Li Y,  
471 Baslanti TO, Bihorac A, et al: Aged mice are unable to mount an effective myeloid response  
472 to sepsis. *J Immunol* 192 (2): 612-622, 2014.

473

## Figure Legends

### **Figure 1. Administration of IL-15 attenuates body weight loss and improves survival in sepsis-induced mice.**

A) Schematic figure of the sepsis model generated using CS and treatment of IL-15 in this study (Experiment 1). Sepsis- induced female young (6 weeks-old) and aged (18-24 months-old) C57BL/6N mice were treated with or without 1.5  $\mu$ g of mouse recombinant IL-15 by subcutaneous injection at day 3, 7 and 10.

B) Effect of administration with/without IL-15 on body weight in young and aged mice. Young slurry: slurry-injected young mice without IL-15 treatment (n = 11, blue broken line and open circle); Young slurry+IL-15: slurry-injected young mice with IL-15 treatment (n = 8, blue line and closed circle). Aged slurry: slurry-injected aged mice without IL-15 treatment (n = 13, red broken line and open triangle); Aged slurry+IL-15: slurry-injected aged mice with IL-15 treatment (n = 6, red line and closed triangle).

C) Survival study of with/without IL-15-treated mice monitored for 50 days after sepsis induction.

The data are expressed as means  $\pm$  SD from three individual experiments. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

**Figure 2. Effect of IL-15 on CD4<sup>+</sup> T cells and its subpopulation after inducing sepsis.**

Peripheral blood was collected from their cheek at various timepoints and separated to perform FACS analysis of peripheral CD4<sup>+</sup> T cells. A) Gating strategy used for the identification of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and their subpopulations in mouse peripheral blood leukocytes. B) CD4<sup>+</sup> T cells, C) percentage of CD62L<sup>High</sup> within CD4<sup>+</sup> T cells: naïve CD4<sup>+</sup> T cells, D) PD-1<sup>+</sup> within CD4<sup>+</sup> T cells, and E) Tregs (CD25<sup>+</sup> CD127<sup>-</sup> within CD4<sup>+</sup> T cells) were analyzed by flow cytometry. Young slurry: slurry-injected young mice without IL-15 treatment (n = 11, blue broken line and open circle); Young slurry+IL-15: slurry-injected young mice with IL-15 treatment (n = 8, blue line and closed circle). Aged slurry: slurry-injected aged mice without IL-15 treatment (n = 13, red broken line and open triangle); Aged slurry+IL-15: slurry-injected aged mice with IL-15 treatment (n = 6, red line and closed triangle).

The data are expressed as the means  $\pm$  SD from three individual experiments. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

**Figure 3. Effect of IL-15 on CD8<sup>+</sup> T cell and its subpopulation after inducing sepsis**

Mice blood was from their cheek at indicated times and separated to perform FACS analysis of peripheral CD8<sup>+</sup> T cells. A) CD8<sup>+</sup> T cells, B) percentage of CD62L<sup>High</sup> within CD8<sup>+</sup> T cells: naïve CD8<sup>+</sup> T cells, and C) PD-1<sup>+</sup> within CD8<sup>+</sup> T cells were analyzed by flowcytometry. Young slurry: slurry-injected young mice without IL-15 treatment (n = 11, blue broken line and open circle); Young slurry+IL-15: slurry-injected young mice with IL-15 treatment (n = 8, blue line and closed circle). Aged slurry: slurry-injected aged mice without IL-15 treatment (n = 13, red broken line and open triangle); Aged slurry+IL-15: slurry-injected aged mice with IL-15 treatment (n = 6, red line and closed triangle). The data are expressed as the means ± SD from three individual experiments. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

**Supplemental Figure 1. IL-15 increases spleen CD4 T cells and NK cells and enhances phagocytosis activity in aged mice**

A) Schematic diagram of the sepsis model generated using CS and treatment of IL-15 (Experiment 2). Sepsis-induced, female, young (6 weeks-old) and aged (18 months-old) C57BL/6N mice were untreated or treated with 1.5 µg mouse recombinant IL-15 by subcutaneous

522 injection at day 3, 7, and 10. Young and aged mice injected 50  $\mu$ L CS and were sacrificed 12 days  
523 after (Experiment 2). B) Spleens were harvested, and the distribution of T cells and NK cells  
524 (defined as CD3<sup>-</sup>NK1.1<sup>+</sup>) in those tissues was analyzed by flow cytometry. C, D) Peritoneal lavage  
525 was aseptically collected. C) Proportion of macrophages in PBMCs. D) Separated peritoneal  
526 lavage cells were cultured with PE-labeled *E. coli* beads for 30 min to evaluate the phagocytic  
527 activity. E) Bacterial colonies were determined from serial dilutions of peritoneal lavage fluid.  
528 The data are expressed as the mean  $\pm$  SD from three individual experiments. \*,  $p < 0.05$ , \*\*,  $p$   
529  $< 0.01$