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

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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 μg) 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 $CD4^+$ and $CD8^+$ 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,

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

INTRODUCTION

Sepsis is defined as a life-threating 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 ≥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 programed 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

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

64 Interleukin (IL)-15 is an attractive therapeutic target in sepsis because it plays an essential
65 role in the development and homeostasis of naive $CD8^+$ T cells, memory T cells and natural killer
66 (NK) cells, which have a key role in pathogen elimination (10-13). In addition, our previous study
67 revealed that IL-15 inhibited $CD8^+$ T cell apoptosis and improved survival rate in septic mice (14).
68 Although very few studies have been conducted, the above mentioned facts indicate the
69 contributory role of IL-15 to improve T cell exhaustion on a long-term prognosis. Clinically
70 relevant septic model was induced by using cecal slurry (CS) model, which is established by
71 Wynn et al. (15) and modified by Starr et al. (16) in this study. Cecal ligation and puncture (CLP)
72 is regarded as the “gold standard” for establishing an experimental sepsis model in rodents
73 because it closely mimics the clinical course of intra-abdominal sepsis. However, variability in
74 this model has been shown as severity of sepsis is highly dependent on the cecal content (e.g
75 quantity or microflora), wound-healing capability, skill of the researcher (e.g accuracy or rapidity),
76 etc. (15, 17). Unlike CLP model, CS model can induce intra-abdominal sepsis by injection of

77 suspended cecum contents, therefore, this model may have high reproducible method regardless
78 of the skill of researcher or individuality of mice. This advantage of in CS method enables us to
79 mimic the repeated clinically-relevant infection with minimally invasive technique on mice.
80 The first purpose of this study was to clarify the trend of sepsis-induced T cell exhaustion
81 among young and aged mice with clinically-relevant repeated sepsis over 50 days. The second
82 purpose was to investigate the effects of IL-15 on sepsis-induced T cell exhaustion in young and
83 aged septic mice over an extended duration.
84

MATERIALS AND METHODS

Chemicals and recombinant mouse IL-15

Ammonium chloride (NH_4Cl), 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-α), APC-conjugated mouse anti-CD11b, and Pacific blue-conjugated mouse anti-Ly6G. Mouse Fc-blocker was procured from BD Pharmingen (San Jose, CA, USA).

101 ***Animals and housing***

102 **Experiment 1.** Young (5-weeks old; n = 19) and aged (18-24 months old; n = 19) female
103 C57BL6/J mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed in
104 groups of 4 to 6 per cage and allowed to acclimatize for a week before the initiation of the test.
105 Mice were maintained under specific pathogen-free conditions with a 12 h light-dark cycle in the
106 Department of Laboratory Animal Science at Tokai University. The experimental protocol was
107 approved by the Institutional Animal Care and Use Committee at Tokai University (#181068 and
108 #181070). All experiments followed the recommendations of the International Expert Consensus
109 Initiative for Improvement of Animal Modeling in Sepsis as previously described (18).

110 **Experiment 2.** In another experimental set, young female and male (5-weeks old; 10
111 females, 10 males) and 18-month old female C57BL6/J mice (n = 10) were obtained from CLEA
112 Japan, Inc. (Tokyo, Japan). The mice were housed in groups of four per cage and allowed to
113 acclimatize for a week before the initiation of the test. Mice were maintained under specific
114 pathogen-free conditions with a 12-h light-dark cycle in the Department of Laboratory Animal
115 Science at Kobe University. The experimental protocol was approved by the Institutional Animal
116 Care and Use Committee at Kobe University (#P180806-R1).

Preparation of cecal slurry

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

Study design

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

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

133 macrophages and to estimate bacterial load. This experiment was conducted as described in

134 Figure 1A, except for the timing of sacrifice (Supplemental Figure 1A).

135 ***Phagocytosis assay***

136 The mice were anesthetized and their peritoneal lavage fluid was collected with 10 mL of

137 phosphate buffered saline containing 0.1% BSA (0.1% BSA /PBS). Peritoneal exudate cells

138 (2×10^5/100 μL) were incubated with 100 μL of 0.2 mg/ml pHrodo™ *Escherichia coli*

139 BioParticles™ conjugate for phagocytosis (REF:P35361; Life Technologies, Carlsbad, CA,

140 USA) for 30 min at 37ºC. After a 10-min incubation with Fc blocker, the cells were stained to

141 identify F4/80$^+$ macrophages and the expression of intra-cellular PE-labeled *E. coli* was

142 quantified by flow cytometry using a FACS Verse device (BD Biosciences, San Jose, CA,

143 USA).

144 ***Bacterial colony counting***

145 The mice were anesthetized and lavage of their peritoneal cavities was carried out using

146 10 mL of warm saline on day 12. Peritoneal lavage fluids were placed in sterile vials and equal

147 volumes were used for bacterial culture. Ten-fold serial dilutions of each lavage fluid were made

148 and cells were plated; next, colonies were counted after 24 h of incubation.

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

150 Blood samples were collected from mice cheek, and murine peripheral blood mononuclear

151 cells (PBMC) were isolated by density gradient cell separation using Histopaque1083™ (Sigma-

152 Aldrich, St Louis, MO, USA). Separated PBMC were treated with red blood cell lysis buffer

153 containing 139.5 mM NH_4Cl and 1.7 mM Tris-HCl (pH 7.65) at 37ºC for 10 min, and then washed
154 with 0.1% BSA /PBS. Murine PBMC were incubated with Abs mixture for 30 min at 4ºC after
155 treating with mouse Fc-blocker to block non-specific binding sites. Stained cells were analyzed
156 using FACS Verse. The proportion of the designated cell fraction was determined by recording
157 10,000 events (Figure 1B), and data files were analyzed using the FlowJo software (Tree Star,
158 OR, USA).

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

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

Statistical analysis

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

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$^+$ and naïve CD4 T cell reduction in septic mice

To investigate whether IL-15 administration influenced CD4$^+$ T cells in septic mice, we monitored CD4$^+$ T cell and subpopulations in peripheral blood (Figure 2A). As shown in Figure 2B, the distribution of CD4$^+$ T cell was consistently lower in aged septic mice for at least 50 days than in young septic mice (p <0.01). Although CD4$^+$ 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$^+$ T cells than non-treated mice (p =0.03). In addition, in both IL-15-treated young and aged septic mice, naive CD4$^+$ 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$^+$ 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$^+$ CD4$^+$ T cells and Treg cells, which are well known hallmarks of T cell exhaustion. Flow cytometric analysis revealed that PD-1$^+$ CD4$^+$ 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$^+$ T cells of aged septic mice as compared to young septic mice

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

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

218 We examined whether IL-15 prevents CD8^{+} T cell exhaustion in septic mice over an
219 extended duration. In young septic mice, the frequency of CD8^{+} T cell population upon IL-15-
220 administration was consistently higher than that in not-treated young septic mice (p =0.03; Figure
221 3A). In contrast, IL-15 did not influence the frequency of CD8^{+} T cells in the peripheral blood of
222 aged septic mice (p =0.91; Figure 3A). In addition, the frequency of naïve CD8 T cells was
223 significantly higher in IL-15-treated aged septic mice in comparison with non-treated aged septic
224 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 $^{+}$ CD8^{+} T cells population in aged septic mice

Flow cytometric analysis revealed that PD-1^{+}CD8^{+} T cell population drastically increased after primary injection of CS in both aged and young septic mice, as well as PD-1^{+}CD4^{+} T cell (Figure 3C). Although PD-1^{+} CD8^{+} 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^{+} 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^{+} 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^{+} 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

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

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

248 We examined the phagocytic activity and bacterial clearance in IL-15 treated mice. First,
249 we analyzed the distribution of macrophages in PBMCs in septic mice. We observed that the
250 frequency of macrophages was statistically different between young and aged septic mice (p
251 <0.01, Supplemental Figure 1C). Moreover, significantly more circulating macrophages were
252 evident in IL-15-treated aged septic mice than in non-treated aged mice (p =0.03, Supplemental
253 Figure 1C). We next examined whether IL-15 affected phagocytic activity, which was defined as
254 the percentage of cells with one or more engulfed PE-conjugated *E. coli* beads within the
255 phagocytic cell population in peritoneal lavage. The phagocytic activity of aged mice was
256 significantly reduced compared to young septic mice (p <0.01, data not shown). The phagocytic

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

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

267

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 $CD4^+$ T cells and naïve $CD4^+$ 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).

284 Hence, our data is important to provide evidence that sepsis-induced T cell exhaustion might be
285 more prolonged than previously assumed. In the case of young septic mice, their body weight and
286 $CD8^+$ T cell populations were recovered for 50 days (Figure 1B and 3A). On the contrary, $CD4^+$
287 T cells of young septic mice were only recovered to approximately 81% of the initial frequency
288 on the last day (day0: 45.6 ± 2.4% → day50: 37.0 ± 2.8%, Figure 2B). These results indicate that
289 host immune function needs time to recover from sepsis induced T cell exhaustion even in young
290 mice. In aged septic mice, we observed consistent reduction of $CD4^+$ and $CD8^+$ T cells (Figure
291 2B and 3A), and up-regulation of Treg and PD-1^+ T cells during 50 days (Figure 2D, 2E and 3C).
292 These results suggest that elderly septic mice show severe and prolonged T cell exhaustion than
293 young septic mice, leading to secondary infection after sepsis in elderly survivors. Rossi et al.
294 have published several studies regarding lymphopenia with skewed differentiation of
295 hematopoietic stem cells in the elderly (25-26). This age-dependent skewed distribution of aged
296 HSCs may be related to the reduction of T cells in the elderly.
297 The term exhaustion has been used to describe the state of functional unresponsiveness,
298 replicative senescence, and ultimate physical deletion of T cells during chronic infection in mice
299 and humans (4,7,27). It is also well known that the population of PD-1^+CD4^+ 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-γ (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-

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

320 IL-15 is secreted primarily by dendritic cells, monocytes, and epithelial cells during
321 infection. Since it shows IL-2-like activity, IL-15 is not only essential for development and
322 activation on T cells and NK cells (10-13), but also it can prevent sepsis-induced T cell apoptosis
323 by regulating of apoptotic associated molecules (14). Thus, it has attracted attention as a potential
324 therapeutic for patients with chronic infection including sepsis (14). In fact, no deaths occurred
325 in IL-15-treated young and aged septic mice (Figure 1C). In addition, IL-15 significantly inhibited
326 reduction of $CD4^+$ T cell (Figure 2B), and continuously up-regulated naive $CD4^+$ and $CD8^+$ T cell
327 in aged septic mice after injection of CS (Figure 2C and 3B). Furthermore, PD-1 expression was
328 suppressed on $CD4^+$ and $CD8^+$ T cells, and Treg cell expansion by IL-15 treatment in aged septic
329 mouse group (Figure 2D, 2E and 3C). Taken together, IL-15 could improve sepsis-induced T cell
330 exhaustion on long-term prognosis and reinvigorate immune response against pathogen in aged
331 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-γ and TNF-α, 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 sever and prolonged in aged mice. IL-15 could improve sepsis-induced T exhaustion by increasing the frequency of naïve $CD4^+$ and $CD8^+$ 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.

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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 μg 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 ± SD from three individual experiments. *, $p < 0.05$, **, $p < 0.01$.

Figure 2. Effect of IL-15 on $CD4^+$ 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 $CD4^+$ T cells**.** A) Gating strategy used for the identification of $CD4^+$ and $CD8^+$ T cells and their subpopulations in mouse peripheral blood leukocytes. B) $CD4^+$ T cells, C) percentage of $CD62L^{High}$ within $CD4^+$ T cells: naïve $CD4^+$ T cells, D) PD-1^+ within $CD4^+$ T cells, and E) Tregs ($CD25^+$ $CD127^-$ within $CD4^+$ 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 ± SD from three individual experiments. *, p <0.05, **, p <0.01.

Figure 3. Effect of IL-15 on $CD8^+$ 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 $CD8^+$ T cells. A) $CD8^+$ T cells, B) percentage of $CD62L^{High}$ within $CD8^+$ T cells: naïve $CD8^+$ T cells, and C) PD-1^+ within $CD8^+$ 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

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