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TITLE:

Design of Cecal Ligation and Puncture and Intranasal Infection Dual Model of Sepsis-Induced Immunosuppression

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KEYWORDS:

cecal ligation and puncture, intranasal infection, sepsis-induced immunosuppression, immune suppression sepsis, sepsis, *Staphylococcus aureus*, nosocomial pneumonias, mice, dual model, double-hit model

SUMMARY:

This protocol describes techniques to measure infectious outcomes underlying secondary hospital-acquired infections in the immunosuppressive condition, first by establishing cecal ligation/puncture mice then challenging them with intranasal infection to create a clinically relevant model of immunosuppression sepsis.

ABSTRACT:

Sepsis, a severe and complicated life-threatening infection, is characterized by an imbalance between pro- and anti-inflammatory responses in multiple organs. With the development of therapies, most patients survive the hyperinflammatory phase but progress to an immunosuppressive phase, which increases the emergence of secondary infections. Therefore, improved understanding of the pathogenesis underlying secondary hospital-acquired infections in the immunosuppressive phase during sepsis is of tremendous importance. Reported here is a model to test infectious outcomes by creating double-hit infections in mice. A standard surgical procedure is used to induce polymicrobial peritonitis by cecal ligation and puncture (CLP) and

followed by intranasal infection of *Staphylococcus aureus* to simulate pneumonia occurring in immune suppression that is frequently seen in septic patients. This dual model can reflect the immunosuppressive state occurring in patients with protracted sepsis and susceptibility to secondary infection from nosocomial pneumonia. Hence, this model provides a simple experimental approach to investigate the pathophysiology of sepsis-induced secondary bacterial pneumonia, which may be used for discovering novel treatments for sepsis and its complications.

INTRODUCTION:

Sepsis initiates a complex interplay of host pro-inflammatory and anti-inflammatory processes and is characterized by a hyperinflammatory response and subsequent immune dysfunction[1,2]. Sepsis represents a global health priority and causes a high number of deaths in intensive care units (ICUs)[3]. The incidence of sepsis is estimated to exceed 30 million cases worldwide per year, with mortality rates as high as 30% despite advances in ICU management[4,5]. In 2017, the World Health Organization adopted a resolution to improve the prevention, diagnosis, and management of this deadly disease[5]. However, recent studies have illustrated that death does not result from primary infection in severe septic patients but rather from secondary nosocomial infection (particularly pneumonia) that caused by immunosuppression[6,7]. Therefore, understanding the mechanisms of why septic patients develop secondary infection and discovering more effective treatments are urgently required. Herein, a dual model, also known as a double-hit model, to study the immunosuppressive phenomenon occurring in patients with protracted sepsis is described.

As the gold standard experimental model in research on polymicrobial sepsis, cecal ligation and puncture (CLP) is a surgery characterized by cecum ligation and perforation, which contributes to polymicrobial peritonitis and sepsis[8,9]. The pathophysiological process and cytokine profiles, along with the kinetics and magnitude, are similar to clinical sepsis. The position of the ligation, needle size used for the puncture, and number of cecal punctures are major factors that impact the mortality following CLP.

The nosocomial pneumonia is the leading cause of mortality among critically ill patients with sepsis. The major type of organisms causing severe sepsis includes *Staphylococcus aureus* (20.5%), *Pseudomonas* species (19.9%), *Enterobacteriacae* (mainly *E. coli*, 16.0%), and fungi (19%). Meanwhile, recent studies have suggested an increasing incidence of gram-positive organisms, which are now almost as common as gram-negative infections[3].

The method described in this protocol involves CLP, performed as the “first hit” to induce sublethal polymicrobial peritonitis, which manifests an immunosuppression condition. The procedure also involves subsequent intranasal instillation of *S. aureus* as the “second hit” to provide a clinically relevant research platform.

PROTOCOL:

All methods described here were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by the University of North Dakota Institutional Animal Care and Use Committee (IACUC).

1. Cecal ligation and puncture

NOTE: Female C57BL/6 mice (weight, 18–22 g; age, 6–8 weeks) are randomly divided into six groups: control group (Ctrl), infection group (SA for *S. aureus*), two sham groups, and two CLP groups. Ctrl animals are left without surgery and secondary infection injuries. SA animals are subjected to *S. aureus* lung infection without the operation. Sham-operated animals undergo the same laparotomy with an exposition of the cecum (except the cecum is neither ligated nor punctured). Eight mice per group are used for survival analysis, and three to five mice are used for assessment of inflammation at various timepoints.

1.1. Prior to surgery, sterilize all surgical instruments and materials by autoclaving for 20 min. Clean and disinfect the operating table with disinfectant wipes. To establish sterile conditions during the surgery, cover the entire operative fields with proper sterile surgical drapes, and wear a hair cover, surgical mask, protective eyewear, surgical gown, and sterile gloves.

1.2. Weigh and anesthetize the mouse using 0.1 mL/mouse of ketamine (80 mg/kg), xylazine (10 mg/kg) mixture intraperitoneally administered. With the thumb and index finger of the left hand forming a U-shaped position, approach the neck of the mouse from behind smoothly and gently, then hold the neck immediately behind the ears so that the mouse head is highly restrained. Use a pinky to hold the mouse's right back leg and use the middle and ring fingers (all fingers from left hand) to hold the body.

1.3. Check the intensity of anesthesia by toe pinch until no flexion of the extremity.

1.4. Shave the lower quadrants of the abdomen using an electric razor and disinfect the area with iodine.

1.5. Place the mouse onto the sterile surface in a supine position, with the head oriented away from the operator. Drape the mouse with sterile towel with hole over the planned surgical incision to maintain sterile fields.

1.6. Make a longitudinal skin incision (about 0.5 cm long) with a scalpel in the left lower abdomen. Use small scissors to extend the incision (1–2 cm).

CAUTION: Be careful not to penetrate the peritoneal cavity.

1.7. Make the intramuscular incision to gain access into the peritoneal cavity and allow exposure of the cecum with the adjoining intestine.

1.8. Isolate the cecum on the left side of the abdomen (in most cases, this is done by using

blunt anatomical forceps) and remove it on a sterile drape, leaving the small and large intestines in the peritoneal cavity.

CAUTION: Avoid damaging the mesenteric blood vessels.

1.9. Ligate the cecum at the distal 25% position to create a prolonged infection with relatively low mortality.

CAUTION: Make sure not to ligate the ileocecal valve.

1.10. Perforate the cecum with a 21 G 1 ½ needle by single through-and-through puncture (two holes) midway between the ligation and tip of the cecum at the least-vascularized area.

CAUTION: Make sure not to puncture blood vessels.

1.11. Remove the needle. Gently extrude a small amount of feces from the penetration holes to ensure full thickness perforation.

CAUTION: Take care not to obstruct the bowel continuity during the procedure.

1.12. Replace the cecum into the abdominal cavity.

1.13. Close the abdomen in two layers with 4-0 nylon surgical sutures. Close the abdominal musculature by applying simple running sutures and close the skin by applying simple interrupted sutures. Clean the skin with Iodine.

1.14. Inject 1 mL of prewarmed (37 °C) 0.9% sterile normal saline (NS) solution on the back subcutaneously to replace third space loss and place it on a warm pad to recover.

1.15. Return the mice in cage in a temperature-controlled room (22 °C) with 12 h light/dark cycles and free access to food and water. Monitor the mice every 0.5 hours for at least 2 h, then every 6 h for at least 2 d, then every 12 h for 3 days, or euthanize them when moribund for survival analysis.

NOTE: Pre- and post-operative analgesia (buprenorphine, 50 µg/kg subcutaneously every 12 h) are recommended for 24 h before surgery until 48 h after surgery[9,10].

2. Secondary lung infection with *S. aureus*

NOTE: Except for the Ctrl and SA groups, surviving mice at 3 days post-CLP in the sham and CLP groups should be administered intranasally with 30 µL of a bacterial suspension or NS, respectively. The surviving mice in the SA group should be instilled intranasally with the bacterial suspension.

2.1. Anesthetize the mouse by intraperitoneally injecting 100 µL solution of ketamine (45mg/kg) and xylazine (10 mg/kg) and wait until the mouse breaths slowly. The puncturing angle between the needle and abdominal wall is less than 30° for slow injection.

2.2. With the help of a micropipette, slowly and intranasally instill with 30 µL of 1 x 10^7 colony forming unit (CFU) of *S. aureus* to be aspirated on inhalation. Holding the mouse by its ears in an upright position, slowly drop 2–3 µL of bacterial suspension into both nostrils each time. Adjust the rate of release to allow the mouse to inhale without forming bubbles and dropping off.

2.3. Raise the mouse up and down, with its head up and tail down, to help the bacteria enter the trachea. Move the mouse up quickly and strongly, while moving it down gently and slowly to increase the gravity. Repeat instillation about 15x until all bacteria are inhaled, which should take 10–15 min for each mouse.

2.4. Lay the mouse on its back and head up on the angled bedding (about 35°) and watch the mouse until its breathing returns to normal and it is completely recovered from the anesthesia and procedures.

2.5. Place the mouse back in the cage in a temperature-controlled room (22 °C) with 12 h light/dark cycles and unlimited access to food and water.

2.6. Check mouse conditions every 2 h for the first day and every 12 h afterwards for 4 days after the surgery. Euthanize them when moribund for survival analysis or 24 h after infection for assessment of inflammation.

3. Analyzed parameters

3.1. Mice survival

3.1.1. Monitor the mice for 7 days. Euthanize when moribund and generate survival curves using Kaplan-Meier methods[11].

3.2. Bacterial counts

3.2.1. 24 h after SA injection, harvest the blood, bronchoalveolar lavage fluid (BALF), and peritoneal lavage fluid (PLF) from the mice. Subsequently, add 100 µL of the diluents to nutrient agar plates and culture them at 37 °C for 24 h to determine the bacterial colony counts[11-13].

3.3. Cytokines

NOTE: Pro-inflammatory cytokine concentrations of IL-1β, IL-6, and TNFα in serum and BALF from mice should be assessed using ELISA kits according to the manufacturer's instructions.

3.3.1. Coat the 96 well ELISA plate with 100 µL/well of different capture antibody working solution to run the standards in duplicate and samples in triplicate. Seal the plate and leave it overnight at 4 °C.

3.3.2. Wash the plate three times with 255 µL/well wash buffer (1x PBS, 0.05% Tween-20).

3.3.3. To block nonspecific binding, add 200 µL/well diluent. Seal the plate and incubate at room temperature (RT) for 1 h.

3.3.4. Prepare standard solution: Eight two-fold serial dilutions from 1000 pg/mL.

3.3.5. Add 100 µL/well of standards or samples to the appropriate wells and 100 µL/well of diluent to the blank wells. Seal the plate and incubate at RT for 2 h or overnight at 4 °C.

3.3.6. Wash the plate 5x with 255 µL/well wash buffer.

3.3.7. Add 100 µL/well of different detection antibody working solution to the appropriate wells. Seal the plate and incubate at RT for 1 h.

3.3.8. Wash the plate 5x with 255 µL/well wash buffer.

3.3.9. Add 100 µL/well of diluted HRP solution to all wells. Seal the plate and incubate at RT for 30 min.

3.3.10. Wash the plate 5x with 255 µL/well wash buffer.

3.3.11. Add 100 µL/well diluted TMB solution. Incubate the plate away from light at RT for 15 min.

3.3.12. Add 50 µL/well stop solution (1 M H_3PO_4). Read absorbance at 450 nm with a plate reader.

3.4. Flow cytometry

3.4.1. Obtain the total number of cells from BALF. Lyse the erythrocytes using ACK lysing buffer and wash 2x using PBS.

3.4.2. Analyze then the single-cell suspensions by flow cytometry as previously described[11]. For surface staining, stain neutrophils (CD11b+GR-1+) with APC anti-mouse CD11b and anti-mouse Gr-1 (Ly-6G/Ly-6C) antibodies. Collect a minimum of 3 x 10^4 live, non-debris cells for analysis.

REPRESENTATIVE RESULTS:

Depending on the experimental design and procedures, C57BL/6 mice were subjected to CLP,

and after 3 days, they were administered bacteria intranasally (**Figure 1**). As shown in **Figure 2**, the mice began to die at ~12 h after induction of peritonitis. Two mice in the CLP+SA group and three mice in the CLP+NS group died before intranasal *S. aureus* instillation. No mortality was detected in uninfected non- or sham-operated mice. Therefore, when mice had CLP before pneumonia, mortality was much higher ($p < 0.05$). After intranasal bacteria challenge (1×10^7 CFU), three of eight mice survived in both the SA and Sham+SA groups, and four of eight mice survived in the CLP+NS group. However, all eight mice died in the CLP+SA group. In contrast, every mouse in the Ctrl group and Sham+NS group survived. CLP mice showed more mortality when subsequently challenged with *S. aureus.* Mortality of *S. aureus* after CLP (100%) was higher than infected alone (37.5%) or *S. aureus* after sham-operated (37.5%; $p < 0.05$).

As shown in **Figure 3**, severe caecum necrosis was observed in post-CLP animals, but more so from the double-hit group (CLP+SA). However, there was no gross change of the cecum in control or single-hit with bacteria groups. Blood, BALF, and PLF were cultured to assess the lung bacterial clearance of mice infected with *S. aureus* 3 days post-CLP. This high lethality was associated with significantly increased *S. aureus* CFU in the blood and PLF of CLP mice compared with sham mice ($p < 0.05$; **Figure 4A,C**). The number of *S. aureus* CFU in the blood and BALF of CLP+SA mice was markedly greater than CLP+NS mice ($p < 0.01$; **Figure 4A,B**).

For pro-inflammatory cytokines, results showed that the expression levels of serum IL-1β, IL-6, and TNFα significantly increased at 24 h after bacterial instillation in sepsis-surviving mice compared to the mice that underwent CLP alone or to the sham-operated mice challenged with *S. aureus* (**Figure 5A**). However, the pro-inflammatory cytokines increased slightly in BALF of septic mice with secondary infection, different from those in the control-infected mice (SA mice) and Sham+SA. Meanwhile, double-hit mice exhibited significantly decreased levels in BALF IL-1β, IL-6, and TNFα levels compared to both SA and Sham+SA mice ($p < 0.001$; **Figure 5B**).

As shown in **Figure 6**, mice were sacrificed at 24 h after infection, and relative neutrophil percentage in BALF were detected by flow cytometry. Double-hit mice exhibited a significant reduction of neutrophils in the BALF compared to mice that underwent *S. aureus* pneumonia alone. Collectively, these data showed that CLP impairs the host immune responses, resulting in increased susceptibility to secondary bacterial pneumonia.

FIGURE LEGENDS:

Figure 1: Experimental design. Mice were randomly divided into six groups. Two groups underwent cecal ligation and puncture (CLP) at D0, and the others were sham-operated or not operated. Three days after surgery (D3), *S. aureus* [SA, 1×10^7 colony forming units (CFU)] or normal saline (NS) was administered intranasally. The control group (Ctrl) mice were not intranasally instilled. Blood, bronchoalveolar lavage fluid (BALF), and peritoneal lavage fluid (PLF) were harvested 24 h after SA injection for a bacteria count assay. The mortality rate in each group was observed over the course of 7 days for survival analysis.

Figure 2: Mouse survival. C57BL/6 mice submitted to either CLP or sham surgery received *S. aureus* (SA) or normal saline (NS) on the third day after surgery (n = 8 mice per group). The mice were monitored for 7 days, and mortality was recorded every 12 h. Kaplan-Meier survival curves, log-rank test (*p < 0.05) compared with Sham+SA mice and SA mice.

Figure 3: Mouse cecum. Secondary infection was induced 3 days post-CLP. 24 h after SA infection, mice were sacrificed to collect colon tissues. The representative photos of cecal ligation under different injury hit are shown. SA = *S. aureus*; NS = normal saline; CLP = cecal ligation and puncture. Scale bar = 1 cm.

Figure 4: Bacterial counts and representative images of agar plates. 3 days after surgery, mice were intranasally instilled with 1 x 10^7 CFU of *S. aureus* (SA) (n ≥ 3 mice per group). Blood, BALF, and PLF were harvested 24 h after SA injection, and bacterial colony counts were determined after 24 h of incubation. Results are expressed as mean ± SEM. One-way ANOVA (Tukey's post hoc; *p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant).

Figure 5: ELISA detecting cytokine secretion. Secondary infection was induced 3 days post-CLP. Concentrations of IL-1β, IL-6, and TNFα in serum (**A**) and BALF (**B**) from mice after SA infection (n ≥ 3 mice per group). Data are presented as the mean ± SEM of three experiments. One-way ANOVA (Tukey's post hoc; *p < 0.05; **p < 0.01; ****p < 0.0001; ns = not significant).

Figure 6: Representative frequency of neutrophil penetration. 24 h after intranasal infection, mice were sacrificed to obtain BALF (n ≥ 3 mice per group). The percentage of neutrophils ($CD11b^+$, $GR\text{-}1^+$) was quantified by flow cytometry and are shown as means ± SEM of three experiments. One-way ANOVA (Tukey's post hoc; ****p < 0.0001; ns = not significant).

DISCUSSION:

As the gold standard model for sepsis research, CLP has a combination of three insults, including tissue trauma caused by the laparotomy, necrosis due to ligation of the cecum, and infection as a result of microbial leakage that causes peritonitis with translocation of bacteria into blood[8]. Therefore, CLP mimics the complexity of human sepsis better than many other models. However, a major limitation of current CLP model is the inability to reflect the more prolonged phase of sepsis seen in patients in ICU[3-5]. Hence, a clinically relevant dual model (double-hit model) was proposed to reflect delayed mortality of sepsis and investigate the mechanisms underlying pulmonary secondary infection. In this protocol, sepsis was induced by combining CLP with *S. aureus* lung infection at 3 days post-CLP. Two-hit mice exhibited higher mortality, severe cecum damage, weakened blood, BALF bacteria clearances, lower pro-inflammatory cytokines, and lower neutrophils in BALF. This resulted in the severe sepsis, which mimics the immunosuppression status in the clinics.

The following descriptions are critical steps. Shown in detail is how to produce sublethal sepsis under the same conditions. First, female mice were used because female mice are more resistant to CLP than male mice[11]. Second, CLP-induced mortality depends on several technical

parameters, such as the length of the cecum ligated, needle size, and number of cecal punctures[9]. Ligation of approximately 75% of the cecum induces severe sepsis, ligation of 60% of the cecum induces middle-level sepsis, and ligation of 25% or less of the cecum results in minor sepsis[9]. It was chosen to standardize the model by performing a mild CLP (25%, single through-and-through puncture with a 21 G 1 ½ needle) to induce sublethal sepsis[11,12]. Third, fluid resuscitation is recommended to prevent shock and rapid death due to circulation collapse and develop a hyperdynamic animal sepsis model, which more closely mimics hemodynamic profile of human sepsis[13]. Additionally, the use of analgesics, such as buprenorphine, should be considered from an experimental and ethical standpoint[10].

Three days post-CLP was chosen as a timepoint to induce secondary infection to reflect the immunosuppressive phase of sepsis. Most patients with sepsis have a protracted hospital course with most deaths occurring beyond 3 days, and many then enter into secondary hospital-acquired pneumonia, which was consistently shown in a recent study with CLP along with *P. aeruginosa*[14]. Previous results from other laboratories also demonstrate that 3 days after CLP, mice show high sensitivity to secondary instilled bacteria, and one day after infection was the turning point from over-inflammation to immunosuppression[15,16].

In addition, differences in bacteria strains and dosage instilled are significant factors causing variability in the dual model. The selection of strain and dose levels are based on the needs of the experimental design of secondary infection during immunosuppressive status. Based on previous findings, 1×10^7 CFU of *S. aureus* was selected for this study.

To improve the efficiency of intranasal bacterial delivery directly into the mouse lungs, attention should be paid to the instillation volume, time, and body position. There are other operational matters that may immensely influence the outcome of the experiment. These include holding the mouse upright, administering multiple-low-dose bacteria liquid into both nostrils separately during each instillation, watching the inhalation of liquid without forming bubbles, controlling the speed of inhalation; moving the mouse up quickly then down slowly, and laying the mouse at a 45° angle to recover from instillation. Intranasal bacteria administration is noninvasive and helps to prevent choking, increase accessibility, improve safety, and minimize surgical injuries.

However, this method has its limitations. As this is a methodological study, data has not been discussed on the changes of clinical signs; anti-inflammatory cytokines; and quantity and function of monocytes, neutrophils, and lymphocytes in the blood. Laboratory mice are often inbred, have similar ages and weights, are housed in specific pathogen-free facilities, and commonly do not have comorbidities, such as pre-existing immunosuppression. Nevertheless, different gender, age, immune and nutritional status, and possible adjuvant treatment such as antibiotics of patients may result in heterogeneous clinical outcomes. Considering the heterogeneity of human patients, variables such as age, weight, pre-existing diseases, and clinical supportive care should be carefully watched.

The development of this dual model (double-hit model) is timely and important for the

infection and immunity research community because it resembles the progression from the hyper- to hypo-inflammatory phase of human sepsis. It reflects the common clinical scenario of a secondary nosocomial pneumonia in patients with sepsis more accurately. This model may help to develop new therapeutic strategies for sepsis-induced immunosuppression.

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DISCLOSURES:

The authors have no financial conflicts of interest.

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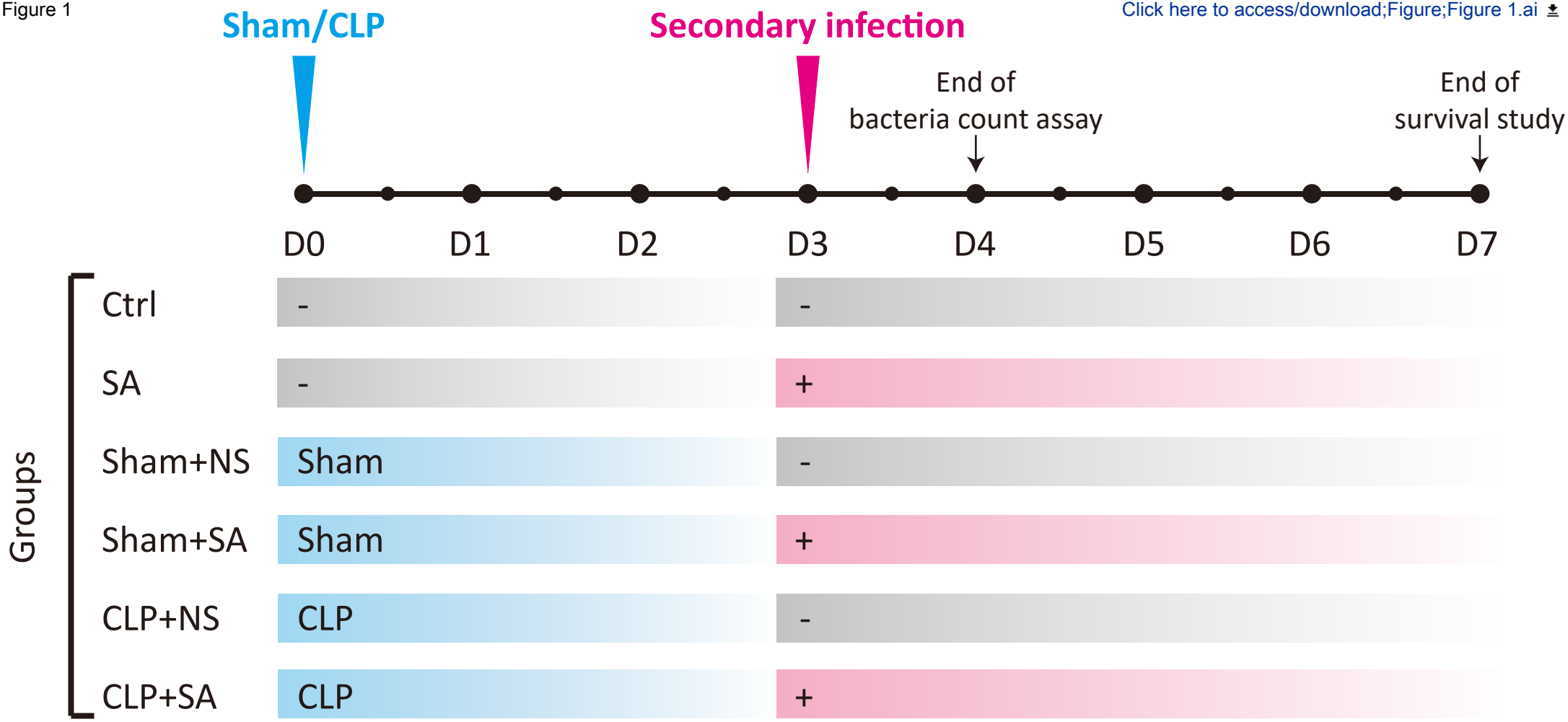
Figure 1



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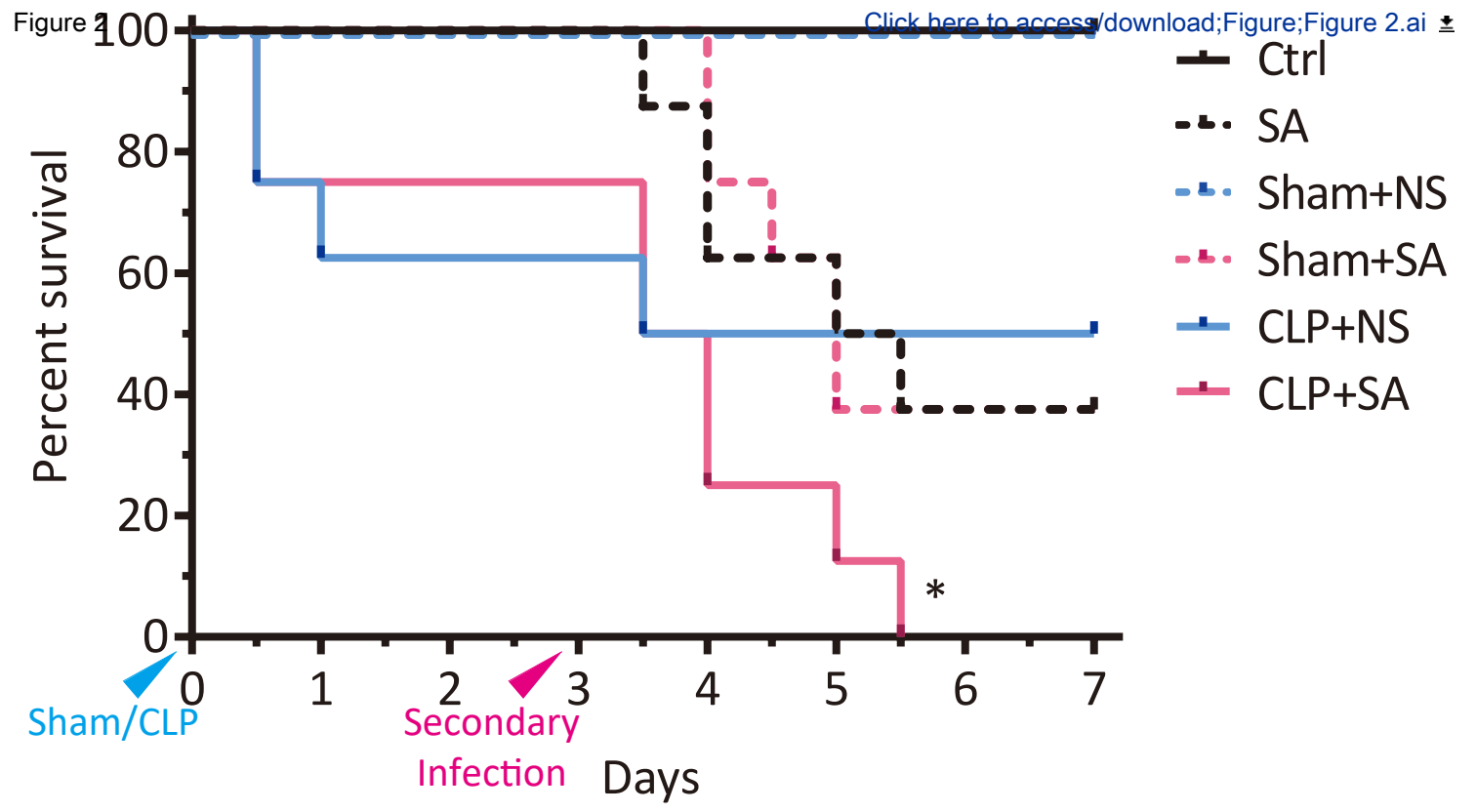

Figure 3

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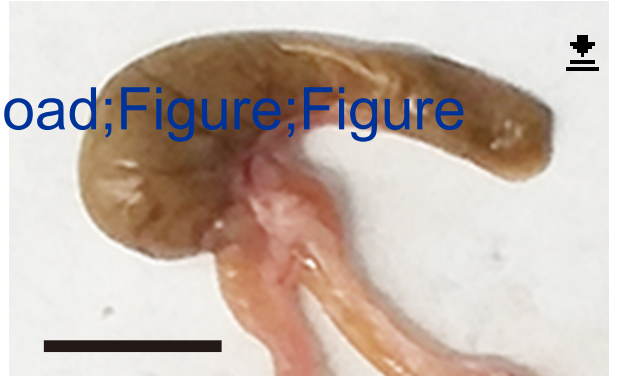

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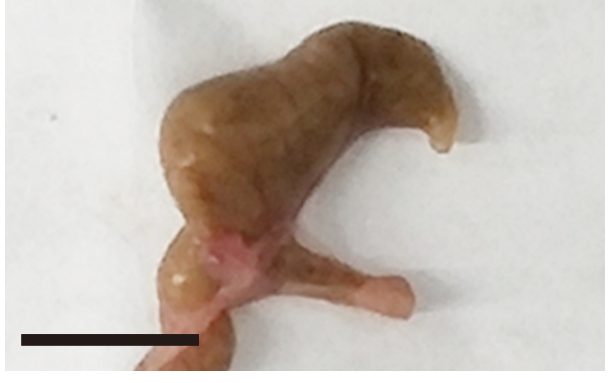

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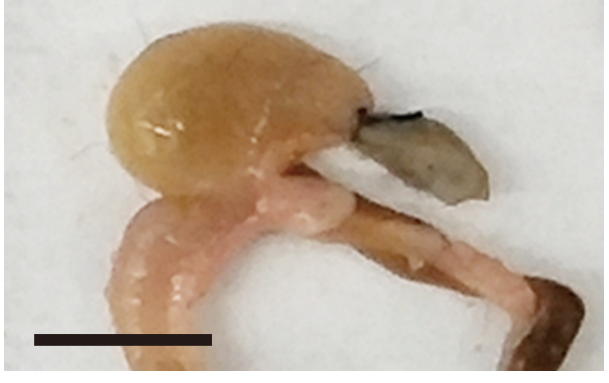

CLP+NS

CLP+SA

Figure 4

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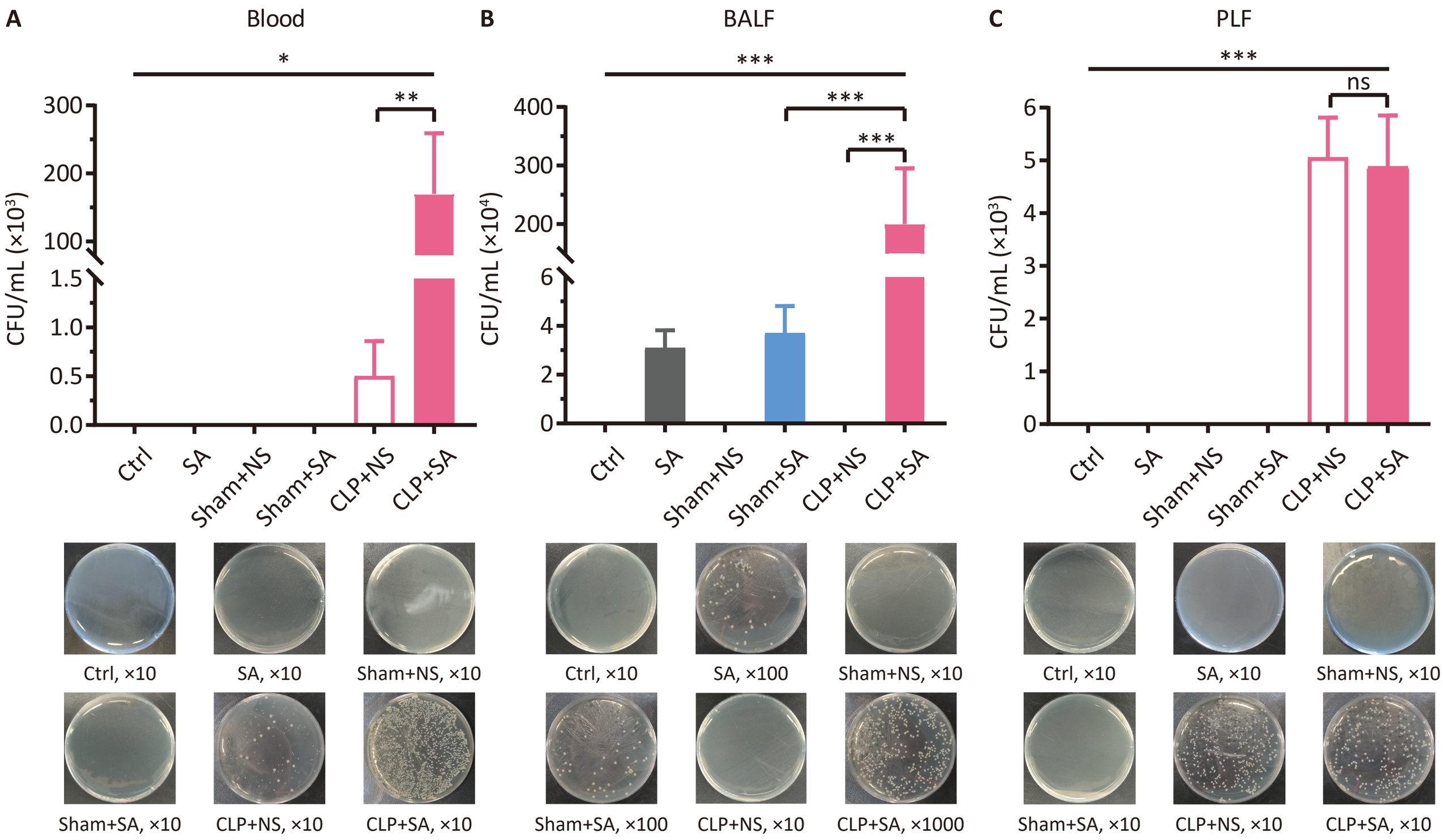

Figure 5

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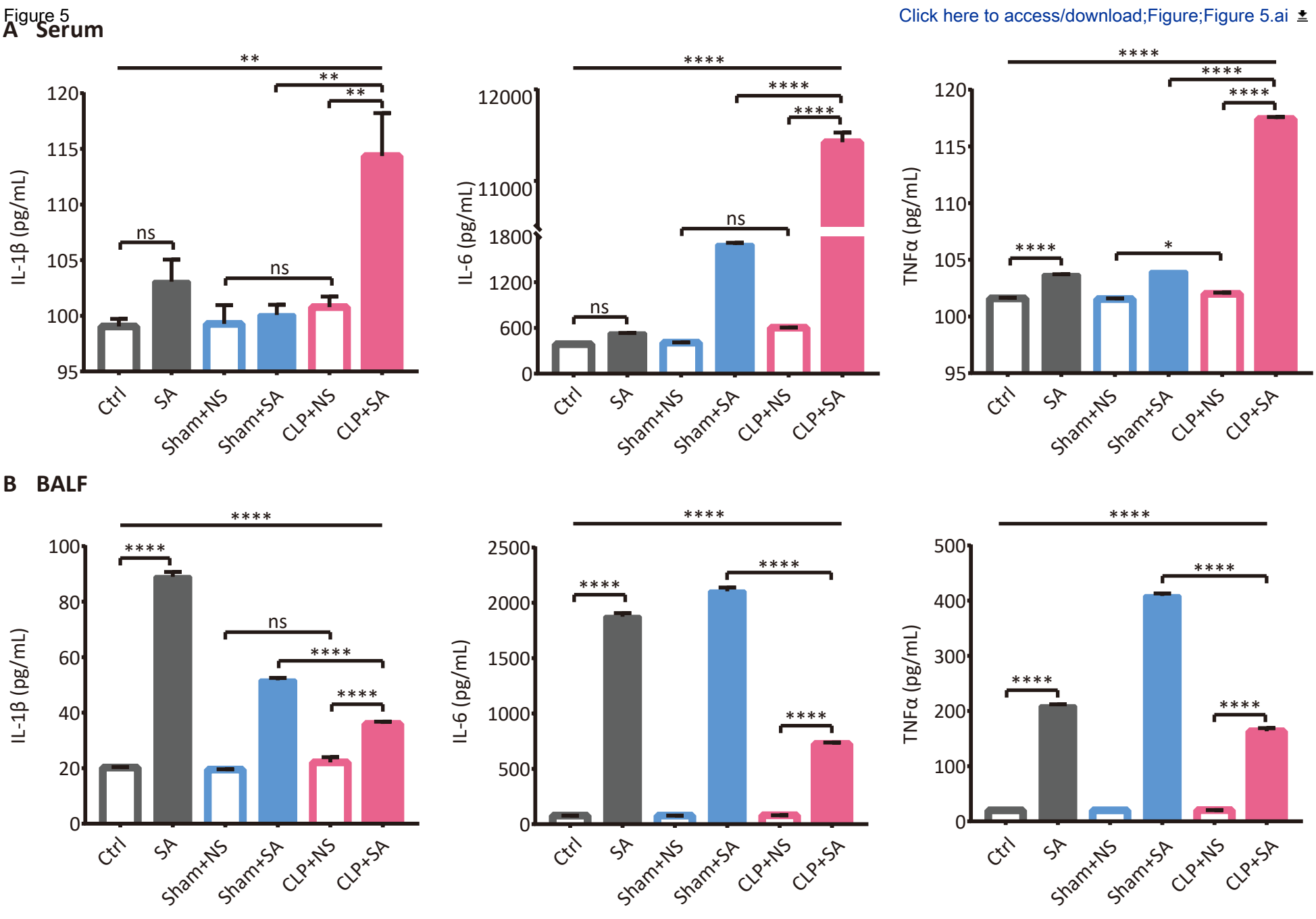

Figure 6

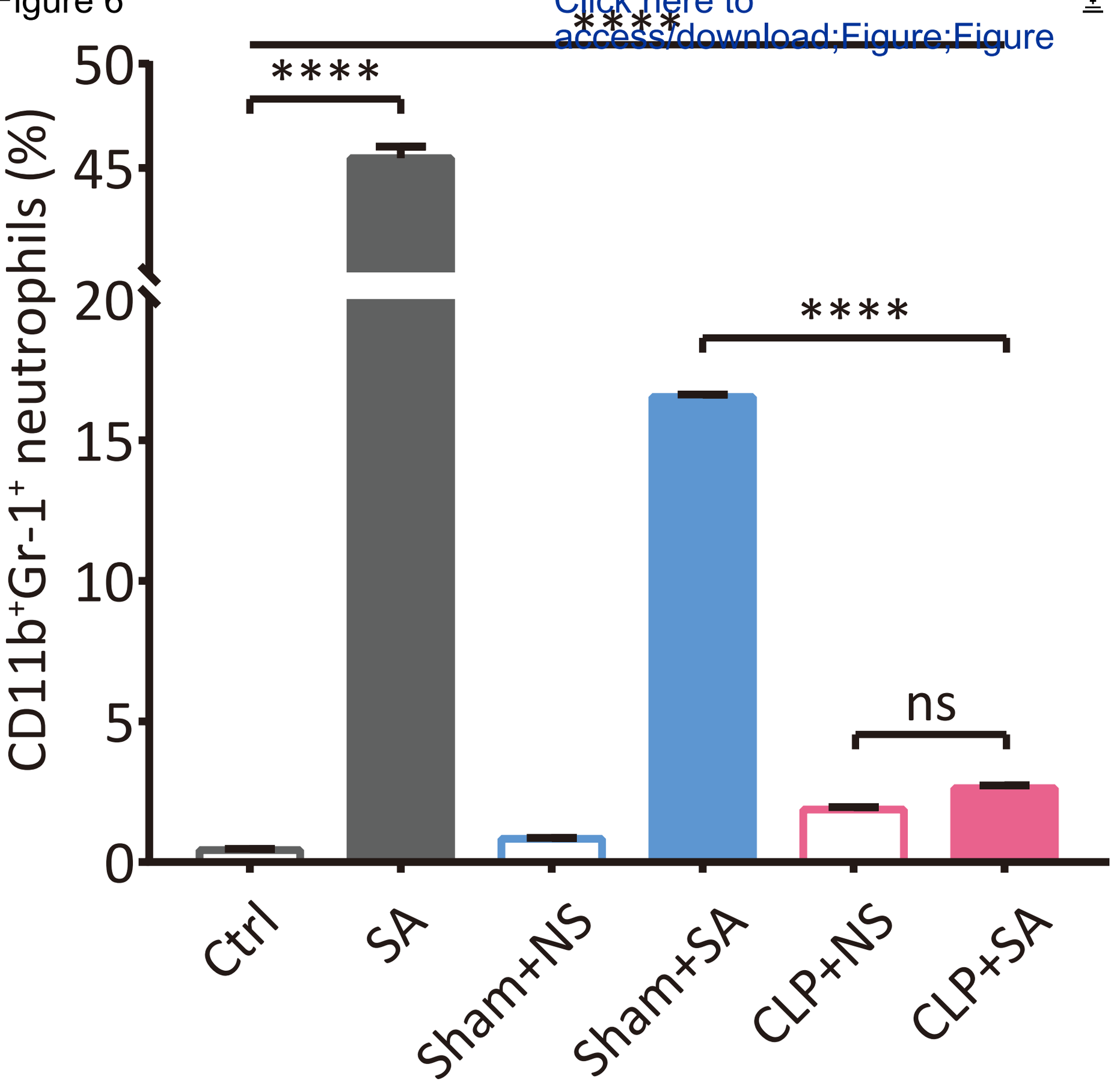

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
21 G 1 ½ Needle	BD	BD305167
ACK lysing buffer	Gibco	A10492-01
Anti-mouse CD11b antibody	Biolegend	101201
Anti-mouse Ly-6G/Ly-6C (Gr-1) antibody	Biolegend	108401
C57BL/6 mice	Harlan (Indianapolis)	C57BL/6NHsd
Desk light	General Supply	General Supply
Disinfecting wipes	Clorox	B07NV5JMCS
Electric razor	General Supply	General Supply
ELISA kits (mouse IL-1β, IL-6 and TNFα)	Invitrogen	88-7013, 88-7064, and 88-
Iodine	Dynarex	B003U463PY
Ketamine	FORT DODGE	NDC 0856-2013-01
Laboratory scale	General Supply	General Supply
LB Agar, Miller	Fisher Scientific	BP1425-500
Micropipette	ErgoOne	7100-1100
Normal saline	General Supply	General Supply
Polylined towel	CardinalHealth, Convertors	3520
Silk suture, 4-0	DAVIS & GECK	1123-31
Small animal needle holder	General Supply	General Supply
Small animal surgery scissors	General Supply	General Supply
Small animal surgical forceps	General Supply	General Supply
Staphylococcus aureus	ATCC	13301
Warm pad	General Supply	General Supply
Xylazine	Alfa Aesar	7361-61-7

Comments/Description

PVP Iodine Wipes
Amine hydrochloride injection

Molecular genetics, powder

Surgical drape, sterile, for single use only

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Title of Article:	Design of Cecal Ligation and Puncture (CLP) and Intranasal Infection Dual Model of Sepsis-induced Immunosuppression
Author(s):	Zhihan Wang, Qinqin Pu, Ping Lin, Changlong Li*, Jianxin Jiang*, and Min Wu*

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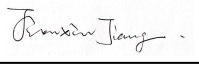
Signature:	
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Dear Dr. Steindel:

Thank you very much for your letter and the veterinary's comments for our manuscript, JoVE59386 "Design of Cecal Ligation and Puncture (CLP) and Intranasal Infection Dual Model of Sepsis-induced Immunosuppression". A revised manuscript with the correction sections red marked was submitted. The new revised version of video has been submitted to the dropbox. The detailed corrections are listed below point-by-point.

Thank you again very much for all your helps and looking forward to hearing from you soon.

Yours sincerely,

Min Wu, MD, PhD, Professor of Immunology

To Veterinary review:

Were animals used humanely and was the appropriate anesthesia or analgesia provided for potentially painful procedures? Unclear – see below

#	*Time in the video*	*comment*	*Change in video required Yes/No*	*Change in text is sufficient Yes/No*	*Suggested Changes*
1	*Introduction (~16 seconds)*	*Surgeon wearing exam gloves, presumably not sterile. Proper garb is shown later in video. Surgical prep is incomplete*	*yes*		*Delete this clip*
Response: Thank you for your comment. We have deleted this clip.					

#	Time in the video	comment	Change in video required Yes/No	Change in text is sufficient Yes/No	Suggested Changes
2	3:37 (and line 133)	Not appropriate to use silk suture in skin because it wicks bacteria	Yes	Yes	Delete section of clip showing use of silk in skin (it is ok in body wall and to ligate cecum). Modify line 133 in narrative – ok to close skin with wound clips or non-braided suture eg nylon.
Response: Thank you for your reminder. We have deleted this clip and changed line 133 into nylon surgical sutures in our revised manuscript.					
3	Line 102	150 ul volume of anesthetic given IM may be excessive		yes	Guidelines recommend not greater than 10-50 ul/site – total volume must be divided into multiple muscle groups and/or concentration increased to reduce injection volume (https://www.jove.com/science-education/10198/compound-administration-i)
Response: Thanks for your suggestion. We have changed to use 0.1 mL/mouse of ketamine (80 mg/kg), xylazine (10 mg/kg) mixture intraperitoneally administered on Lines 102-103.					
4	Line 133	Typo "mesenterial"		yes	"mesenteric"
Response: We are very sorry for our incorrect writing. We have corrected this word.					
5	Line 142			Yes	Buprenorphine dose is incorrect. Should be 0.05 mg/kg BID/TID or 1.2 mg/kg of SR-buprenorphine. Provide scientific justification for not giving it, or delete statement that it was not given.
Response: Thank you for your comment. We have changed to 50 μg/kg every 12 h and deleted the statement "not used in this experiment".					
6	Line 315	Statement about fluid therapy is unclear – is it recommended or not		Yes	clarify

Response: So sorry for this mistake. We have re-written this paragraph on lines 315-317. Fluid resuscitation is considered to prevent shock and rapid death due to circulation collapse and develop a hyperdynamic animal sepsis model which more closely mimics hemodynamic profile of human sepsis.					
7	*Line 344-349*	*Placement of cecum on sterile drape not done*		*yes*	*Harmonize with video line 349 which states it should be done*
Response: We are sorry for our careless mistakes. The last revision, we have reshot the clips showing that the mouse was draped during the operation and the cecum was placed on the sterile drape, not exteriorized onto unclipped and non-disinfected skin. This time we have deleted these descriptions about not placement of the cecum on a sterile drape.					