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Targeting Junctional Adhesion Molecule-C Ameliorates Sepsis-induced Acute Lung Injury by Decreasing CXCR4+ Aged Neutrophils

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Abstract

Sepsis is a severe inflammatory condition causing high mortality. Transmigration of neutrophils into tissues increases their lifespan to promote deleterious function. Junctional adhesion molecule-C (JAM-C) plays a pivotal role in neutrophil transmigration into tissues. We aim to study the role of JAM-C on the aging of neutrophils to cause sepsis-induced acute lung injury (ALI). Sepsis was induced in C57BL/6J mice by cecal ligation and puncture (CLP) and JAM-C expression in serum was assessed. Bone marrow-derived neutrophils (BMDN) were treated with recombinant mouse JAM-C (rmJAM-C) *ex vivo* and their viability was assessed. CLP-operated animals were administrated with either isotype IgG or anti-JAM-C Ab at a concentration of 3 mg/kg and after 20 h, aged neutrophils ($CXCR4^+$) were assessed in blood and lungs and correlated with systemic injury and inflammatory markers. Soluble JAM-C level in serum was upregulated during sepsis. Treatment with rmJAM-C inhibited BMDN apoptosis, thereby increasing their lifespan. CLP increased the frequencies of $CXCR4^+$ neutrophils in blood and lungs, while treatment with anti-JAM-C Ab significantly reduced the frequencies of $CXCR4^+$ aged neutrophils. Treatment with anti-JAM-C Ab significantly reduced systemic injury markers (ALT, AST and LDH) as well as systemic and lung inflammatory cytokines (IL-6 and IL-1β) and chemokine (MIP-2). The

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Conflicts of Interest

The authors report no financial conflicts of interest.

AUTHORSHIP

YH, MA designed and performed the experiments. YH, MA analyzed the data and prepared the manuscript. YH, YO, MO performed animal works. MA revised the manuscript. PW conceived the idea, reviewed the manuscript and supervised the whole project. All authors read and approved the final manuscript.

blockade of JAM-C improved lung histology and reduced neutrophil contents in lungs of septic mice. Thus, reduction of the pro-inflammatory aged neutrophils by blockade of JAM-C has a novel therapeutic potential in sepsis-induced ALI.

Keywords

Neutrophil Aging; Junctional Adhesion Molecule-C (JAM-C); CXCR4; Sepsis; Apoptosis; Transmigration; Acute Lung Injury

INTRODUCTION

Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection [1], remains a considerable challenge to critical care medicine. According to the new clinical criteria of sepsis as represented by an increase in the Sequential Organ Failure Assessment (SOFA) score of 2 points or more, in-hospital mortality rate of sepsis is recorded as over 10% [1]. However, it grows up to greater than 40% when septic shock develops [2]. In the United States, more than 1 million cases of septicemia are reported annually [3, 4]. Despite resources incurred in treatment, over 200,000 patients die annually due to this condition, making septicemia as one of the leading causes of death in the United States [3]. Due to this unacceptably high mortality rate and frequent admission of septic patients in the intensive care unit [2], scientists and the clinicians are desperately looking for an effective strategy to treat sepsis [2]. Although more than 100 clinical trials tested for sepsis resulted in failure in the past [5], additional efforts and novel therapeutic ideas should be implemented to reduce sepsis-induced death.

Neutrophil infiltration in the lungs is a pathological hallmark of sepsis-induced acute lung injury (ALI), or acute respiratory distress syndrome (ARDS) [6]. Therefore, controlling neutrophil recruitment and activation is considered to be one of the main therapeutic strategies to treat ALI in sepsis [7]. Neutrophils are produced in the bone marrow and released into the circulation as the first responders of the innate immune system during acute inflammatory conditions [8]. After the sequential processes between the circulatory neutrophils and the vascular endothelium represented by capture, rolling, adhesion, crawling and transmigration [9], they infiltrate into the inflamed tissues and eliminate invading pathogens by releasing proteolytic enzymes such as myeloperoxidase (MPO) and reactive oxygen species (ROS), forming neutrophil extracellular traps (NETs), and promoting phagocytosis [7, 10, 11]. However, overwhelming migration and exaggerated function of activated neutrophils in the inflamed tissues not only kill the bacteria but also cause surrounding tissue injury and unrestrained inflammation, resulting in organ dysfunction and death [12, 13]. Thus, regulating the function of neutrophils and their uncontrolled infiltration into tissues could serve as an effective therapeutic tool during sepsis.

Under steady-state conditions, the heterogeneity of neutrophils arises from their ageing and replenishment by naïve bone marrow-derived neutrophils. A growing body of literatures demonstrated the phenotype of aged neutrophils which express CXCR4 at a high level on their cell surface compared to freshly isolated neutrophils [14–17]. The chemokine receptor CXCR4 expressed on the surface of aged neutrophils helps their clearance in the bone

marrow [16, 17]. Recently, the $CXCR4^+$ neutrophils are shown as a pro-inflammatory phenotype of neutrophils whilst in circulation [15]. $CXCR4^+$ aged neutrophils represent an excessively active subset exhibiting enhanced $\alpha_M\beta_2$-integrin activation and NET formation under inflammatory conditions [15]. The NET forming neutrophils were previously shown to be deleterious in sepsis [18], thus suggesting the fact that $CXCR4^+$ neutrophils could be detrimental in sepsis as $CXCR4^+$ neutrophils form excessive NET [15]. Zhang *et al.* also have shown that the neutrophil ageing is driven by the microbiota via Toll-like receptor (TLR) and myeloid differentiation factor 88 (MyD88)-mediated signaling pathways [15]. Depletion of the microbiota significantly reduced the number of circulating aged neutrophils and dramatically improved the pathogenesis and inflammation-related organ damage in endotoxin-induced septic shock [15]. Therefore, the above literatures clearly suggest that, i) $CXCR4^+$ neutrophils are named “aged” neutrophils, ii) sepsis can increase the contents of CXCR4 expressing aged neutrophils, and iii) $CXCR4^+$ neutrophils display pro-inflammatory roles in sepsis.

Junctional adhesion molecule-C (JAM-C) is a glycoprotein which belongs to the immunoglobulin superfamily with two extracellular immunoglobulin-like domains, one transmembrane segment, and a short cytoplasmic tail [19]. JAM-C is expressed in a wide variety of cells including endothelial cells, fibroblasts, intestinal epithelial cells and smooth muscle cells [20]. JAM-C promotes neutrophil transendothelial migration (TEM) from circulation to the inflamed tissues by binding to its receptor integrin $\alpha_M\beta_2$ (Mac-1) [21]. By contrast, the cleavage of endothelial JAM-C promotes neutrophils to facilitate their migration from tissues to the circulation through a mechanism called reverse transendothelial migration (rTEM) [22, 23]. While much attention was paid to the function of JAM-C on neutrophil chemotaxis, no description of JAM-C on neutrophil aging was given previously. Since several reports demonstrate that the apoptosis of neutrophils is inhibited as they travel through the transendothelial axis [24, 25], it is interesting to reveal that JAM-C not only regulates neutrophil chemotaxis but also prolongs their survival by promoting anti-apoptotic function.

In this study, we aimed to investigate the role of JAM-C on the aging of neutrophils during sepsis. We hypothesized that JAM-C could prolong the survival of neutrophils through the inhibition of apoptosis, thus increasing the pro-inflammatory aged population of neutrophils during sepsis. We also aimed to assess the therapeutic potential of the blockade of JAM-C by administrating its neutralizing antibody (Ab) to ameliorate inflammation and injury to the lungs during sepsis.

MATERIALS AND METHODS

Animal model of sepsis and administration of anti-JAM-C Ab

Eight-weeks-old male C57BL/6J mice purchased from Taconic (Albany, NY) were housed in a temperature-controlled room on a 12 h light/dark cycle and fed a standard laboratory diet. Experimental sepsis was induced in mice using cecal ligation and puncture (CLP) method. Briefly, mice were anesthetized by isoflurane inhalation, and the abdomen was shaved and wiped with 10% povidone iodine. A 1-cm abdominal incision was performed to expose the cecum. The cecum was tightly ligated with a 4-0 silk suture 1.5 cm away from

the tip and the puncture was made in one pass, through and through both sides of the bowel wall with a 22-gauge needle to eject a small amount of feces from the perforation sites by gentle squeezing. The cecum was returned to the abdominal cavity and the laparotomy site was closed with a 4-0 silk suture in two layers. In sham-operated animals laparotomy was performed, but the cecum was neither ligated nor punctured. Animals were resuscitated with 1 ml of normal saline subcutaneously. At 4 h after operation, mice were anesthetized again and a small incision on the neck was performed to expose the internal jugular vein. Affinity purified polyclonal anti-mouse JAM-C antibody (anti-JAM-C Ab, R&D Systems, Minneapolis, MN, Catalog No.: AF-1213) at a dose of 3 mg/kg body weight (BW) in 100 μl volumes was delivered by bolus injection through the jugular vein to serve as the treatment group. In the same way, normal goat IgG control (R&D Systems, Catalog No.: AB-108-C) at a dose of 3 mg/kg in 100 μl volumes was injected in septic animals to serve as the non-immunized IgG control group. The anti-JAM-C Ab used in our study is a neutralizing Ab because it efficiently blocks JAM-C interaction with its ligand JAM-B (R&D Systems, Catalog No.: AF-1213). We chose the dose (3 mg/kg BW) of anti-JAM-C Ab or isotype IgG control to inject mice intravenously based on previous studies on murine models of acute inflammatory diseases [20, 26]. The proximal and distal ends of the injected jugular vein were tightly ligated with 5-0 silk suture. The wound was closed with one interrupted 6-0 silk suture. To validate our *in vivo* approach of using polyclonal anti-mouse JAM-C Ab in mice, previous studies by others also used the same polyclonal goat anti-mouse JAM-C Ab (R&D systems) *in vivo* in a murine model of allergic contact dermatitis and demonstrated enormous efficacy of this polyclonal Abs in inhibiting allergic contact dermatitis [27]. At 20 h after operation, mice were anesthetized and blood and lung samples were collected. Blood samples were centrifuged at 3,000 g for 10 min to collect plasma. The plasma and tissue samples were frozen immediately in liquid nitrogen and stored at −80°C until analysis. A section of lung tissue was preserved in formalin for histopathological analysis. All experiments were performed in accordance with the guidelines for the use of experimental animals by the National Institutes of Health (Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee (IACUC) at the Feinstein Institute for Medical Research.

Isolation of neutrophils from bone marrow and blood

To isolate bone marrow-derived neutrophils (BMDN), mice were euthanized and femurs from both hind legs were removed. The distal tip of each edge was cut off and bone marrow cells were isolated by flushing Hanks' Balanced Salt Solution (HBSS without Ca^{2+} and Mg^{2+}, Mediatech Inc., Manassas, VA) into the femur from each end of the bone. Cell suspensions were filtered through the 70μm cell strainer (Corning Inc., Corning, NY) and centrifuged at 300 g for 10 minutes. After cell pellets were lysed with ACK lysing buffer (Quality Biological Inc., Gaithersburg, MD) for 5 min and washed with cold PBS twice, BMDN were isolated magnetically by using the commercial kit (Stem Cell Technology, Vancouver, Canada, Cat. No.:19762). For the isolation of circulatory neutrophils, blood samples were collected from the sham, isotype IgG control-treated CLP and anti-JAM-C Ab-treated CLP groups with 8 unit/ml of heparin. The whole blood samples were then lysed with ACK lysing buffer for 10 min and washed with cold PBS twice, followed by the magnetic isolation of neutrophil. The purity of neutrophil isolation was confirmed by

staining cells with Alexa-Fluor 488-Ly-6G/Ly-6C Abs (Biolegend, San Diego, CA, Cat. No.: 108417) using LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA), which guaranteed the high purity of neutrophils over 97% (data not shown).

Detection of neutrophil apoptosis by flow cytometry

For the assessment of neutrophil apoptosis *in vitro*, isolated BMDN were resuspended in RPMI medium 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, glutamine, penicillin and streptomycin and incubated with PBS or recombinant mouse JAM-C Fc Chimera (rmJAM-C, R&D Systems, Catalog No.: 1213-J3) at 5 μg/ml for 20 h to allow BMDN to go through intrinsic apoptosis. Equivalent concentration of rat anti-mouse CD11b blocking antibody (Biolegend, Cat. No.:101213) or rat IgG2bκ isotype control (Biolegend, Cat. No.: 400621) or anti-JAM-C Ab were also added in the medium together with rmJAM-C. We used 5 μg/ml of anti-JAM-C Ab, because at this concentration this antibody blocks >50% of the binding of JAM-C with its ligand JAM-B *in vitro* (R&D Systems, Catalog No.: AF-1213). Then after the washing process, the total amount of 1×10^6 BMDN were stained with FITC-Annexin V (BD Biosciences, Cat. No.: 556420, San Jose, CA) and propidium iodide (Apoptosis Detection Kit I, BD Biosciences, San Jose, CA). Freshly isolated BMDNs were also stained similarly for the comparison. The all stained cells were subjected to flow cytometric analysis by LSR Fortessa (BD Biosciences). Data were analyzed by FlowJo software (Tree Star, Ashland, OR) with 20,000 events per sample. Fc-receptor blockers (BioLegend) were used for all the samples. We interpreted the results as non-viable cells, which included i) only Annexin V^+ early apoptotic cells, ii) Annexin V and PI double positive end stage/late apoptotic cells, and iii) only PI^+ necrotic/dead cells. The viable cells were represented as Annexin V and PI double negative cells.

Flow cytometric analysis of CXCR4 expression on neutrophils

Lung tissues were minced and digested in complete RPMI medium containing 100 U/ml Collagenase type 1 (Worthington Biochemical, Lakewood, NJ) and 20 U/ml DNase 1 (Roche Diagnostics, Mannheim, Germany) for 30 min at 37 °C in a shaker incubator followed by filtering through 70 μm nylon strainer and washed in PBS with 1% FBS. Cells were then resuspended in 44% Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), underlaid with 66% Percoll, and centrifuged for 20 min at 2000 rpm. Cell suspension surrounding the interface were collected, washed, and stained with Alexa-Fluor 488-Ly-6G/Ly-6C Abs and PerCP-Cy5.5-CXCR4 Abs (Biolegend, Cat. No.: 146510). Isolated BMDN incubated with PBS for 0, 2, 4, 20 h and isolated circulatory neutrophils were stained with PerCP-Cy5.5-CXCR4 Abs. All stained cells were subjected to flow cytometric analysis and analyzed as previously described. Fc-receptor blockers were used for all the samples.

Western blot analysis

Isolated BMDN (1.5×10^6 cells) were treated with PBS or 5 μg/ml of rmJAM-C for 30 min and homogenized in lysis buffer (10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Protein concentration was determined by Bio-Rad protein assay reagent. Total lysates were fractionated on Bis-Tris gels (4%–12%) and transferred to nitrocellulose membrane. The membranes were then blocked with 5%

nonfat dry milk in Tris-buffered saline with tween-20 (TBST) and incubated overnight at 4°C with anti-IκB-α Ab (Santa Cruz biotechnology, Dallas, Texas, Cat. No.: sc-371) or anti-β-actin Ab (Sigma-Aldrich, St Louis, MO). Serum samples collected at 5 and 20 h after CLP and sham operation were similarly fractionated, transferred, blocked and incubated overnight with anti-mouse JAM-C Ab (R&D Systems, Cat. No.: AF-1213). The Western blots of the serum samples were stained by Ponceau red to ensure equal loading of the samples.

Quantitative real-time RT-PCR analysis

Total RNA was extracted from lung tissues by using TRIzol (Invitrogen, Carlsbad, CA) and was reverse-transcribed into cDNA using reverse transcriptase (Applied Biosystems, Foster City, CA). A Polymerase chain reaction (PCR) was carried out in 20 μl of final volume containing 0.08 μM of each forward and reverse primer, cDNA and 10.5 μl SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY). Amplification was conducted using an Applied Biosystems Step One Plus real-time PCR machine under the thermal profile of 50°C for 2 min, 95°C for 10 min followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 min. For relative quantization, 2^{-ddCt} method normalized to mouse β-actin mRNA was used. Relative expression of mRNA was expressed as the fold change in comparison with the sham tissues. The primers used for this study are: IL-6 (NM_031168) Forward: CCGGAGAGGAGACTTCACAG, Reverse: GGAAATTGGGGTAGGAAGGA; IL-1β (NM_008361) Forward: CAGGATGAGGACATGA GCACC, Reverse: CTCTGCAGACTCAAACTCCAC; MIP-2 (NM_009140) Forward: CCCTGGTTCAGAAAATCATCCA, Reverse: GCTCCTCCTTTCCAGGTCAGT; β-Actin (NM_00739 3): Forward: CGTGAAAAGATGACCCAGATCA, Reverse: TGGTACGACCA GAGGCATACAG.

Measurement of organ injury markers

Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured using commercial assay kits (Pointe Scientific, Lincoln Park, MI) according to the manufacturer's instructions.

Measurement of pro-inflammatory cytokines and chemokine

Pro-inflammatory cytokines interleukin-6 (IL-6) and IL-1β in plasma and lung tissues were quantified by using the mouse ELISA kits (BD Biosciences, Franklin Lakes, NJ). Macrophage inflammatory protein (MIP)-2 in plasma and lung tissues was measured by using the mouse ELISA kit (R&D Systems).

Histological examination

The lung tissue was fixed in 10% formalin and then embedded in paraffin. Later, the tissue blocks were cut into 5-μm sections, placed onto glass slides and stained with hematoxylin-eosin (H&E), dehydrated, and mounted. Morphologic examinations in these tissues were evaluated by light microscopy in a blinded fashion. The lung injury score system was adapted from Matute-Bello *et al*, which represented an overall score of between 0 and 1 [28]. To examine the extent of lung injury we evaluated its five pathological features, these

were *i.* neutrophils in the alveolar space, *ii.* neutrophils in the interstitial space, *iii.* hyaline membranes, *iv.* proteinaceous debris filling the airspaces, and *v.* alveolar septal thickening. The severity of each of these pathological features was evaluated by a score indicating 0 as absent or none, 1 as mild, and 2 as moderate to severe injury. We acquired histological images of each slide from at least 10 random areas/fields at an original magnification ×200. The final injury score was derived from the following calculation: Score = [20*(*i*) + 14*(*ii*) + 7*(*iii*) + 7*(*iv*) + 2*(*v*)]/(number of fields*100).

For neutrophil staining, the 10% formalin-fixed, paraffin-embedded lung tissues were dewaxed in xylene and rehydrated in a graded series of ethanol. The slides were heated in 0.92% citric acid buffer (Vector, Burlingame, CA) at 95°C for 30 min. After cooling to room temperature, the slides were incubated with 2% H_2O_2 in 60% methanol and blocked in 2% normal rabbit serum/Tris-buffered saline. Anti-Gr-1 antibody (BioLegend) was then applied and incubated overnight. Vectastain ABC reagent and DAB kit (Vector) were used to detect the immunohistochemical reaction. Slides were counterstained with 4′, 6-diamidino-2-phenylindole and examined under a phase contrast light microscope (Eclipse Ti-S; Nikon, Melville, NY). Gr-1-positive staining cells were counted in 20 visual fields/section at ×400 magnification, and averaged number was calculated.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) and analyzed using Sigma Plot12.5 graphing and statistical analysis software (Systat Software Inc., San Jose, CA). Multiple groups were compared by one-way analysis of variance (ANOVA) using the Student-Newman-Keuls' (SNK) test. Student's t test was used for two-group analysis. The normality test was assessed using Shapiro-Wilk test, and the samples which passed the normality test were analyzed for statistical significance. Differences in values were considered significant if $P < 0.05$.

RESULTS

Treatment with rmJAM-C attenuates neutrophil apoptosis in vitro via Mac-1 receptor

Soluble JAM-C exists in circulation because of the cleavage of endothelial JAM-C by a disintegrin and metalloprotease-10 (ADAM10), ADAM17 and neutrophil elastases [23]. Soluble JAM-C levels in the serum were increased by 65.2% and 107% at 5 and 20 h after CLP operation, respectively, as compared to sham-operated mice (Fig 1A). According to Fig 1B, we found that the freshly isolated neutrophils (0 h) represented 9.9±0.62% non-viable cells. On the other hand, after 20 h of their culture, the neutrophils underwent spontaneous apoptosis/necrosis which gave rise to 25.9±1.67% non-viable cells. Therefore, considering the 20 h time point would undergo spontaneous apoptosis of BMDN, we studied the effect of rmJAM-C on BMDN viability *in vitro*. We pre-treated BMDN with either isotype IgG control or anti-CD11b (Mac-1) Ab as the receptor for JAM-C or anti-JAM-C Ab and then stimulated the BMDN with rmJAM-C protein. Stimulation with rmJAM-C in isotype IgG control-treated BMDN significantly reduced their death by a mean value of 47.2% compared to PBS-treated BMDN at 20 h, while the BMDN pre-treated with either anti-CD11b or anti-JAM-C Abs did not show rmJAM-C-stimulated inhibition of BMDN apoptosis/necrosis (Fig

1C). Since CD11b exists in the form of the heterodimeric integrin $\alpha_M\beta_2$ (Mac-1) on neutrophils, we suggested that the apoptosis of neutrophils was inhibited by JAM-C through binding to Mac-1 receptors.

The blockade of JAM-C decreases aged neutrophil population in blood and lungs after sepsis

Neutrophils underwent aging spontaneously over time as assessed by their significantly higher expression of surface CXCR4 at 20 h of culture *in vitro* (Fig 2A, B). By considering the fact that JAM-C had an inhibitory effect on neutrophil apoptosis as shown in Fig 1B, C, neutrophils interacting with soluble and/or endothelial JAM-C while circulating in the blood or transmigrating into the lungs could prolong their survival by inhibition of apoptosis, which could lead to increased numbers of aged neutrophils in the blood and in inflamed lungs during sepsis. As expected, aged neutrophil population characterized by high surface expression of CXCR4 was significantly increased in the blood and lungs after CLP in isotype IgG control-treated group compared to the sham-operated group (Fig 2C–F). In contrast, anti-JAM-C Ab significantly decreased the percentages of aged neutrophils in the blood and lungs by 59.3%, and 67.3%, respectively, as compared to isotype IgG control-treated group after CLP (Fig 2C–F), indicating that the populations of aged neutrophils in the blood and lung tissues were regulated by JAM-C in sepsis.

JAM-C inhibition attenuates tissue injury and systemic inflammation in septic mice

We assessed systemic levels of organ injury markers (ALT, AST and LDH), inflammatory cytokines (IL-6 and IL-1β), and chemokine (MIP-2) following treatment with either anti-JAM-C Ab or Isotype IgG control during sepsis. Plasma levels of ALT, AST and LDH were significantly elevated in the IgG-treated control group as compared to the sham-operated group (Fig 3A–C). By contrast, the treatment with anti-JAM-C Ab significantly reduced the levels of ALT, AST, and LDH by 37%, 25%, and 58%, respectively, as compared to the IgG control group (Fig 3A–C). Similarly, the plasma levels of IL-6, IL-1β, and MIP-2 were significantly increased in IgG control group as compared to the sham-operated group, whereas the treatment with anti-JAM-C Ab significantly decreased the plasma levels of IL-6, IL-1β, and MIP-2 by 50%, 68%, and 58%, respectively, as compared to the isotype IgG control-treated animals (Fig 3D–F).

Inhibition of JAM-C improves sepsis-induced ALI in mice

To assess whether the attenuation of aged neutrophil population in the lungs via inhibition of JAM-C can lead to the improvement of the ALI during sepsis, histological examination of lung tissue as well as neutrophil infiltration assays were performed. The lung tissues in the isotype IgG control group showed substantial morphological changes including edema, hemorrhage, alveolar collapse, and inflammatory cell infiltrations as compared with the sham group (Fig 4A, B). On the other hand, the treatment with anti-JAM-C Ab dramatically reduced the microscopic deterioration which revealed 35% decrease in terms of injury score in comparison with the isotype control group (Fig 4A, B).

Anti-JAM-C Ab treatment attenuates neutrophil infiltration in lungs in sepsis

We examined Gr-1 immunostaining, a surface marker of activated neutrophils in the lungs. The number of Gr-1-positive cells in the lung tissues was significantly increased in the isotype IgG control group as compared to the sham-operated mice (Fig 5A, B). However, the number of Gr-1 positive cells in the JAM-C Ab treated group was significantly reduced by 71.5% compared to the isotype IgG control-treated mice with sepsis (Fig 5A, B).

Treatment with anti-JAM-C Ab improves sepsis-induced lung inflammation in mice

We further assessed the expression of proinflammatory cytokines (IL-6, IL-1β) and chemokine MIP-2 at their mRNA and protein levels. Both the mRNA and protein levels of IL-6 in the lung tissues were significantly increased in the isotype IgG control group as compared to sham-operated mice, while the treatment with anti-JAM-C Ab significantly inhibited its mRNA and protein levels by 81% and 61%, respectively, as compared with the isotype IgG control mice (Fig 6A, D). Although we could not find any statistically significant reduction in the expression levels of IL-1β in both mRNA and protein in anti-JAM-C Ab-treated animals compared to isotype IgG control-treated septic animals, we noticed 44% and 35% decrease in the levels of IL-1β mRNA and protein, respectively in the lung tissues in anti-JAM-C Ab-treated mice compared to IgG-treated septic mice (Fig 6B, E). The expression levels of MIP-2 in both mRNA and protein in the lung tissues were significantly up-regulated in the isotype IgG control-treated animals as compared with the sham-operated mice and they were significantly decreased in anti-JAM-C Ab-treated animals by 92% and 61%, respectively, relative to the isotype IgG control-treated mice (Fig 6C, F).

DISCUSSION

In this study, we demonstrated that soluble JAM-C levels in serum were increased in sepsis. Soluble JAM-C promoted neutrophil aging as confirmed by increased surface expression of CXCR4. Post-treatment of mice with anti-JAM-C Ab significantly attenuated systemic inflammation and lung tissue injury and inflammation during sepsis. Here, we used CXCR4 as one of the key surface markers to detect aged neutrophils during sepsis. Among other surface markers of aging of neutrophils only CXCR4 expression was spontaneously upregulated without any stimulation by incubating isolated neutrophils from human blood *in vitro* [29]. In our study, murine bone marrow neutrophils also showed an upregulation of CXCR4 expression in a time-dependent manner, indicating CXCR4 is a reliable marker for detecting aged neutrophils. Although we found spontaneous upregulation of surface expression of CXCR4 in aged neutrophils, the surface expression of CD62L another marker of aged neutrophils was not reduced spontaneously while incubating the cells without any stimuli (data not shown). Since CD62L expression is down-regulated by proteolytic cleavage by ADAM-17 and other shedders [30, 31], we assume that the decreased levels of CD62L in aged neutrophil population as described previously [15] could be the result of external factors.

A growing body of literature refers to the consequences of delayed apoptosis and prolonged lifespan of neutrophils at the site of infection during inflammation [10, 24, 32], which

appears to correlate with disease severity and outcome in critical illness such as ARDS, sepsis, burn, and acute coronary artery disease [33–36]. Our study also supports these findings by showing increased cytokine production and exaggerated tissue injury with upregulation of aged neutrophils in lungs following sepsis. Among the various signaling pathways regulating neutrophil apoptosis discovered [37], outside-in signaling induced by the interaction between integrin receptors on neutrophils and their ligands on endothelial cells during the adhesion and transmigration process of neutrophils before their infiltration into the inflamed tissues seems to be critical to inhibit their apoptosis [24, 28, 38]. Adhesive molecules on endothelial cells such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) had been shown as inhibitory regulators of the apoptosis of neutrophils [24, 39–41], indicating that the neutrophil adhesion and transmigration is the process not only for their extravasation but for the control of their aging as well.

In this study, we for the first time, showed that JAM-C, an endothelial molecule which is known to regulate neutrophil transmigration, had an inhibitory effect on neutrophil apoptosis through Mac-1 (CD11b) signaling pathway *in vitro*. Furthermore, we were able to downregulate aged neutrophil population in the lungs during sepsis in mice by the blockade of JAM-C using its neutralizing Ab which might accelerate neutrophil apoptosis. Subsequently, the treatment with anti-JAM-C Ab reduced local inflammation by inhibiting cytokine and chemokine production and microscopic tissue injury in lungs during sepsis. Thus, regulating aged neutrophil population at the inflamed tissues could help attenuate sepsis-induced ALI.

We injected mice with anti-JAM-C Ab post CLP to mimic clinical situation. We chose to inject mice with anti-JAM-C Ab or IgG at 4 h after CLP because this time period is known as the hyperdynamic phase of sepsis where the expression of the pro-inflammatory cytokines and chemokines are robustly up-regulated [42, 43]. We selected a single time point to inject anti-JAM-C Ab or IgG in septic mice because antibody drugs typically possess several desirable pharmacological characteristics, such as long serum half-lives, high potency, and limited off-target toxicity [44]. With this single dose of anti-JAM-C Ab treatment we obtained significant protection from sepsis-induced acute lung injury in mice.

In our study, IL-1β levels in the serum were significantly decreased following treatment with anti-JAM-C Ab, while in the lungs the decrease of IL-1β expression after treatment of septic mice with anti-JAM-C Ab was not statistically significant, despite the decrease was profound. This discrepancy could be due to the diverse pattern of the induction of various cytokines in the circulation and in tissues with respect to time course change in sepsis [45–48]. In the present study, we assessed the pro-inflammatory cytokines in serum and in lungs at a single time point, which might lead to omission of the original peak of induction of a specific cytokine or its inhibition by the treatment with anti-JAM-C Ab in sepsis. Future studies including multiple time-points after CLP to generate a kinetic plot for each cytokine following treatment with anti-JAM-C Ab in sepsis will provide valuable information about the inhibitory effect of this drug during sepsis.

Endothelial JAM-C is cleaved by metalloproteinases ADAM10, ADAM17, and neutrophil elastases in inflammation and exists in circulation in its soluble form [23, 49]. Since sepsis causes induction of the expression of ADAM10 and ADAM17 proteins [50], it is therefore reasonable to obtain elevated levels of circulatory JAM-C protein following sepsis. Similar to lung tissues, treatment with anti-JAM-C Ab also decreased circulatory aged neutrophil population, indicating that soluble JAM-C possesses an anti-apoptotic effect on circulatory neutrophils and can prolong their survival as endothelial JAM-C performs. Thus, neutralization of JAM-C contributed to not only the improvement of local inflammation but also systemic inflammation in septic mice as illustrated in Fig 7.

Aside from inhibiting $CXCR4^{+}$ aged neutrophils in blood and lungs, our data showing reduced neutrophil infiltration in the lungs following treatment with anti-JAM-C Ab in sepsis also implicates endothelial JAM-C induces neutrophil infiltration in the inflamed tissues [21]. Thus, the downregulation of the number of infiltrating neutrophils into the lungs by inhibition of JAM-C could be another mechanism for improving the outcomes in sepsis-induced ALI. In the present study, we demonstrated the inhibition of aged neutrophils by treatment with anti-JAM-C Ab in circulation and lung tissues at a single time point of 20 h after CLP operation. However, it is well-recognized that the pathophysiology of sepsis changes with time course from the onset of the disease which is best described as systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS) [42]. Hence, investigation of the kinetics of aged neutrophil population will provide additional insights on the pathophysiology of sepsis.

The most authentic evidence of whether ALI has occurred in an animal is provided by histological images, which define neutrophil infiltration into the lungs and measure the integrity of the alveolar capillary barrier showing the lung injury. Similarly, the key pathological features of human ALI/ARDS are a severe neutrophilic alveolitis with deposition of hyaline membrane and formation of microthrombi [51]. In CLP-induced sepsis model, increased epithelial permeability, PMN accumulation in the interstitium and alveolar spaces are the hallmarks of ALI. In our study, in addition to the assessment of pro-inflammatory cytokines and chemokines in the lung tissues, we performed histological assessments and neutrophil infiltrations in lungs which clearly defined the extent of lung injury and inflammation in sepsis. In the present study, we did not assess the blood gas levels in mice after CLP-induced sepsis which serves as a pivotal marker of ALI. Further studies to assess lung function test focusing on blood gases and other clinical parameters during sepsis will help provide additional information regarding pathopgysiology and therapeutic potential of anti-JAM-C Ab in sepsis-induced ALI.

It is interesting to know whether or not the improvement of systemic inflammation and lung injury after treatment with anti-JAM-C Ab in septic mice can improve their survival outcomes in mice. A recent study demonstrated the beneficial outcomes in the survival of JAM-$A^{-/-}$ mice in sepsis [52]. It was previously shown that the JAM-A protein is closely related to the structure and function of JAM-C protein [53], thus pointing out the possibility of JAM-$C^{-/-}$ mice to exhibit similar beneficial outcomes in the survival in sepsis as those of JAM-$A^{-/-}$ mice. A future study demonstrating the outcomes of survival in JAM-$C^{-/-}$ mice

or following treatment with anti-JAM-C Ab in WT sepsis mice will help establish JAM-C to further use in clinical aspects of sepsis-induced ALI.

During inflammation, neutrophils are released from the bone marrow into circulation and then migrate toward inflamed tissues to play an important role in host defense against pathogen [54]. However, the excess accumulation and activation of neutrophils can cause severe tissue injury [54]. The apoptosis of neutrophils and their phagocytic engulfment by macrophages are important for the resolution of inflammation to prevent tissue injury [55]. $CXCR4^+$ neutrophils play pro-inflammatory role and have longer life span [15, 55]. Prolonged neutrophil infiltration in lungs are deleterious, therefore decrease of these neutrophils by anti-JAM-C Ab treatment reflected beneficial outcomes in sepsis. On the other side, since neutrophils play effector function against invading pathogen, it may be a concern whether or not the reduction of $CXCR4^+$ neutrophils impairs the host response to infection at the early stage of septic infection. In the present study, we delivered anti-JAM-C Ab at 4 h after CLP, by this time the innate immune response might have launched to defend host against pathogen. The above concern can also be resolved by the fact that CXCR4 serves as a pivotal receptor for negatively controlling neutrophil release from bone marrow [17]. It has been shown that higher levels of CXCR4 causes less neutrophil release from bone marrow [17, 56]. Our approach of using anti-JAM-C Ab to decrease the frequencies of pro-inflammatory $CXCR4^+$ neutrophils might help neutrophils to get released from bone marrow, ensuring efficient host defense against infection at the early phase of sepsis. Therefore, treatment with anti-JAM-C Ab not only decreased the pro-inflammatory $CXCR4^+$ phenotype of neutrophils to lessen overall inflammation, but also might promote neutrophil release from bone marrow into the circulation to maintain homeostasis.

In conclusion, we found that the blockade of JAM-C by its neutralizing Ab reduced the contents of aged neutrophil population in both the lung tissue and circulation of septic mice. We also found that the inhibition of JAM-C improved ALI as well as systemic inflammation in septic mice, suggesting that the reduction of proinflammatory population of aged neutrophil by blocking anti-apoptotic molecule JAM-C could have therapeutic benefits in sepsis. Although there are still questions worth addressing, such as the detailed inflammatory properties of aged neutrophil population and the difference of neutrophil phenotypes between animals and humans, we believe that further research in the aged neutrophil population merits the struggle in order to elucidate sepsis treatment.

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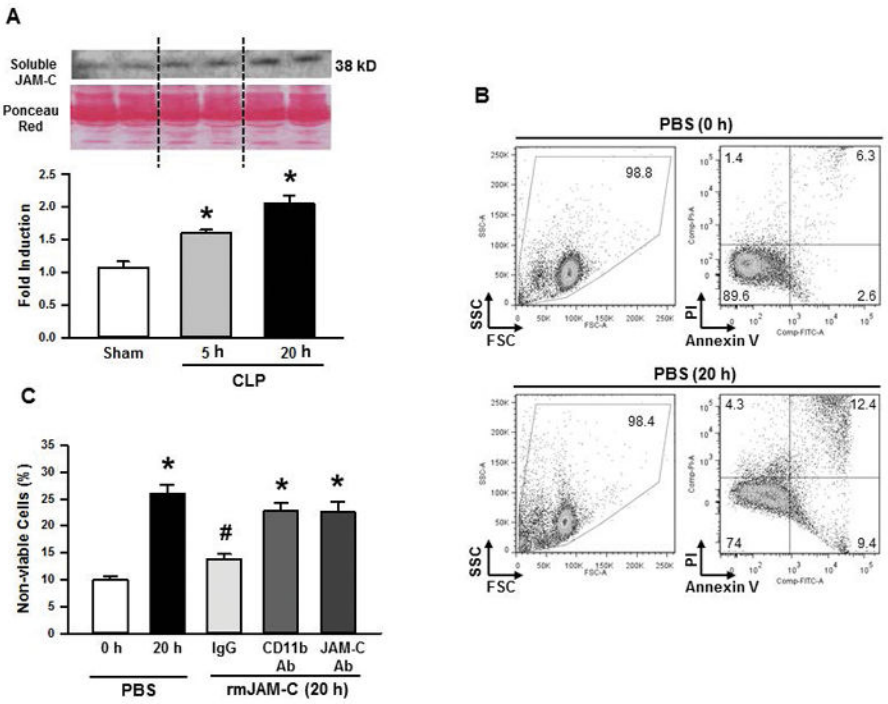

Figure 1. Inhibition of neutrophil apoptosis by stimulation with rmJAM-C

(A) Blood samples were collected at 5 and 20 h after CLP and then serum was separated from each sample. Soluble JAM-C levels in serum from sham and CLP animals were determined by Western blot. A representative blot and densitometric analysis are presented. As control, Western blots were stained by Ponceau red to ensure equal loading of each sample. Data are expressed as means ± SE and compared by one-way ANOVA and SNK method. $^{*}p<0.05$ *vs.* sham-operated animals. (n=7 mice/group). **(B)** BMDN (1×10^6 cells) were cultured for 20 h to assess their viability. The cells were stained with Annexin V (FITC) and propidium iodide (PI). Freshly isolated BMDN (0 h) were also stained similarly. Representative flow cytometric dot blots showing the Annexin V and PI staining of BMDN cultured at 0 h or 20 h are presented. **(C)** After pre-treatment with equivalent concentration of isotype IgG control or anti-CD11b (Mac-1) blocking Ab or anti-JAM-C Ab, BMDN (1×10^6 cells) were stimulated with rmJAM-C (5 μg/ml) for 20 h. The average frequencies (%) of non-viable cells as described in the Materials and Methods of respective groups are shown in bar diagram. Data are expressed as means ± SE. $^{*}p<0.05$ *vs.* PBS 0 h, $^{\#}p<0.05$ *vs.* PBS 20 h. (n = 7 samples/group). BMDN, bone marrow-derived neutrophils; rm-JAM-C, recombinant mouse junctional adhesion molecule; SNK, Student-Newman-Keuls.

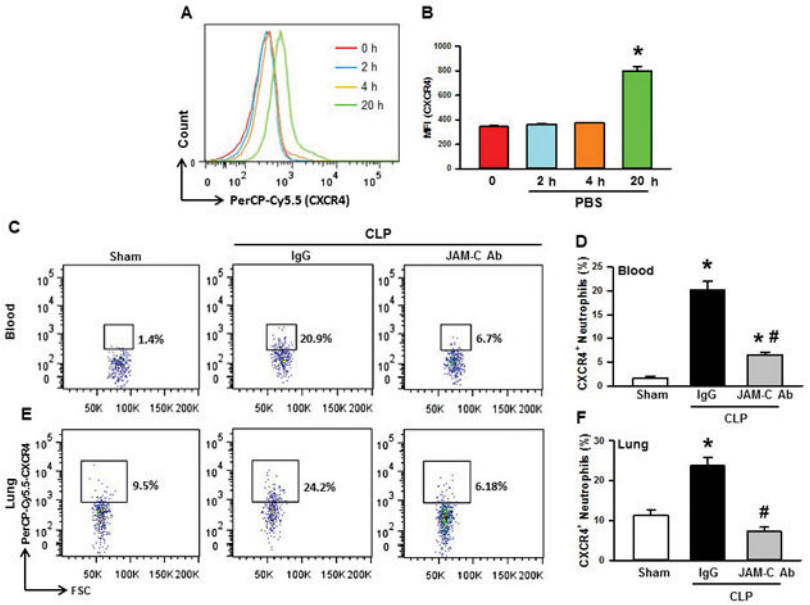

Figure 2. Inhibition of CXCR4 expressing aged neutrophils by treatment with anti-JAM-C Ab

Isolated BMDN were incubated with PBS for 0, 2, 4, 20 h and then stained with PerCP-Cy5.5-CXCR4 Ab for flow cytometric analysis. **(A)** Representative flow cytometric histograms for each group are shown. **(B)** The MFI for each group is compared. Data are represented as means ± SE and compared by one-way ANOVA and SNK method. $^{*}p<0.05$ *vs.* 0 h. (n=9/group from 3 independent experiments). **(C)** Neutrophils were isolated from blood at 20 h after CLP, and then stained with PerCP-Cy5.5-CXCR4 Ab. The stained cells were subjected to flow cytometric analysis to measure the frequency of $CXCR4^{+}$ population among neutrophils. The representative data for the sham, isotype IgG control and anti-JAM-C Ab treatment groups are shown. **(D)** The average of the frequency of $CXCR4^{+}$ population among neutrophils for the sham, isotype IgG control and anti-JAM-C Ab treatment groups are shown in bar diagram. Data are represented as means ± SE and compared by one-way ANOVA and SNK method. $^{*}p<0.05$ *vs.* sham; $^{\#}p<0.05$ *vs.* isotype IgG control. (n=6 mice/group). **(E)** Neutrophils were isolated from lungs harvested at 20 h after CLP, then stained with PerCP-Cy5.5-CXCR4 Ab. The stained cells were subjected to flow cytometric analysis to measure the percentage of $CXCR4^{+}$ population among neutrophils. The representative data for the sham, isotype IgG control and anti-JAM-C Ab treatment groups are shown. **(F)** The bar diagram showing the average percentage of $CXCR4^{+}$ population among neutrophils for the sham, IgG control and anti-JAM-C Ab treatment groups are presented. Data are represented as means ± SE and compared by one-way ANOVA and SNK method. $^{*}p < 0.05$ *vs.* sham; $^{\#}p < 0.05$ *vs.* IgG. (n=6 mice/group). BMDN, bone marrow-derived neutrophils; CLP, cecal ligation and puncture; JAM-C, junctional adhesion molecule; MFI, mean fluorescence intensities; SNK, Student-Newman-Keuls.

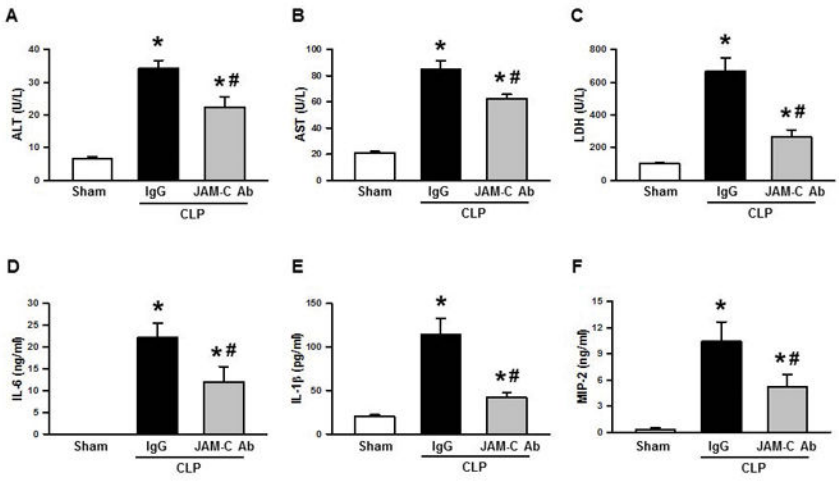

Figure 3. Reduction of systemic injury and inflammatory markers by the treatment with anti-JAM-C Ab in sepsis

Sepsis was induced in mice by CLP. At 4 h after CLP operation, mice were injected with either anti-JAM-C Ab or isotype IgG control at a dose of 3 mg/kg BW in 100 μl volumes through the jugular vein. After 20 h of CLP, plasma levels of injury markers **(A)** ALT, **(B)** AST, **(C)** LDH and inflammatory markers **(D)** IL-6, **(E)** IL-1β and **(F)** MIP-2 were assessed. Data are expressed as means ± SE and compared by one-way ANOVA and SNK method. $^{*}p < 0.05$ *vs.* sham; $^{\#}p < 0.05$ *vs.* isotype IgG control. (n=6–13 mice/group). CLP, cecal ligation and puncture; JAM-C, junctional adhesion molecule; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; IL, interleukin; MIP-2, macrophage inflammatory protein-2; SNK, Student-Newman-Keuls.

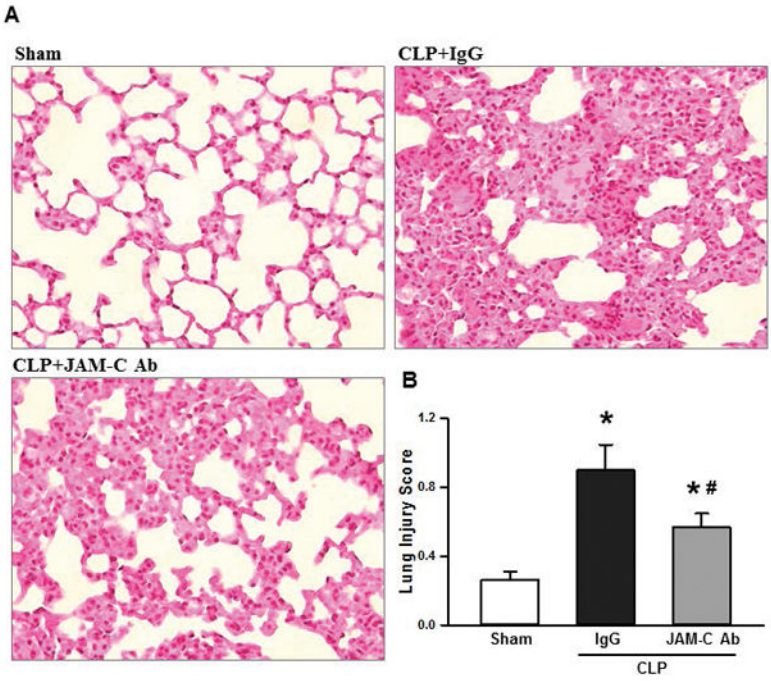

Figure 4. Improvement of lung histology by the treatment with anti-JAM-C Ab in sepsis

(A) Lung tissues harvested at 20 h after CLP were stained with H&E, and examined under light microscopy at ×200 original magnifications. Representative images for the sham, isotype IgG control and anti-JAM-C Ab treatment groups are shown. **(B)** The severity of histological injury in different groups was assessed by using a scoring system as described in the Materials and Methods. Data are expressed as means ± SE and compared by one-way ANOVA and SNK method. $^{*}p < 0.05$ *vs.* sham; $^{\#}p < 0.05$ *vs.* isotype IgG control. (n=5 mice/group). CLP, cecal ligation and puncture; JAM-C, junctional adhesion molecule; H&E, hematoxylin & eosin; SNK, Student-Newman-Keuls.

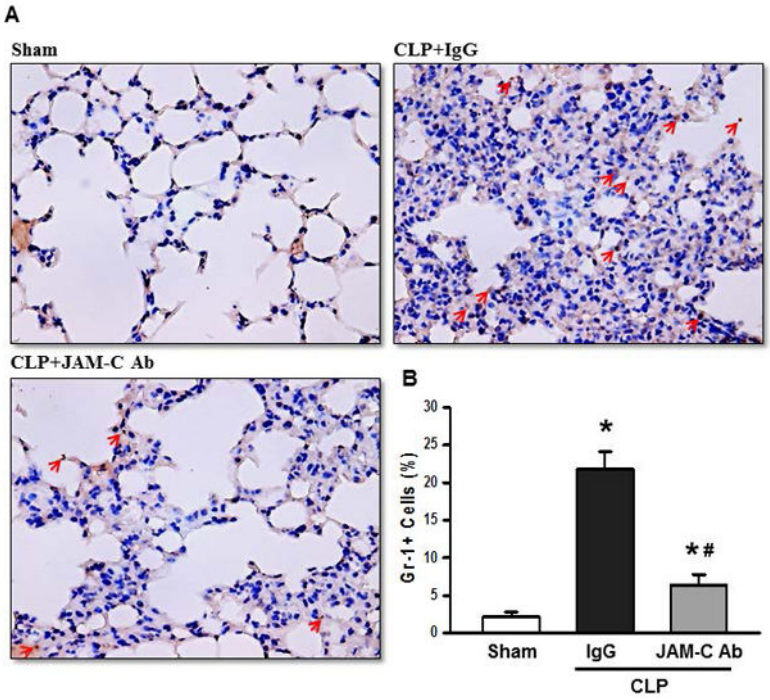

Figure 5. Decreased neutrophil infiltration in lungs in anti-JAM-C Ab-treated mice in sepsis

Lung tissues harvested at 20 h after CLP were immunostained using anti-Gr-1 Ab and examined under light microscopy at ×400 original magnifications. **(A)** Representative images for the sham, isotype IgG control and anti-JAM-C Ab treatment groups are shown. Arrows indicate examples of Gr-1 positive cells. **(D)** The numbers of Gr-1-positive cells averaged over 5 microscopic fields at ×400 magnification per animal's lung are shown. Data are expressed as means ± SE and compared by one-way ANOVA and SNK method. $^{*}p < 0.05$ *vs.* sham; $^{\#}p < 0.05$ *vs.* isotype IgG control. (n=5 mice/group). CLP, cecal ligation and puncture; JAM-C, junctional adhesion molecule; SNK, Student-Newman-Keuls.

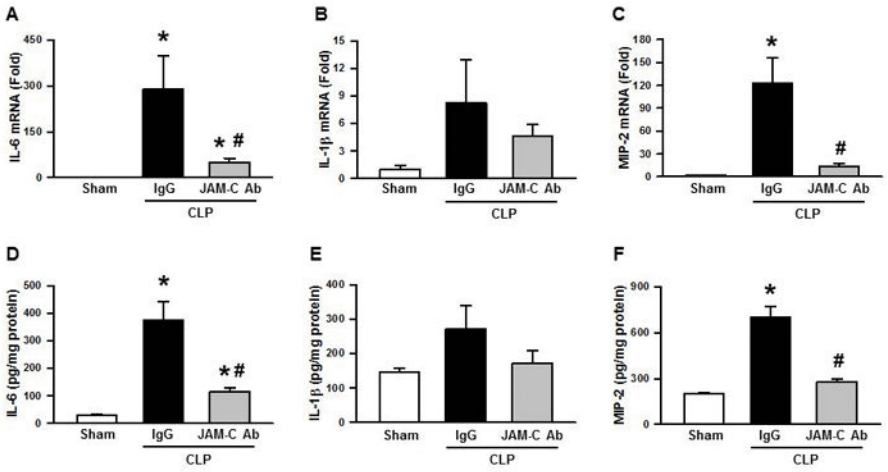

Figure 6. Inhibition of pro-inflammatory cytokines and chemokine production in lungs by the treatment with anti-JAM-C Ab during sepsis

At 4 h of CLP operation, mice were injected with either anti-JAM-C Ab or isotype IgG control at a dose of 3 mg/kg BW in 100 μl volumes through the jugular vein. The expression of **(A, D)** IL-6, **(B, E)** IL-1β and **(C, F)** MIP-2 at its mRNA and protein levels in the lung tissues harvested at 20 h after CLP were measured by using real-time qRT-PCR and ELISA, respectively. Gene expression was normalized to β-actin. The sham expression level was designated as 1 for comparison. Data are represented as means ± SE and compared by one-way ANOVA and SNK method. $^{*}p<0.05$ *vs.* sham; $^{\#}p<0.05$ *vs.* IgG. (n=6–9 mice/group). CLP, cecal ligation and puncture; JAM-C, junctional adhesion molecule; IL, interleukin; MIP-2, macrophage inflammatory protein-2; SNK, Student-Newman-Keuls.

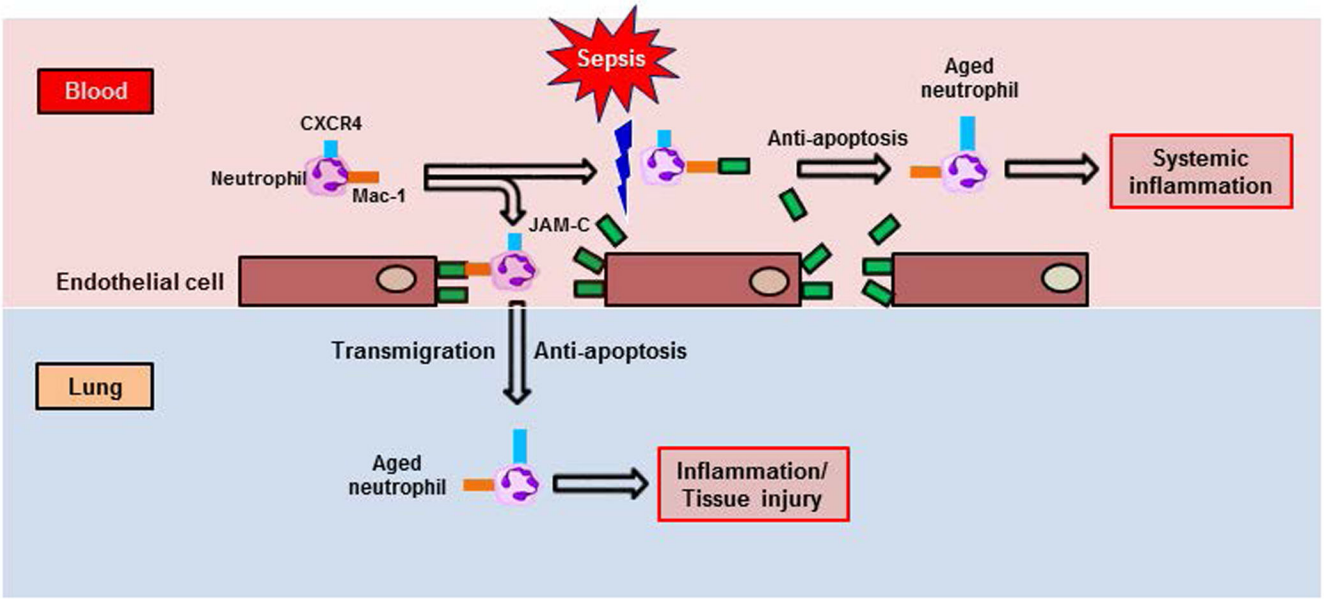

Figure 7. Mechanism of actions of JAM-C during sepsis

Neutrophils transmigrate from circulation to the inflamed tissues through the interaction with their Mac-1 receptor and endothelial JAM-C molecule. The interaction between Mac-1 on neutrophils and JAM-C on endothelial cells prolongs the survival of neutrophils by inhibiting apoptosis, resulting in the increase of aged neutrophil population. Aged neutrophil populations are characterized by high surface expression of CXCR4. $CXCR4^{+}$ neutrophils induce inflammation and injury to the lung tissues. Sepsis causes cleavage of endothelial JAM-C, which interacts with Mac-1 on circulatory neutrophils and prolongs their survival, leading to systemic inflammation. JAM-C, junctional adhesion molecule.