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Old Mice Demonstrate Organ Dysfunction as well as Prolonged Inflammation, Immunosuppression, and Weight Loss in a Modified Surgical Sepsis Model

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Abstract

Objectives: Our goal was to “reverse translate” the human response to surgical sepsis into the mouse by modifying a widely adopted murine intra-abdominal sepsis model to engender a phenotype that conforms to current sepsis definitions and follows the most recent expert recommendations for animal preclinical sepsis research. Furthermore, we aimed to create a model that allows the study of aging on the long-term host response to sepsis.

Design: Experimental study.

Setting: Research laboratory.

Subjects: Young (3–5 mo) and old (18–22 mo) C57BL/6j mice.

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Interventions: Mice received no intervention or were subjected to polymicrobial sepsis with cecal ligation and puncture followed by fluid resuscitation, analgesia, and antibiotics. Subsets of mice received daily chronic stress after cecal ligation and puncture for 14 days. Additionally, modifications were made to ensure that "Minimum Quality Threshold in Pre-Clinical Sepsis Studies" recommendations were followed.

Measurements and Main Results: Old mice exhibited increased mortality following both cecal ligation and puncture and cecal ligation and puncture + daily chronic stress when compared with young mice. Old mice developed marked hepatic and/or renal dysfunction, supported by elevations in plasma aspartate aminotransferase, blood urea nitrogen, and creatinine, 8 and 24 hours following cecal ligation and puncture. Similar to human sepsis, old mice demonstrated low-grade systemic inflammation 14 days after cecal ligation and puncture + daily chronic stress and evidence of immunosuppression, as determined by increased serum concentrations of multiple pro- and anti-inflammatory cytokines and chemokines when compared with young septic mice. In addition, old mice demonstrated expansion of myeloid-derived suppressor cell populations and sustained weight loss following cecal ligation and puncture + daily chronic stress, again similar to the human condition.

Conclusions: The results indicate that this murine cecal ligation and puncture + daily chronic stress model of surgical sepsis in old mice adhered to current Minimum Quality Threshold in Pre-Clinical Sepsis Studies guidelines and met Sepsis-3 criteria. In addition, it effectively created a state of persistent inflammation, immunosuppression, and weight loss, thought to be a key aspect of chronic sepsis pathobiology and increasingly more prevalent after human sepsis.

Keywords

chronic; inflammation; mouse; organ dysfunction; sepsis

Sepsis is currently the leading cause of death in U.S. hospitals and accounts for greater than $20 billion of total hospital costs in the United States (1, 2). Although sepsis can affect patients of all ages, older adults exhibit increased susceptibility and mortality to sepsis (3). Explanations for this predisposition in older adults largely revolve around differences in the host immune response, with studies supporting the concept of "immunosenescence," which is the inability of the aged immune system to mount an effective host-protective response to pathogens (4). There is evidence for deficiencies in both innate and adaptive immunity in the elderly, and our laboratory has revealed comparable immune dysfunction in old mice after severe infection (5).

Despite older adults being most vulnerable to sepsis, researchers frequently use young mice (i.e., 2–5 mo) to study sepsis and to develop potential immunotherapies (6–8). Although an appropriate and accepted approach to murine sepsis research, these young mice are equivalent to human postpubertal teens or young adults (9) and have increased survival compared with old mice when subjected to equivalent septic insults (10). In addition, it is understood that from progenitor hematopoietic stem cell to terminal effector leukocytes, the immune response to severe infection in older mammals deviates from the young (5). Thus, studying age-dependent differences is important in this era of precision and personalized

medicine and could potentially contribute to improvements in the translation of murine sepsis findings to humans (11, 12).

Importantly, human sepsis has now been redefined as “life-threatening organ dysfunction caused by a dysregulated host response to infection” (13). However, some rodent sepsis studies do not exhibit or confirm organ injury. Furthermore, improved in-hospital survival has yielded a rapidly expanding population of sepsis survivors who develop chronic critical illness (CCI) rather than succumbing to acute mortality (14). These survivors are often discharged to long-term acute care (LTAC) facilities, where they experience dismal outcomes, frequent readmissions, and exhibit approximately 40% post-discharge mortality at 1 year (15, 16). These outcomes are even worse for older adults (17). Our laboratory has described these patients as entering a state of persistent inflammation, immunosuppression, and cachexia/catabolism (PICS) (18). Despite this evolution in sepsis outcomes, many models of murine sepsis continue to focus primarily on the early hyperinflammatory phase (11, 12).

Experts in the field have stated that improved, reproducible, and standardized sepsis models will be a key aspect to the development and implementation of therapies to the disease (19). We hypothesized that it would be possible to “reverse translate” the human response to sepsis in mice to accomplish this goal (11). First, we evaluated our previous cecal ligation and puncture (CLP) model in both young and old mice to determine whether this model produces similar organ dysfunction. Second, we modified this model to guarantee adherence to the “Minimum Quality Threshold in Pre-Clinical Sepsis Studies (MQTiPSS): An International Expert Consensus Initiative for Improvement of Animal Modeling in Sepsis (6–8).” Finally, we added a component of daily chronic stress (DCS) to mimic the ICU stay of human patients who develop CCI. Using this novel model, we examined the physiologic and immunologic response in young and old mice with intra-abdominal surgical sepsis.

MATERIALS AND METHODS

Animals

Young (3–5 mo) and old (18–22 mo) C57BL/6j (B6) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were cared for by the University of Florida Animal Care Services (Gainesville, FL) and housed in transparent cages (four animals of same sex/age per cage) within specific pathogen-free facilities at ambient room temperatures (23°C). The animals were provided standard rodent chow and water ad libitum for the duration of the study. Prior to initiation of the experiment, mice were acclimated to the humidity-controlled housing room programmed for a 12-hour light-dark cycle for 1 week. The animals were cared for and used according to the Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC protocols: #201608141, #201807157, #201708454).

Experimental Design

Young and old female (50%) and male (50%) mice underwent CLP followed by DCS. Plasma cytokines, markers of organ dysfunction, and myeloid-derived suppressor cell

(MDSC) populations were compared with appropriate age-matched naive controls, DCS controls, or CLP only mice. Approximately eight to 12 mice were used per experimental group. Mice were euthanized at specified time intervals (8, 24, and 48 hr; 7 and 14 d) post CLP + DCS, whereas DCS or CLP-only mice were euthanized at 14 days. Experimental mice were euthanized in compliance with predetermined IACUC Body Condition Score (BCS) criteria. A modification to this included the following: greater than or equal to 20% weight loss from baseline or from age-matched controls if the animals are still growing during the study or greater than or equal to 20% weight loss from baseline in old mice who have a BCS of greater than 2 in the first 5 days after CLP, or greater than 40% weight loss from baseline in old mice with a BCS greater than 2 after 5 days following CLP. Based on these criteria, no mice had to be euthanized for excessive weight loss during the course of the study.

Intra-Abdominal Sepsis Model

CLP was conducted under isoflurane anesthesia as previously described (5). The cecum was ligated 1 cm from its tip and a 22- or 25-gauge needle was used to puncture the cecum depending on the desired experimental mortality. DCS-only mice underwent sham surgery, which involved a midline laparotomy with exteriorization of the cecum only. CLP-only mice had no subsequent DCS. Buprenorphine analgesia was provided for 48 hours postsurgery.

Antibiotics

Antibiotics (imipenem monohydrate; 25 mg/kg diluted in 1 mL 0.9% sodium chloride [NaCl]) were administered 2 hours post-CLP then continued bid for 72 hours. DCS-only mice received normal saline (0.9% NaCl) at similar time intervals.

DCS

DCS was conducted as previously described (20). Briefly, this involved placing mice in weighted plexiglass animal restraint holders (Kent Scientific, Torrington, CT) for 2 hours daily starting the day after CLP. The purpose was to simulate the ICU environment, in which patients are often bedbound with limited mobility. Episodic random noise was not used in our model as it was considered a potential confounder as B6 mice lose their hearing with age (21).

Weight and Blood Pressure Measurements

Baseline and postoperative weights were obtained in all murine subjects prior to kill at 8 hours, 24 hours, 48 hours, 7 days, and 14 days to determine the percent total body weight loss over the given time period. Daily weights were also recorded in mice subjected to CLP, DCS, or CLP + DCS. Noninvasive blood pressure (BP) measurements were collected as previously described using the CODA tail-cuff BP system (Kent Scientific, Torrington, CT), both at baseline and prior to kill at 24 hours (22).

Blood Sample Collection and Laboratory Analysis

Mice were euthanized following isoflurane inhalation, after which mixed arteriovenous blood was collected in a Becton Dickinson sodium heparin vacutainer tube (Franklin Lakes,

NJ) from the neck vasculature. Blood was analyzed for the following tests at the University of Florida College of Veterinary Medicine Clinical Pathology Laboratory: total bilirubin (mg/dL), alkaline phosphatase (U/L), alanine aminotransferase (U/L), aspartate aminotransferase (AST) (U/L), blood urea nitrogen (mg/dL), and creatinine (mg/dL). Complete blood counts with differential analysis were determined using the VetScan HM5 hematology analyzer (Abaxis, Union City, CA).

Plasma Cytokines

Human and mouse plasma was isolated as previously described (5, 17). Whole blood was centrifuged at 1,800*g* at 4°C for 10 minutes, then plasma was aliquoted and stored at −80°C until analysis. Concentrations of granulocyte colony-stimulating factor (G-CSF)/macrophage colony-stimulating factor (M-CSF)/granulocyte M-CSF (GM-CSF), interleukin-1α and β, interleukin-2, interleukin-4, interleukin-6, interleukin-10, interleukin-12p70, interleukin-17, tumor necrosis factor (TNF), interferon-γ, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and keratinocyte chemoattractant (KC) were determined using commercially available multiplexed Luminex kits (Millipore, Bellirica, MA). Similarly, within the human cohort, concentrations of G-CSF, GM-CSF, interleukin-6, interleukin-8, interleukin-10, interleukin-12p70, interferon-γ, TNF-α, MCP-1, MIP-1α, and interferon-γ–induced protein (IP)-10 were measured. All assays were performed according to the manufacturer's protocols, and cytokine concentrations were determined using BeadView software (Millipore, Bellirica, MA).

Lung Histopathology

Murine lungs were harvested from septic and control mice at 24 hours and processed as previously described (5, 23, 24). In brief, formalin-fixed lung tissue was embedded in paraffin wax and sectioned for routine histology. The tissue specimens were plated on slides, stained with hematoxylin and eosin, and evaluated by two board-certified pathologists in a blinded fashion. All slides were examined on an Olympus BX43 microscope using a 400× (numerical aperture 0.95) Olympus UPlanSApo magnification objective (Olympus, Center Valley, PA). An Olympus DP27 camera was used to capture the images, which were then acquired using Olympus cellSens standard software. Each specimen was examined for evidence of acute lung injury and assigned a lung injury score between 0 and 1 based on a previously published scoring system (24).

Flow Cytometry

Spleens were harvested from naive mice and septic mice at days 7 and 14. Single-cell suspensions were created by passing cells through 70-μm pore-sized cell strainers (BD Falcon, Durham, NC). Erythrocytes were then lysed using ammonium chloride lysis buffer and washed twice using phosphate-buffered saline. Cells were stained with the following antibodies for flow cytometric studies: PE Cy7 anti-CD11b, PB anti-Ly6C, APC Cy7 anti-Ly6G, PerCpCy5.5 anti-MHCII, FITC anti-F4/80, and APC anti-CD11c (BD Pharmigen, Billerica, MA). Sytox Blue (Invitrogen, Carlsbad, CA) was used for cell viability analysis, and samples were acquired and analyzed using a LSRII flow cytometer (BD Biosciences) and FACSDiva (BD Biosciences, San Jose, CA).

T Cell Suppression Assays

Similar to phenotyping, spleens were harvested from septic and nonseptic mice, and single cell suspensions were collected by passing the cells through 70-μm pore-sized cell strainers (BD Falcon, Durham, NC). T cells were collected by negative immunomagnetic selection using EasySep Mouse T Cell Isolation Kit (Stemcell Technologies, Vancouver, BC, Canada). MDSCs were enriched by positive immunomagnetic separation using anti-Gr-1 biotin followed by anti-Biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were stained with cell trace violet to detect cell proliferation then plated with and without MDSCs in a 1:1 ratio, stimulated with anti-$CD3^+$/$CD28^+$ plate-bound antibodies at a concentration of 1 μg/mL, and allowed to incubate for 4 days at 37°C. T cell proliferation was then measured in the presence and absence of MDSCs. The proliferation index and percent $CD4^+$ and $CD8^+$ T cell suppression were calculated for every murine subject at each time point.

Human Subjects

Human subject data were derived from a prospective observational study conducted at the University of Florida by the Sepsis and Critical Illness Research Center. The study was approved by the Institutional Review Board and the study design, procurement of data, and data analysis has been published elsewhere (25). The cohort included 102 surgical ICU patients who were either admitted with or subsequently developed sepsis. Written informed consent was obtained from all patients or legal proxy. Sepsis screening was performed using the Modified Early Warning Signs Sepsis Recognition System, and all patients who were enrolled were managed with standardized protocols based on the Surviving Sepsis guidelines. Blood samples were collected at 14 days after sepsis protocol onset and analyzed for plasma cytokine concentrations. Patients were determined to have one of three potential clinical trajectories: 1) early death—death within 14 days of sepsis protocol onset; 2) CCI—ICU length of stay greater than or equal to 14 days with evidence of persistent organ dysfunction, determined using components of the Sequential Organ Failure Assessment (SOFA) score; or 3) rapid recovery (RAP)—those who recovered and did not meet criteria for CCI or early death.

Statistical Analysis

Data were analyzed using Prism 7 (GraphPad Software, San Diego, CA). Results are reported as median ± interquartile range unless otherwise noted. Nonparametric Mann-Whitney *U* test was used to compare continuous variables, whereas a two-way analysis of variance with either Sidak or Tukey multiple-comparison test was used to compare the markers of organ dysfunction, cytokines, proportions of MDSCs, and weight loss based on age and timing after sepsis. Kaplan-Meier survival curves are presented with significance determined using log rank (Mantel-Cox) test.

RESULTS

Traditional CLP Reveals Increased Organ Dysfunction in Old Mice

In humans, sepsis-induced organ dysfunction is assessed using changes in SOFA score (Table 1) (13). Therefore, similar measures of organ dysfunction were examined in mice subjected to CLP. Initially, CLP was performed with a 22-gauge needle on both young and old mice and significant hemodynamic changes were observed after sepsis (Supplemental Fig. 1A, Supplemental Digital Content 1, http://links.lww.com/CCM/E820; and legend, Supplemental Digital Content 7, http://links.lww.com/CCM/E826). Although not significantly different between ages, young mice, on average, experienced a 40 mm Hg reduction in mean arterial pressure (MAP), whereas old mice experienced a 53 mm Hg decline in MAP after CLP.

Interestingly, only old septic mice developed hepatic and/or renal dysfunction after CLP with a 22-gauge needle (Supplemental Fig. 1B–D, Supplemental Digital Content 1, http://links.lww.com/CCM/E820; and legend, Supplemental Digital Content 7, http://links.lww.com/CCM/E826). Total bilirubin levels were also higher in old mice compared with their age-matched controls, although within the normal reported reference range (Supplemental Fig. 1E, Supplemental Digital Content 1, http://links.lww.com/CCM/E820; and legend, Supplemental Digital Content 7, http://links.lww.com/CCM/E826). Platelet counts in septic animals were similar to that of age-matched controls (Supplemental Table 1, Supplemental Digital Content 2, http://links.lww.com/CCM/E821).

Femoral artery cannulation under general anesthesia after CLP resulted in hemodynamic instability and mortality in old mice, preventing arterial blood gas/Pao_2-to-Fio_2 ratio measurements. Hence, histologic evaluation of the lung tissue was performed as a surrogate means to assess acute lung injury (Supplemental Fig. 2, Supplemental Digital Content 3, http://links.lww.com/CCM/E822; and legend, Supplemental Digital Content 7, http://links.lww.com/CCM/E826) (24). Twenty-four hours after CLP, no significant differences were observed in the acute lung injury score between young and old septic mice and their age-matched controls (Supplemental Fig. 2A, Supplemental Digital Content 3, http://links.lww.com/CCM/E822; and legend, Supplemental Digital Content 7, http://links.lww.com/CCM/E826). However, histologic differences were appreciated between septic and control mice (Supplemental Fig. 2, B–E, Supplemental Digital Content 3, http://links.lww.com/CCM/E822; and legend, Supplemental Digital Content 7, http://links.lww.com/CCM/E826).

Old Mice Have High Mortality After a Septic Insult

As previously demonstrated (5, 26), old mice have increased mortality versus young mice with similar CLP insults (Supplemental Fig. 1F, Supplemental Digital Content 1, http://links.lww.com/CCM/E820; and legend, Supplemental Digital Content 7, http://links.lww.com/CCM/E826). However, this acute mortality using a 22-gauge needle was too great for subacute and chronic analysis, as well as for evaluating PICS (27). Thus, we modified the model to reduce mortality and induce PICS while adhering to the MQTiPSS guidelines (6–8).

Novel Murine Sepsis Model Adheres to the MQTiPSS Consensus Guidelines

Supplemental Table 2 (Supplemental Digital Content 4, http://links.lww.com/CCM/E823) provides a composite list of the 20 recommendations and nine considerations established by MQTiPSS. Our murine model satisfies almost all of the stated considerations and all but one nonapplicable recommendation since no specific treatment was being tested in the model (6–8).

Young and Old Septic Mice Demonstrate Similar Hemodynamic Changes but Only Old Mice Have Acute Organ Dysfunction in a Novel Model of CLP + DCS

After substituting the 22-gauge needle with a 25-gauge needle to reduce overall mortality, we experimented with the delivery of antibiotics to mice at different post-CLP time-points. Attempts to give antibiotics later than 2 hours after CLP resulted in high/ acute lethality in the old mice (≥ 50% mortality at 4 d). Hence, a delay of 2 hours was chosen for delivery of antibiotics because this resulted in a mortality in old mice which is more similar to the mortality rates observed in clinical practice (28). Because Sepsis-3 refers to organ failure acutely after infection, we studied early time-points for organ failure, specifically 8, 24, and 48 hours post-CLP. Subsequently, mice underwent daily chronic restraint stress (DCS) 24 hours post-CLP.

In the CLP + DCS model using a 25-gauge needle, a greater proportion of old mice survived out to 4 days (63%) compared with the 22-gauge CLP model (50%) (Fig. 1A vs Supplemental Fig. 1F, Supplemental Digital Content 1, http://links.lww.com/CCM/E820; and legend, Supplemental Digital Content 7, http://links.lww.com/CCM/E826), although this difference was not statistically significant. Although both young and old mice had similar reductions in MAP (Fig. 1B), only old mice demonstrated renal insufficiency 8 hours post-CLP, which resolved by 24 and 48 hours (Fig. 1C; and Supplemental Table 3, Supplemental Digital Content 5, http://links.lww.com/CCM/E824). Both young and old mice had elevated AST levels around 24 and 48 hours (Fig. 1E; and Supplemental Table 3, Supplemental Digital Content 5, http://links.lww.com/CCM/E824). Additional markers of organ dysfunction are illustrated in Figure 1. As platelet counts and lung histology did not reveal differences between septic versus control mice and young versus old septic mice with the 22-gauge needle, respectively, we refrained from repeating these analyses in the 25-gauge needle CLP + DCS model. This was to maintain the “reduction” aspect of the three R’s of research (replacement, reduction, and refinement) as required by our animal ethics committee (29) because additional mice are required to obtain enough blood and lung tissue for these multiple tests.

Old Mice 14 Days After CLP + DCS Better Reflect the Low Grade Systemic Inflammation Displayed in Human Septic Patients

We analyzed our human sepsis database (25), determining the serum cytokine/chemokine levels of patients 14 days after sepsis who entered CCI versus having a RAP. Early death patients were excluded. Median age of the human sepsis cohort was 63 years with 58% being male. Acute Physiology and Chronic Health Evaluation II scores at 24 hours indicated severe physiologic derangement (median CCI 21 vs RAP 16; $p < 0.05$). Approximately 32% of the cohort developed septic shock. Median hospital and ICU lengths of stay were 21 and

9 versus 32 and 24 for patients with RAP and CCI, respectively. Patients with CCI were more likely to require mechanical ventilation (96% vs 67%; $p < 0.05$) and almost twice as likely to progress to multiple organ failure (84% vs 46%; $p < 0.05$). Furthermore, they were frequently discharged to facilities associated with poor outcomes such as skilled nursing facilities, LTAC facilities, and hospice (70% vs 27%; $p < 0.05$). Additionally, those with CCI were more likely to die at 1 year (41% vs 6%; $p < 0.05$). Of note, age was an independent risk factor for CCI. Importantly, those patients who progressed to a state of CCI had significantly increased serum cytokine/chemokine concentrations versus RAP patients at 14 days, including levels of interleukin-6, interleukin-8, IP-10, and MCP-1 (Fig. 2A).

Plasma isolated 14 days after CLP + DCS revealed that old mice had significantly increased concentrations of GM-CSF, interferon-γ, interleukin-1α, interleukin-12p70, interleukin-17, MIP-1α, TNF-α, and M-CSF (Fig. 2B). Interleukin-6 was not significantly greater in old mice ($p = 0.14$) (Fig. 2B), and G-CSF was elevated in both young and old compared with nonseptic mice (Supplemental Table 4, Supplemental Digital Content 6, http://links.lww.com/CCM/E825). Interleukin-1β, interleukin-2, MCP-1 (CC chemokine ligand 2), and KC (C-X-C motif ligand-1) were not different between old septic and nonseptic mice (Supplemental Table 4, Supplemental Digital Content 6, http://links.lww.com/CCM/E825).

Old Mice 14 Days After CLP + DCS Better Reflect the Immunosuppression Displayed in Human Septic Patients

We also examined anti-inflammatory cytokine serum concentrations in septic mice. Both interleukin-10 and interleukin-4 levels were greater in old mice 14 days after CLP + DCS versus young mice (Fig. 2B). Although the current study was not significant and only demonstrated a trend toward increased interleukin-10 in CCI and RAP patients (Fig. 2A), our previous work revealed human patients with CCI after sepsis had significantly increased interleukin-10 concentrations versus RAP in a larger cohort of patients (30).

Additional evidence for immune suppression included expansion of MDSCs in both young and old septic mice at 7 and 14 days (Fig. 3A), as well as increased MDSC-mediated $CD8^+$ T cell suppression at 7 days in old mice when compared with the young (Fig. 3B). Similarly, septic humans exhibit markedly increased percentages of MDSCs after sepsis out to 28 days, and these cells have also been shown to suppress T cells (31).

Old Mice Have Increased and Sustained Weight Loss After CLP + DCS, Which Is More Similar to Human Sepsis

Most septic patients, particularly those with CCI, lose body weight, and muscle mass (32). Both old and young mice had initial loss of body weight after CLP + DCS when adjusted for starting body weight differences (Fig. 4A). However, by 14 days, the body weight of young mice returned to baseline, whereas old mice maintained their weight loss. Interesting, this weight loss can be induced by DCS alone in old mice (Fig. 4B), demonstrating its importance to the model. However, DCS by itself had a much decreased overall effect on serum cytokine levels, revealing the importance of the CLP with antibiotics to the model (Supplemental Table 4, Supplemental Digital Content 6, http://links.lww.com/CCM/E825). Finally, CLP alone without DCS also induced some weight loss, but the addition of DCS

significantly increased the weight loss of old mice in the postsepsis period (Fig. 4B), again illustrating the importance of the combined model.

DISCUSSION

Sepsis biology and research are changed landscapes. International consensus has altered the definition of sepsis to “infection that results in organ insufficiency or failure” (13). The phenotype of the septic patient has also evolved. Although the study of the acute response of the host to sepsis remains important, the majority of patients now survive their acute insult and subacute hospitalization (14, 16, 17, 30, 33). Many of these patients recover, whereas others enter a state of CCI, which is associated with dismal long-term outcomes (16, 34, 35). Thus, the study of the nonacute pathophysiology of sepsis is becoming increasingly relevant.

A working group was created to develop recommendations for “best practices, management guidelines, and standardization” in animal modeling of sepsis (6–8). It is hoped that by following this group’s recommendations (MQTiPSS), more effective animal modeling can occur with sepsis, similar to what has already been accomplished for other diseases such as stroke, heart failure, and pulmonary fibrosis (6–8). Because mice will never completely represent the human condition after sepsis, the intent of these recommendations was to create a framework for investigators to improve upon animal modeling. The goal, however, is not perfection nor restriction of animal models that do not conform to the recommendations. Many investigators would agree that maintaining mice in a true ICU setting with ventilation, vasopressor capabilities or renal replacement therapy is not feasible or affordable for most laboratories; therefore, we created an improved murine surgical sepsis model that follows the MQTiPSS recommendations without requiring these interventions (6–8). Our results indicate that our model engenders a murine phenotype in-line with current sepsis definitions and better recapitulates the human septic response in this model. Overall, we have determined that old mice have a unique response to CLP + DCS, and this appears to reflect older septic patients in many regards. In addition, the inclusion of DCS in this model leads to the development of PICS (18).

This surgical sepsis model incorporates several important aspects that relate it to human sepsis. First, it includes rapid volume resuscitation and antibiotic treatment 2 hours after induction of sepsis, which are best practice measures for the treatment of sepsis (36) and currently required by the Centers for Medicare and Medicaid Services and The Joint Commission in the United States (36). Second, we have added a DCS component to the animals to reproduce the stress of the human ICU environment (20). DCS has been successfully added to other animal models, inducing continuously elevated levels of stress hormones and negatively affecting the host (i.e., immune surveillance, gastrointestinal integrity, coronary artery disease, and wound healing) (37–40). Furthermore, DCS contributes to cachexia, particularly in old mice, which is a necessary component of PICS. Finally, MQTiPSS recommends that sepsis models “include [comorbidities] and/or other biological variables (i.e., age, gender, diabetes, cancer, immuno-suppression, genetic background, and others)” (6–8). Thus, we elected to use old mice, as sepsis predominantly affects the aged, and it is clear that older individuals respond differently to infection than the young. Age is not only an independent predictor of mortality to sepsis (3, 41), but older

patients are also more likely to have poor acute, subacute, and chronic outcomes after sepsis than their young counterparts (42, 43). This includes greater healthcare needs after hospitalization (3).

Importantly, age is only one of several comorbidities that are prevalent in humans with sepsis and are not recapitulated by most murine models of sepsis that employ young adult animals. Studies have shown that dietary-induced type II diabetes in mice also increases morbidity and mortality (44, 45). Mice with chronic renal dysfunction have increased inflammatory responses and organ injury to sepsis (46). Similar results are seen in mice with cardiomyopathies and underlying pulmonary disease (47, 48).

The multifactorial mechanisms that drive the poor outcomes of elderly patients to sepsis are still being defined. For example, modern medicine allows individuals with chronic diseases to survive into old age and elderly patients are more likely to have comorbidities than younger individuals (49). In addition to comorbidities, preadmission status, malnutrition, medications, mitochondrial dysfunction, and intestinal microbiota are all thought to contribute to the increased risk for and outcome of older adults with sepsis (50, 51). However, two of the key phenotypes of older adults are inflammaging (chronic subclinical systemic inflammation) and immunosenescence (42, 43, 51). Immunosenescence is defined as the gradual deterioration of protective immunity associated with aging, which affects the host's response to infections (42, 43, 51). The young are more capable of returning to a homeostatic state of restored innate and adaptive immunity after infection than older adults (52). In contrast, old adults have prolonged defects in their immune cells, thought to be a major contributor to increased morbidity and mortality (5, 23, 53).

Other authors have worked toward similar goals regarding examining the late effects of sepsis, which is becoming increasingly relevant in the human population. Nomellini and Caldwell have examined young mice 8 days after CLP for aspects of PICS using historical CLP methodology (54). Osuchowski et al (55) and Remick et al (19) performed fairly sophisticated analyses of late murine CLP outcomes in young mice. Singer et al (13) created a young adult rat model of sepsis that induces organ dysfunction, in-line with Sepsis-3, and followed these animals through the acute and subacute periods (56). Steele et al (57) created a cecal stent model with delayed antibiotics in middle-aged mice and were able to follow these mice long term. Finally, Lyons et al (58) used a cancer-sepsis model, as cancer patients are at a much greater risk for sepsis. Importantly, the latter two models simulate the human condition, either with delayed antibiotics and prolonged survival, or by looking at a cohort that is at significant risk for sepsis (cancer) (59). We have built upon these models to further improve murine surgical sepsis modeling. Although our novel CLP + DCS model in old mice is not a perfect analogy of surgical sepsis in humans, we believe that our approach follows the current recommendations for preclinical sepsis animal research, is accomplished in an easily reproducible manner, and may allow improved translational sepsis studies to occur (11).

Despite our attempts to develop a murine model that accurately reflects surgical sepsis in humans, there are several limitations to our model which are worth mentioning. The MQTiPSS guidelines recommend considering source control, and we did not perform

cecectomy or washout of the intra-abdominal abscess following CLP. The reason we did not perform source control was that the sterile abscess is required to drive the protracted inflammatory response seen in CCI patients within the surgical ICU. Additionally, one human study by Torgersen et al (60) determined that most septic patients in the surgical ICU die with a residual septic focus, either undetected or despite attempted surgical source control. Another limitation is the somewhat narrow scope of our study. Rather than analyzing a broad subset of murine sepsis models, which would undoubtedly better reflect the heterogeneous human condition, we focused on a single reproducible intra-abdominal sepsis model and examined the impact of age and stress on the model. Our goal was to create a surgical sepsis model that reflects the human disease, and we believe that we have accomplished that by taking into consideration age, organ dysfunction, immune status, and environmental factors that may impact sepsis outcomes. Still, it is clear that additional studies will be required to assess the ability of other sepsis models (e.g., pneumonia and urosepsis models) to reproduce the organ dysfunction and prolonged inflammatory and immunosuppressive effects seen in septic patients.

Another potential controversial aspect of the study include is the use of both sexes. It is unclear the role that sex, and the estrous cycle, may play in differences observed between young and old mice. Therefore, future directions to enhance the translatability of the model should include analyzing sex-specific differences in organ dysfunction and the immune response to sepsis. Finally, the measures we used to assess organ dysfunction and immune status are by no means fully comprehensive. Unfortunately, investigators can be limited by the number of old adult mice that can be obtained from either commercial sources or the National Institute of Aging, as well as the number of analytes (i.e., measures of organ dysfunction) that can be run on a single mouse's blood volume. Therefore, our study may be underpowered regarding some analytes as well as determining sex-related differences. However, despite these constraints, we attempted to include analytes that best reflect the individual components of the SOFA score, which is used to diagnose sepsis in humans. Although some of these measures are not identical to those included in SOFA, we employed reasonable surrogates. We also selected measures of immune function that have been previously researched in humans to allow for comparison.

Ultimately, we anticipate that future researchers will make further modifications to our model, and perhaps surpass it in their effectiveness. Regardless, the consideration of age and the incorporation of DCS in the model clearly impacts the host response to sepsis. Attempts to integrate details such as this may further improve murine models of sepsis and lead to the enhanced translatability of current animal models altogether.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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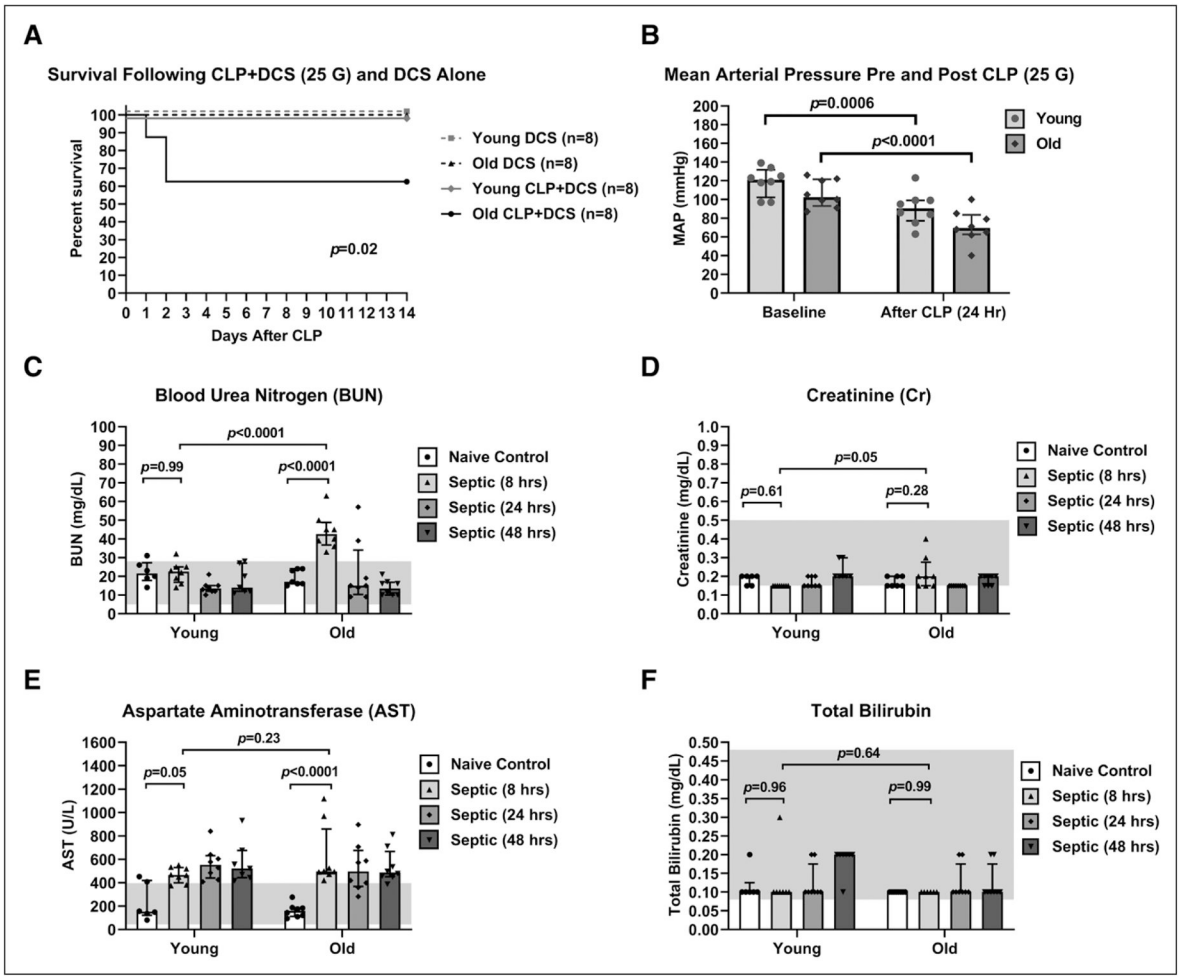

Figure 1.

Organ dysfunction and mortality in young versus old septic mice following cecal ligation and puncture (CLP) + daily chronic stress (DCS) with a 25-gauge needle (low lethality model). **A**, Mean arterial pressure (MAP) was measured in young and old mice at baseline, prior to CLP, and 24 hr after CLP using a noninvasive CODA tail-cuff blood pressure system. Both age groups experienced significant reductions in MAP 24 hr after CLP. **B**, Kaplan-Meier survival plot showing survival of young versus old mice subjected to either CLP + DCS or DCS alone following sham laparotomy. Significance was determined using the log rank test. **C**–**F**, Blood of control mice and septic mice at 8, 24, and 48 hr post-CLP was analyzed for the following: **C**, total bilirubin; **D**, aspartate aminotransferase (AST); **E**, blood urea nitrogen (BUN); and **F**, creatinine (Cr) levels. Old mice demonstrate hepatic and renal dysfunction at 8 hr, with significantly increased AST and BUN levels compared with age-matched naive controls. Of note, antibiotics and fluid resuscitation are used in this model for 72 hr post-CLP. Data are reported as median ± interquartile range unless otherwise stated. *Gray-shaded boxes* represent normal laboratory ranges. Corresponding *p* values appear in the figures and were determined using two-way analysis of variance with multiple-comparison testing.

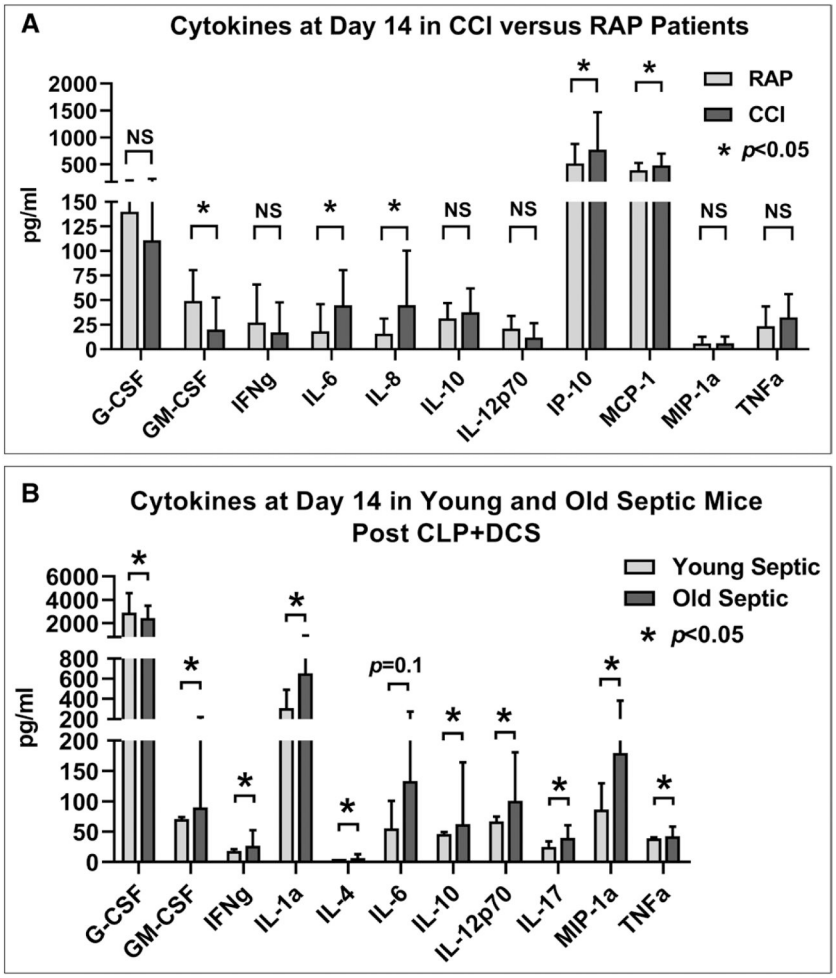

Figure 2.

Plasma cytokine/chemokine concentrations 14 d postsepsis in mice subjected to cecal ligation and puncture (CLP) + daily chronic stress (DCS) and in human patients with chronic critical illness (CCI) versus rapid recovery (RAP) at 14 d. **A**, Septic old mice have significantly increased levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-1α, macrophage colony-stimulating factor, IL-12p70, IL-17, macrophage inflammatory protein (MIP)-1α, tumor necrosis factor (TNF)-α, IL-4, and IL-10 when compared with young septic mice following CLP + DCS. **B**, Patients with CCI exhibit significantly higher concentrations of IFN-γ, IL-6, IL-8, IL-10, and monocyte chemoattractant protein (MCP)-1 when compared with those who experience RAP. *$p < 0.05$. Nonparametric Mann-Whitney U test was used to determine significance. G-CSF = granulocyte colony-stimulating factor, NS = not significant.

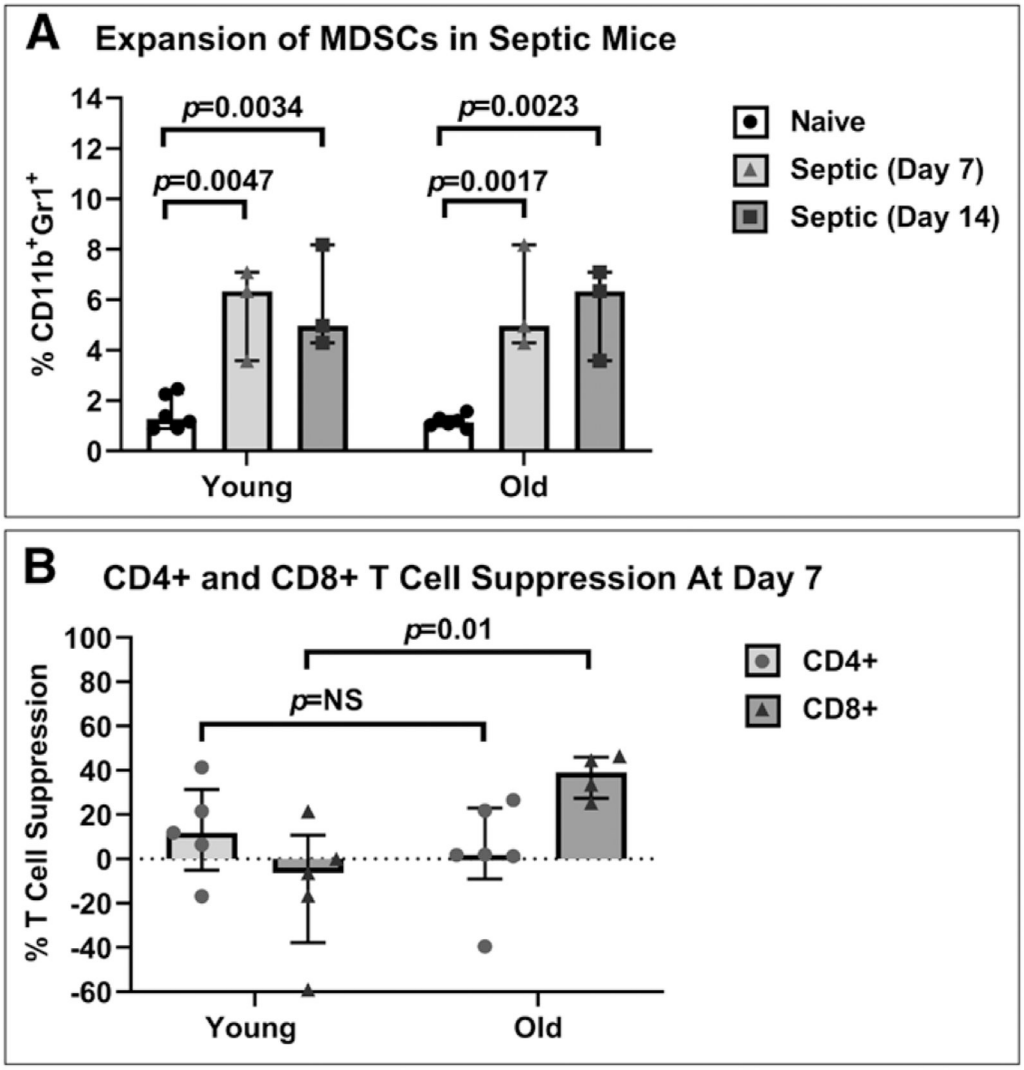

Figure 3.

Evidence of myeloid-derived suppressor cell (MDSC) expansion in septic mice and suppression of CD8+ T cells in old mice at 7 d. **A**, In comparison to controls, both young and old septic mice had increased proportions of $CD11b^{+}Gr\text{-}1^{+}$ cells (MDSCs) at 7 and 14 d post cecal ligation and puncture + daily chronic stress. **B**, However, only MDSCs of septic old mice at 7 d significantly suppressed CD8+ T cell proliferation induced by soluble CD3 and CD28. *p* values are reported in the figures and were determined using two-way analysis of variance with multiple-comparison testing. NS = not significant.

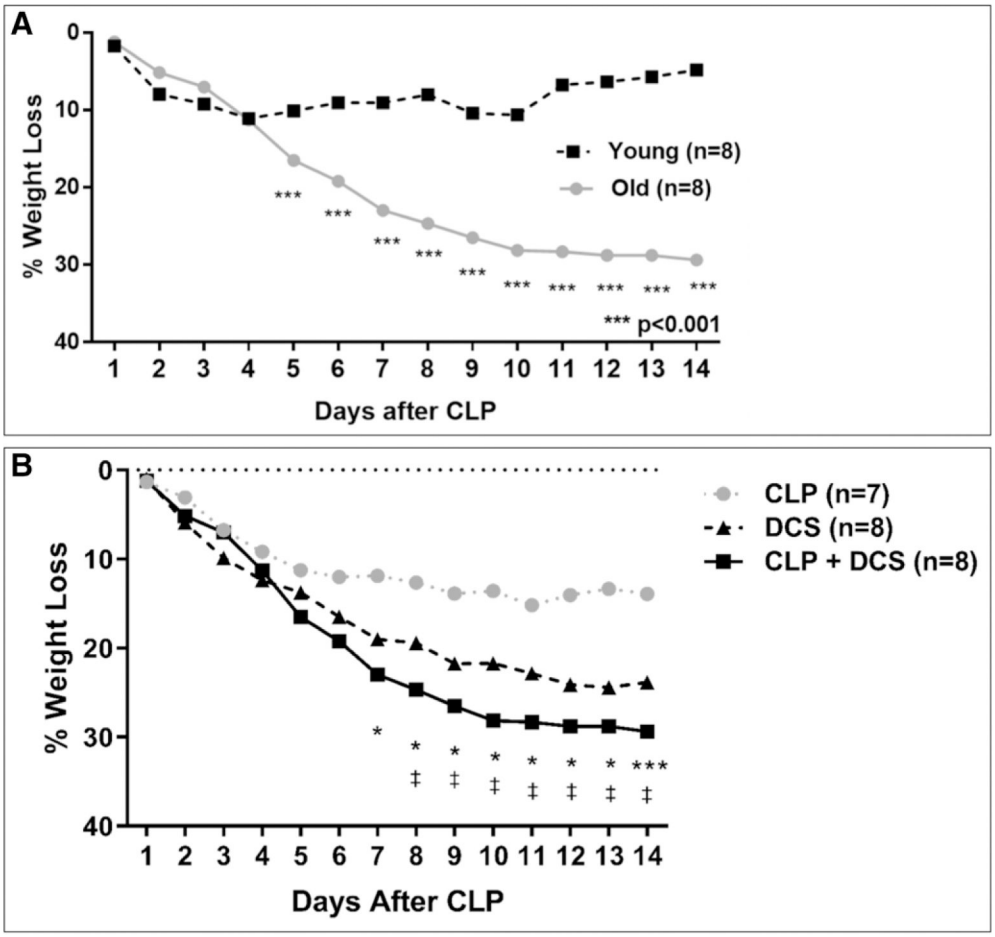

Figure 4.

The effects of daily chronic stress (DCS) and cecal ligation and puncture (CLP) on weight loss in old and young mice. **A**, Both young and old mice had initial loss of body weight after CLP + DCS when adjusted for starting body weight differences, but by 14 d, the body weight of young mice returned to baseline, whereas old mice showed sustained weight loss. Corresponding *p* values are reported in the figure with statistical analysis being performed using the nonparametric Mann-Whitney *U* test. **B**, Weight loss of old mice after CLP + DCS, CLP only, and DCS only (*CLP vs CLP + DCS, $p < 0.05$; ***CLP vs CLP + DCS, $p < 0.001$; ‡DCS vs CLP + DCS, $p < 0.05$).

TABLE 1.

Sequential (Sepsis-Related) Organ Failure Assessment Score

Organ System	Objective Measurement
Respiration	PaO_2/FIO_2 (mm Hg)
CNS	Glasgow coma scale
Cardiovascular	Mean arterial pressure (mm Hg) or use of vasopressors
Liver	Bilirubin (mg/dL)
Coagulation	Platelets × $10^3/\mu L$
Renal	Creatinine (mg/dL) or urine output