

# High mobility group box protein 1 neutralization therapy in ovine bacteremia: Lessons learned from an ovine septic shock model incorporating intensive care support

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**Abstract.** Sepsis is a highly complex and often fatal syndrome which varies widely in its clinical manifestations, and therapies that target the underlying uncontrolled immune status in sepsis are needed. The failure of preclinical approaches to provide significant sepsis survival benefit in the clinic is often attributed to inappropriate animal disease models. It has been demonstrated that high mobility group box protein 1 (HMGB1) blockade can reduce inflammation, mortality and morbidity in experimental sepsis without promoting immunosuppression. Within this study, we explored the use of ovine anti-HMGB1 antibodies in a model of ovine septic shock incorporating intensive care supports (OSSICS). Results: Septic sheep exhibited elevated levels of HMGB1 within 12 h after the induction of sepsis. In this study, sepsis was induced in six anaesthetized adult Border Leicester x Merino ewes via intravenous instillation of *E. coli*

and sheep monitored according to intensive care unit standard protocols for 26 h, with the requirement for noradrenaline as the primary endpoint. Septic sheep exhibited a hyperdynamic circulation, renal dysfunction, deranged coagulation profile and severe metabolic acidosis. Sheep were assigned a severity of illness score, which increased over time. While a therapeutic effect of intravenous anti-HMGB1 antibody could not be observed in this model due to limited animal numbers, a reduced bacterial dose induced a septic syndrome of much lower severity. With modifications including a reduced bacterial dose, a longer timeframe and broad spectrum antibiotics, the OSSICS model may become a robust tool for preclinical assessment of sepsis therapeutics.

## Introduction

Sepsis is broadly defined as a dysregulated immune response to a pathogenic insult which damages host tissues, and represents a highly complex and often fatal syndrome which varies widely in its clinical presentation. Currently, sepsis treatment is primarily limited to supportive medications and broad spectrum antibiotics, however currently no clinically validated approach exists to treat the underlying immune dysregulation in sepsis. The majority of immune-modulating therapeutics that show efficacy in preclinical sepsis models do not exhibit similar clinical benefits (1) which is often attributed to the pervasive use of inappropriate animal models. The use of relevant animal models is imperative to gain insights into sepsis pathophysiology, to improve treatment paradigms and to investigate long-term effects in survivors. Small animal models favor quick, cost effective and easily executed studies but larger animal models are often more appropriate to measure multiple clinical parameters within more relevant physiological contexts to humans.

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*Abbreviations:* CO, cardiac output; CVP, central venous pressure; DAMP, damage-associated molecular pattern; HMGB1, high mobility group box 1; HR, heart rate; MAP, mean arterial pressure; NorA, noradrenaline (Norepinephrine); OSSICS, ovine septic shock incorporating intensive care support; pAb(s), polyclonal antibody(-ies); PCV, packed cell volume; RCT, randomized controlled trial; SOFA, sequential organ failure assessment; SVRI, systemic vascular resistance index

*Key words:* animal models, sepsis, sheep, pAb(s), HMGB1

The availability of genetically defined rodent strains has enabled in-depth evaluation of immunological parameters that are not achievable in large animal species (2). Despite controversy surrounding the applicability of murine studies to human diseases (3), recent evidence suggests that mouse models parallel many aspects of human inflammatory and immunological diseases (4). Nevertheless, large animal models including pigs, sheep and dogs are more likely to recapitulate pathophysiological changes seen in humans (5). The reclassification of sepsis according to Sepsis-3 has ignited a renewed focus on organ damage rather than systemic inflammatory criteria as the most useful diagnostic and prognostic measures for sepsis (6). Consequently, preclinical approaches are adopting a similar shift away from immunological endpoints towards more integrated measures (7,8). Ovine models are particularly advantageous for investigation of physiological outcomes due to the hemodynamic similarity between sheep and humans (5), the availability of sheep, and the ability to repurpose clinical instrumentation. Sheep are comparable to humans for most common coagulation tests (9), and exhibit similar metabolic changes in response to sepsis (10). Instrumented septic ovine models have been used to study acute lung injury (11), fluid resuscitation (12) and renal inflammation (13). Recently, an ovine septic shock incorporating intensive care supports (OSSICS) model which recapitulates multiple pathophysiological hallmarks of human septic shock (14) has been used to investigate renal pathology (15) and thyroid hormone therapy (16) in sepsis. However, the utility of this model for studying the physiologic effects of immunomodulatory therapies has not been established.

High mobility group box protein 1 (HMGB1) is a potential therapeutic target in sepsis with a wide therapeutic window (17). Normally located within the nucleus, HMGB1 is released during sepsis and promotes inflammation as a damage associated molecular pattern (DAMP). Preclinical modelling has shown that methods used to neutralize or inhibit HMGB1 activity reduce mortality (18).

Previously, our laboratory has demonstrated that ovine anti-HMGB1 polyclonal antibodies (pAbs) can reduce mortality in murine endotoxemia (19) and polymicrobial sepsis (20) in a similar manner to other studies (21). While the effects of anti-HMGB1 pAb therapy on the immune phenotype of sepsis may be assessed in the murine model, little can be ascertained about the impact of such therapy on sepsis pathophysiology. Therefore, we aimed to determine the suitability of the OSSICS model for the evaluation of anti-HMGB1 pAb using clinically-relevant parameters.

## Materials and methods

*Ovine polyclonal anti-HMGB1 production.* Ovine anti-HMGB1 pAbs were generated via immunization of sheep with recombinant HMGB1 in Freund's adjuvant and purified from anti-HMGB1 serum via Protein G chromatography as previously described (19). Neutralization capacity of ovine anti-HMGB1 pAbs were evaluated as previously described (19). Purified pAbs were sterile filtered and adjusted to 5 mg/ml in PBS for administration.

*HMGB1 and anti-HMGB1 quantitation by ELISA.* Sheep serum samples were analyzed by a human HMGB-1 ELISA

kit (Cloud-Clone Corp; standard curve range 4,000–63 pg/ml) according to the manufacturer's instructions. Anti-HMGB1 was quantified in sheep serum using a custom ELISA method. Briefly, ELISA plates were coated with recombinant HMGB1 antigen (5 µg/ml) produced as previously described (19) and blocked with BSA. Sheep serum samples were diluted 1:2 and bound ovine antibodies were detected with anti-ovine IgG-HRP (Donkey anti-sheep IgG A3415; Sigma Aldrich) and OPD substrate (SigmaFAST; Sigma Aldrich). Readings were compared to a standard curve prepared from an aliquot of anti-HMGB1 pAb preparation.

*Sheep preparation and monitoring.* All sheep studies were approved by at The South Australian Health and Medical Research Institute (Adelaide, Australia) Animal Ethics Committee and conducted following institutional and national ethical guidelines according to the 'Australian code for the care and use of animals for scientific purposes (2013)'. Border Leicester x Merino ewes (2.4–4 years old) were group housed at The South Australian Health and Medical Research Institute and given free access to food and water. A model of OSSICS was performed on six sheep previously published protocols (15,16). Briefly, anesthesia was induced in overnight fasted randomly selected sheep by intravenous (IV) injection of Thiopentone (15 mg/kg) before intubation and ventilation with isoflurane (2%) in oxygen the morning following fasting (8–10 am). Catheters were placed under surgical conditions: an arterial catheter (Angiocath 1.7x133 mm; Becton Dickinson) was placed into the right carotid artery, and a quad-lumen central venous catheter (8.5 Frx20 cm; Arrow International Inc.) and a large-bore single lumen catheter (8.5 Frx10 cm; Arrow International Inc.) were both inserted into the right jugular vein. The large-bore single lumen catheter was used for the placement of a pulmonary artery catheter (Swan Ganz CCombo 7.5 Frx110 cm; Edwards Lifesciences) performed under radiology imaging.

A tracheostomy tube was inserted into the proximal trachea (Portex, ID 9.0, 24 cm; Smith's Medical) and a rumen tube (16 gauge, Bard Medical) was inserted via the nose. An indwelling urethral catheter (14Fr, Covington; Bard Medical) was placed into the bladder and attached to a burette for measurement. Sheep were moved onto a surgical table, lying prone with head and neck supported in an upright position to prevent edemous fluid accumulation in the lower limbs, and mechanically ventilated (Puritan Bennett 7200). Central venous and arterial catheters were attached to a pressure monitor (Intellivue MP 50; Phillips) and a pulmonary artery catheter to cardiac output monitor (Vigilance Monitor, Edwards Lifesciences). Cardiac output was continuously monitored and indexed to sheep body surface area according to the calculation (weight (kg) 0.67x0.0842).

All sheep were monitored by a dedicated intensive care research nurse throughout the study (Table I), and mean arterial pressure (MAP) was maintained according to the protocol described in Table II. Previous studies have demonstrated that anti-HMGB1 pAbs are efficacious in reducing mortality in murine endotoxemia within 24 h (19), thus a short time frame was adopted in the current study to allow comparison to previous OSSICS cohorts. Sheep were euthanized under

Table I. Sheep monitoring and maintenance.

Parameter	Monitoring	Maintenance
Sedation	Depth of sedation continuously monitored; medication dose recorded hourly.	IV infusion of midazolam (0.1-0.5 mg/kg/h) and ketamine (1-5 mg/kg/h).
Ventilation	Respiratory rate, tidal volume, peak and plateau pressures and O <sub>2</sub> and CO <sub>2</sub> saturation recorded hourly. Tracheal suctioning was performed hourly or as required.	Synchronised intermittent mandatory ventilation with 10 ml/kg tidal volume and positive end expiratory pressure of 5 cm H <sub>2</sub> O. Respiration adjusted to maintain end tidal CO <sub>2</sub> of 30 mmHg. Fraction of inspired oxygen was adjusted to maintain pulse O <sub>2</sub> saturation >95%.
Hemodynamics	HR, CVP and pulmonary arterial pressure were recorded hourly. MAP and CO recorded every 10 min.	Parenteral fluid was administered at 3 ml/kg/h via peristaltic pump. CVP was maintained ≥5 mmHg via fluid bolus administration (250 ml saline). MAP was maintained ≥75 mm/Hg (Table II).
Temperature	Recorded every hour.	Temperature was maintained <42°C with the application of cool damp drapes to the back
Blood analysis	-	Samples taken every 2 h for cytokine and lactate analysis, every 4 h for blood gas analysis and every 6 h for pathology analysis including coagulation, hematological and biochemical tests.
Fluid balance	Fluids administered and urine output recorded hourly.	Total urine collected at 0, 12 and 26 h was subjected to protein and creatinine analysis.

HR, heart rate; CVP, central venous pressure; MAP, mean arterial pressure; CO, cardiac output.

Table II. Ovine model hemodynamic management protocol.

Blood pressure parameters	Action
MAP <75 mmHg and CVP <5 mmHg	250 ml saline bolus
75 mmHg and CVP ≥5 mmHg	NorA infusion as below; adjust every 2 min
MAP <55 mmHg	↑ 10 µg/min NorA
MAP 55-59 mmHg	↑ 5 µg/min NorA
MAP 60-64 mmHg	↑ 3 µg/min NorA
MAP 65-69 mmHg	↑ 2 µg/min NorA
MAP 70-74 mmHg	↑ 1 µg/min NorA
MAP 75-80 mmHg	No change to NorA dose
MAP 81-85 mmHg	↓ 1 µg/min NorA
MAP 86-90 mmHg	↓ 2 µg/min NorA
MAP 91-95 mmHg	↓ 3 µg/min NorA
MAP 96-00 mmHg	↓ 5 µg/min NorA
MAP >100 mmHg	↓ 10 µg/min NorA

MAP, mean arterial pressure; NorA, noradrenaline.

anesthesia at 26 h post-*E. coli* administration by IV injection of pentobarbitone (6.5 g). Death was apparent by unrecordable arterial blood pressure, and confirmed by a trained intensive care nurse. All laboratory and biochemical measurements

were conducted by technicians blinded to the treatment group identity.

*Preparation and administration of E. coli and anti-HMGB1 polyclonal antibodies.* *E. coli* (Serotype O6, Biotype 1; ATCC 25922) inoculum was prepared from colonies (8-10) grown overnight on fresh Columbia horse blood agar (bioMérieux Australia) emulsified in 100 ml sterile saline to an optical density of 0.25 at 550 nm (empirically determined to yield 1.5x10<sup>8</sup> CFU/ml). The number of CFUs/ml of each inoculum was determined by serial dilution and plating onto Columbia Horse Blood agar.

The inoculum (1 ml/kg) was administered to prepared, anaesthetized sheep as an IV infusion in the over one hour to achieve 1-1.5x10<sup>8</sup> CFU/kg based on previous studies (15,16). Anti-HMGB1 antibodies (10 mg/kg) were administered via IV infusion over one hour commencing two hours after the induction of sepsis. This dose rate was based on previous murine studies and is in molar excess (180-fold) of detectable plasma HMGB1. Placebo-treated sheep were administered IV saline in place of the antibody infusion. Bacterial doses and treatments administered to the six experimental sheep are summarized in Table III.

*Statistical analysis.* Analysis was performed using GraphPad Prism V3.0 software. Measurements at individual time points were compared using student's two-tailed t-tests and time course analysis was performed by two-way analysis of variance.

Table III. Characteristics of sheep and survival outcomes within the study as compared to archive data.

Sheep	Weight, kg	<i>E. coli</i> dose, CFU/kg	Treatment	Survival time, h	Cumulative NorA administered, $\mu\text{g}/\text{kg}$	NorA dose rate mean AUC, $\mu\text{g}/\text{kg}$
1	68	$1.08 \times 10^8$	Placebo (normal saline)	26	26.6	13.3 <sup>a</sup>
2	74	$0.94 \times 10^8$	Placebo (normal saline)	26	0	
3	69	$1.47 \times 10^8$	Placebo (normal saline)	26	718.5	715
4	58	$1.62 \times 10^8$	10 mg/kg anti-HMGB1 pAbs	26	556.2	794 <sup>b</sup>
5	71	$1.50 \times 10^8$	10 mg/kg anti-HMGB1 pAbs	12	234.4	
6	76	$1.58 \times 10^8$	10 mg/kg anti-HMGB1 pAbs	25	869.4	
Archive data (n=8; mean $\pm$ SD)	63 $\pm$ 8	$1.08 \pm 0.5 \times 10^8$	Placebo (normal saline)	25 $\pm$ 2.1	330 $\pm$ 403	340

<sup>a</sup>Applies to sheep 1 and 2; <sup>b</sup>applies to sheep 4-6. SD, standard deviation; NorA, noradrenaline; AUC, area under the curve; HMGB1, high mobility group box 1.

## Results

### *Ovine septic shock induces elevation of plasma HMGB1.*

First we determined whether HMGB1 release kinetics were similar between sheep and humans. Banked plasma samples from previous OSSICS subjects (see references 15 and 16) and analyzed for HMGB1 content by commercial ELISA. Elevated HMGB1 was detectable (Fig. 1) from 12 h post *E. coli* infusion ( $1.08 \pm 0.05 \times 10^8$  CFU/kg) while no HMGB1 elevation was observed in non-septic animals. Significantly, plasma HMGB1 levels at 26 h were three-fold higher in septic sheep than non-septic sheep, or those measured at the onset of sepsis (Fig. 1). Septic sheep exhibited HMGB1 levels between 2-6 ng/ml over the course of sepsis. These results support the use of the ovine septic shock model for the study of anti-HMGB1 therapy in sepsis.

### *Anti-HMGB1 therapy did not reduce the development of shock or noradrenaline requirement in the OSSICS model.*

The requirement for vasopressor therapy in the form of noradrenaline (NorA) to maintain adequate blood pressure has previously been used as the primary outcome measure in the OSSICS model. Within this study, shock was generated in six sheep and compared to data from previous studies utilising the same protocol. Characteristics of study sheep and outcomes are depicted in Table III. Initially, two sheep (#1 and #2) were administered the same *E. coli* dose as used in previous studies ( $1 \times 10^8$  CFU/kg) (15,16), however the sheep required little NorA and did not progress to prolonged septic shock. To replicate a degree of sickness severity observed in previous studies, an increased *E. coli* dose ( $1.5 \times 10^8$  CFU/kg) was employed for the remaining sheep within the study (#3-6). Three sheep were administered anti-HMGB1 pAbs (10 mg/kg; sheep 4-6) two hours after the induction of sepsis. Two early deaths occurred within this group, as reflected by the recorded survival time. Hemodynamic parameters were evaluated in sheep for 1 h before the induction of sepsis and for 26 h thereafter (Fig. 2). Placebo-treated sheep from previous studies (n=8; hereafter referred to as archive data) displayed a rapid decrease in MAP and SVRI within 2 h of bacterial infusion before increasing to

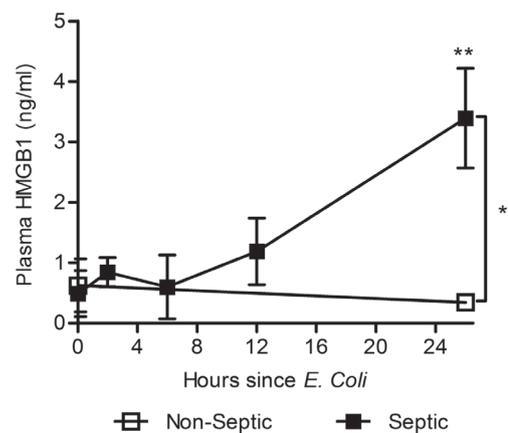


Figure 1. Significant elevation of plasma HMGB1 is detectable at 26 h in septic sheep. Kinetic plasma samples from sheep administered  $1 \times 10^8$  CFU/kg *E. coli* (n=4) and non-septic control animals (n=4) in previous studies were assessed for HMGB1 content via ELISA. The mean  $\pm$  standard error of the mean of individual time points are represented. \*P<0.05, \*\*P<0.01. HMGB1, high mobility group box 1.

a stable level within 4 h of infusion (Fig. 2A and B). Between 6-8 h after infusion, MAP and SVRI gradually declined until a MAP <75 mmHg was achieved and NorA therapy was initiated (Fig. 2C). Two sheep within the current study were administered  $1 \times 10^8$  CFU/kg *E. coli* and these sheep displayed a markedly reduced need for NorA compared to archive data. One sheep with an increased bacterial dose ( $1.5 \times 10^8$  CFU/kg) exhibited consistently low MAP and SVRI and had high NorA requirements. Infusion of anti-HMGB1 pAbs two hours post-challenge in three sheep that received the increased bacterial dose of  $1.5 \times 10^8$  CFU/kg did not appear to impact on NorA requirements compared to the equivalently-dosed control sheep. Urine output (Fig. 2D) in the OSSICS model appeared consistent with archive data, where measured urine output was between 1-4 ml/hour/kg and subsequently fell to less than 1 ml/h/kg by 8 h post-challenge.

*Administration of anti-HMGB1 pAb did not alter detectable HMGB1 levels in septic sheep plasma.* In this preclinical

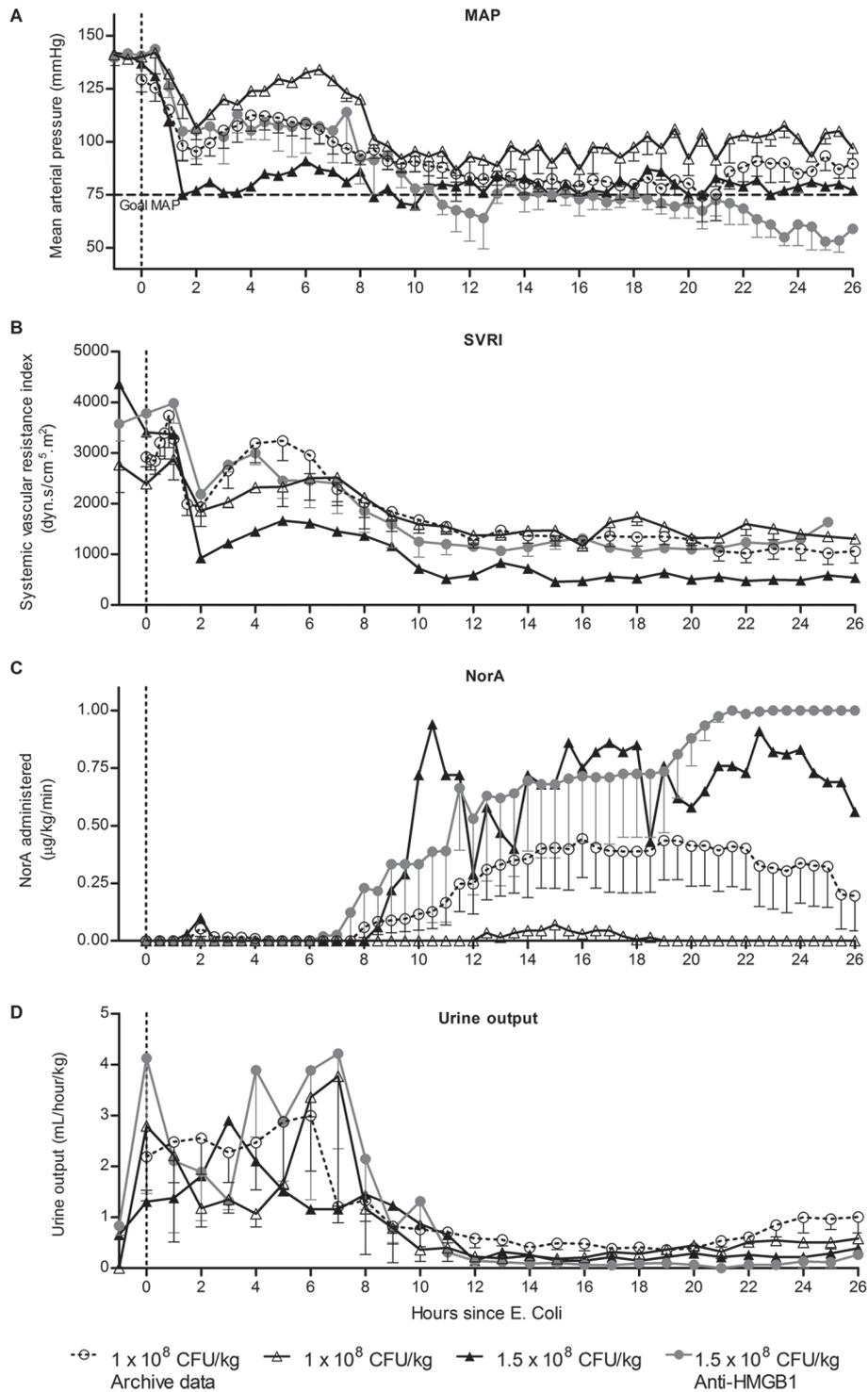


Figure 2. Measured hemodynamic parameters in an intensive care support ovine septic shock model. Sheep were anesthetized and catheterized to enable measurement of vascular pressure and urine output. After 1 h, sepsis was induced (indicated by dotted vertical line) by the IV infusion of  $1 \times 10^8$  CFU/kg *E. coli* (n=2), or  $1.5 \times 10^8$  CFU/kg *E. coli* with placebo (saline) treatment (n=1) or 10 mg/kg anti-HMGB1 pAb (n=3). (A) MAP at 10 min intervals and (B) hourly SVRI to body surface area are represented. (C) NorA requirements were recorded at 10 min intervals and (D) urine output were recorded hourly. Data are presented as the mean-standard error of the mean of measurements for each group with archive data from placebo-treated (saline) sheep (n=8) included for comparison. HMGB1, high mobility group box 1; MAP, mean arterial pressure; NorA, noradrenaline; SVRI, systemic vascular resistance index.

model, a dose rate of 10 mg/kg anti-HMGB1 pAb was selected based on previous murine studies and on concentrations of HMGB1 detected within the circulation of septic sheep. In order to establish whether sufficient anti-HMGB1 pAb was administered in this study to observe effects, kinetic serum samples from the three Ab dosed sheep were analyzed by

ELISA to detect anti-HMGB1 ovine IgG (Fig. 3A) and circulating HMGB1 (Fig. 3B). Anti-HMGB1 pAbs were detectable two hours following Ab infusion at  $18 \mu\text{g/ml}$  (18 mg/l), and decreased thereafter. As one sheep died 11 h following *E. coli* infusion, measurements at later time points are the mean values of two remaining sheep. These sheep exhibited vastly

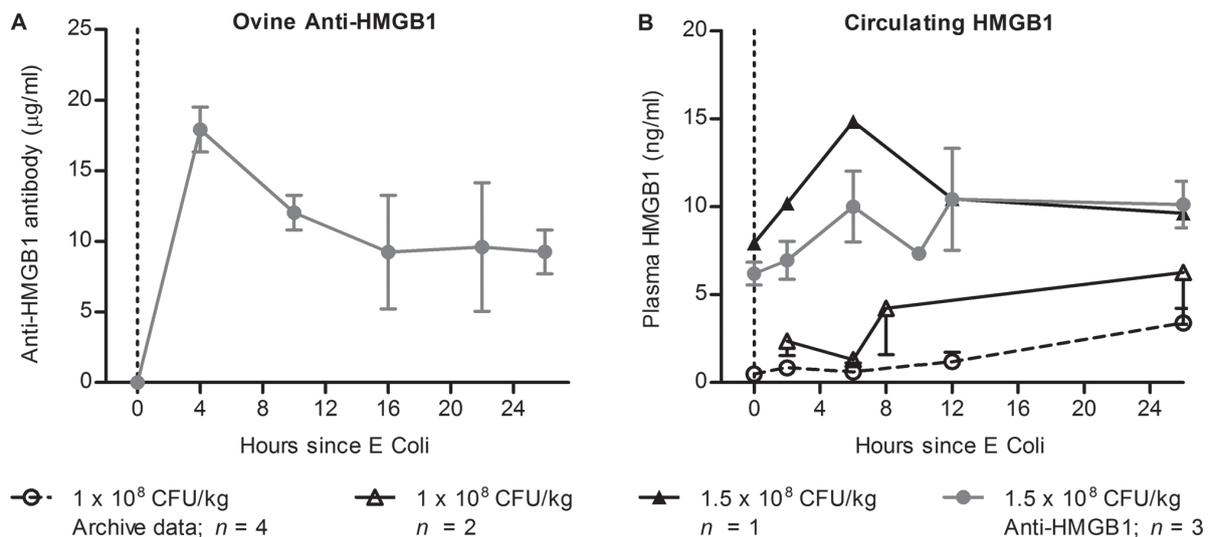


Figure 3. Kinetics of circulating anti-HMGB1 pAb and impact on plasma HMGB1 in the ovine septic shock incorporating intensive care support model. Sheep were anesthetized and sepsis was induced (indicated by dotted vertical line) by the IV infusion of  $1 \times 10^8$  CFU/kg *E. coli* ( $n=2$ ), or  $1.5 \times 10^8$  CFU/kg *E. coli* with placebo treatment ( $n=1$ ) or 10 mg/kg anti-HMGB1 pAb ( $n=3$ ) administered 2 h following bacterial challenge. Levels of circulating (A) ovine anti-HMGB1 pAb and (B) HMGB1 were determined by ELISA analysis. HMGB1, high mobility group box 1; pAb, polyclonal antibody.

different kinetics of pAb clearance, as the detected concentration decreased rapidly in one sheep and remained constant in another, which may be due to metabolic variability in the sheep following sepsis induction.

*Sepsis-associated alterations in hematological, coagulative and metabolic parameters were not improved by anti-HMGB1 treatment.* In the current study, blood and plasma samples drawn from septic sheep were subjected to standard pathology testing and compared to archive values from placebo-treated septic sheep. Ovine septic shock has previously been associated with a progressive decline in packed cell volume (PCV). In the current study, pre-operative samples were drawn which showed increased PCV and hemoglobin (data not shown) compared to pre-septic baseline samples. Measured PCV sharply increased in all groups 2 h post challenge and declined similarly for all groups for the duration of the study (Fig. 4Aa). Conversely to PCV, measured WBC counts increased from pre-operative to baseline time points (Fig. 4Ab), potentially due to inflammation caused by surgical catheter placement or isoflurane exposure. WBC counts decreased sharply by 80% 2 h post challenge before gradually increasing over time. Further analysis showed that this decrease was due to marked depletion of neutrophils, which decreased by 98% from baseline values (Fig. 4Ac), whereas lymphocytes (Fig. 4Ad) and other granulocytes (data not shown) did not change significantly.

Sheep that received the lower *E. coli* dose ( $1 \times 10^8$  CFU/kg) exhibited mild coagulopathy, with only a modest increase in PT (Fig. 4Ba) and APTT (Fig. 4Bb) and mild fibrinogen depletion (Fig. 4Bc) from baseline values compared to archive data. These sheep also exhibited reduced platelet depletion (Fig. 4Bd), although this was not consistent throughout the septic period. Sheep treated with anti-HMGB1 pAbs exhibited more profound clotting dysfunction than a placebo-treated sheep given the same *E. coli* dose, however these did not appear altered from archive data and low subject numbers preclude statistical comparison. As anti-HMGB1 pAb treated sheep exhibited the highest mortality

of the groups, these results support the notion of coagulation function tests as a marker of disease severity in ovine septic shock, however further investigation is required to determine if anti-HMGB1 therapy may influence clotting dysfunction.

Creatinine, lactate and pH were monitored throughout the experimental period as a measure of metabolic status. Plasma creatinine (Fig. 4Ca) increased in all groups from 2 h post challenge, however a marked increase was observed in sheep treated with anti-HMGB1 pAbs. Similarly, the anti-HMGB1 pAb group exhibited the most elevated lactate (Fig. 4Cb) measurements and the highest degree of acidosis (Fig. 4Cc). The group that received a low *E. coli* dose exhibited mild increases in creatinine, and no increase in plasma lactate. Correspondingly, the development of acidosis was largely absent in this group, as pH only decreased 0.06 units below baseline. These results support the notion that metabolic parameters are useful clinical tests in ovine septic shock that reflect disease severity.

*Modified SOFA scores may reflect severity of illness in the OSSICS model.* Clinically, the sequential organ failure assessment (SOFA) score is used to assess the morbidity associated with sepsis and incorporates measures of respiratory, hemostatic, liver, cardiovascular, neurological and renal function. Within this study, data collected from experimental sheep over time were collated into a modified SOFA score representing cardiovascular, hemostatic and renal function using human clinical reference ranges in the absence of validated ovine ranges (Table IV). All sheep exhibited an increase in modified SOFA score over time (Fig. 5), however increases were most apparent in sheep administered the highest bacterial dose. These results demonstrate that combined morbidity scores may have value as endpoints for preclinical sepsis studies.

## Discussion

Sepsis still causes significant mortality in developed nations and remains the primary cause of death from infection (22).

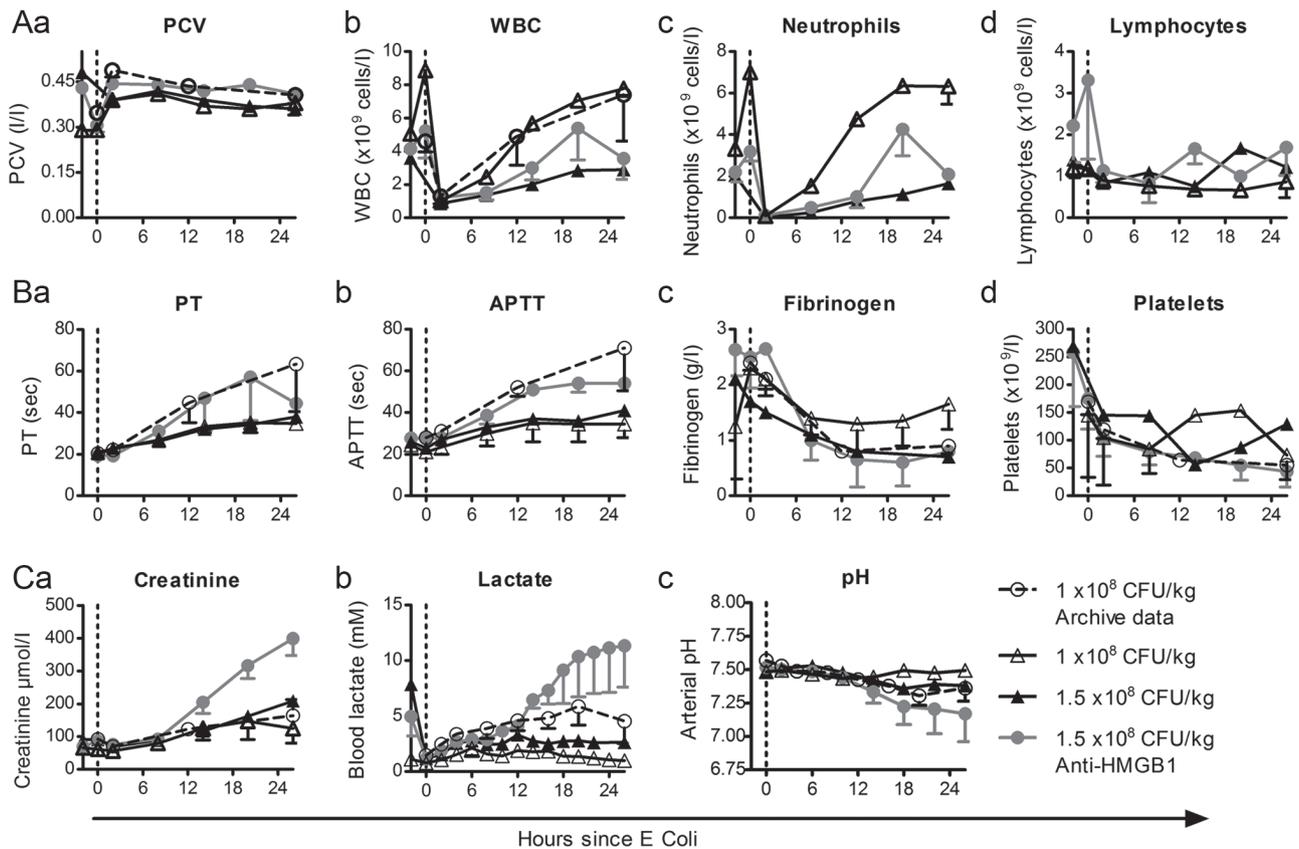


Figure 4. Measured hematological, coagulative and metabolic parameters in experimental sheep. Sheep were administered  $1.5 \times 10^8$  CFU/ml *E. coli* (IV) to induce sepsis and either treated with anti-HMGB1 2 h post challenge ( $n=3$ ) or placebo (saline) treated ( $n=1$ ). A total of two sheep were administered a lower *E. coli* dose ( $1 \times 10^8$  CFU/ml). Hematological parameters were measured from whole blood samples collected every 6 h, with (Aa) PCV, (Ab) WBC, (Ac) neutrophil count and (Ad) lymphocyte count are presented. Coagulation tests were performed on plasma samples collected every 6 h, with (Ba) PT, (Bb) APTT, (Bc) fibrinogen level and (Bd) platelet count from whole blood represented. Metabolic parameters were assessed via (Ca) creatinine measurement from six-hourly plasma samples with (Cb) lactate measurement from two-hourly snap-frozen plasma samples and (Cc) pH assessment via bedside blood gas analysis (Cc). Experimental groups were compared to archive data ( $n=8$ ). Data are presented as the mean-standard error of the mean of treatment groups. PCV, packed cell volume; WBC, white blood count; PT, prothrombin time; APTT, activated partial thromboplastin time; HMGB1, high mobility group box 1.

The reclassification of sepsis definitions (Sepsis-3) have highlighted the importance of organ dysfunction for diagnosis<sup>6</sup>. Consequently, there is an increased need for animal models than can recapitulate septic organ damage including circulatory, metabolic, renal and coagulative dysfunction and efforts to assess therapeutics should investigate multiple organ systems (8).

Within the present study, the feasibility of using the OSSICS model for evaluating the impact of anti-HMGB1 pAb therapy on physiological manifestations of septic shock was assessed in six sheep. Analysis of banked plasma samples collected from septic and non-septic OSSICS sheep revealed similar septic HMGB1 release profiles to that found in septic humans. Ovine HMGB1 has been demonstrated to have inflammatory effects and release kinetics that mirror those found in humans in studies of chorioamnionitis (23), cell migration (24) and fetal brain injury (25). Consequently, sheep may respond to HMGB1 blockade in the context of sepsis in a similar manner to humans.

Initially, trials aimed to reproduce a sickness severity achieved in previous studies (see references 15 and 16), however administration of the same *E. coli* dose ( $1 \times 10^8$  CFU/kg) resulted in decreased morbidity with little requirement for NorA. It was subsequently hypothesized that a higher *E. coli*

dose ( $1.5 \times 10^8$  CFU/kg) may increase the sickness severity to a level observed in previous studies, thus the remainder of trials were performed with this higher *E. coli* dose. An increased bacterial dose increased requirement for NorA to maintain blood pressure, considered the primary study endpoint, however these sheep exhibited a very rapid disease course which culminated in two early deaths. Three sheep were treated with anti-HMGB1 antibodies via IV injection at a rate of 10 mg/kg based on previous murine studies. Anti-HMGB1 therapy was associated with no improvement to MAP, NorA requirement or vascular resistance in anti-HMGB1 pAb treated sheep compared to controls, however a high degree of variation within this group, a low number of subjects and an early death complicated data interpretation. Additional studies incorporating non-specific pAb control groups would provide information as to whether the increased hypotension and morbidity in anti-HMGB1 treated sheep was specific to anti-HMGB1 or a consequence of IV pAb administration in this model.

Kinetic analysis of administered anti-HMGB1 pAb in treated sheep revealed that circulating pAb concentration decreased over the study period. Similar fluctuations in mAb concentration in the context of tumor therapy have been attributed to several mechanisms including Ab deposition, FcR binding

Table IV. Parameters used to calculate the modified sequential organ failure assessment for experimental sheep.

Parameter	Score assigned				
	0	1	2	3	4
Respiratory PaO <sub>2</sub> /FiO <sub>2</sub> , mmHg	>400	≤400	≤300	≤200	≤100
Coagulation platelets, x10 <sup>3</sup> /μl	>150	≤150	≤100	≤50	≤20
Cardiovascular MAP, mmHg or NorA, μg/kg/min	None	MAP <70		NorA ≤0.1	NorA >0.1
Renal Creat, μmol/l or urine output, ml/day	Creat <106.1	Creat ≥106.1	Creat ≥176.85	Creat ≥309.5 or UO <500	Creat ≥442.1 or UO <200

MAP, mean arterial pressure; NorA, noradrenaline; Creat, creatine.

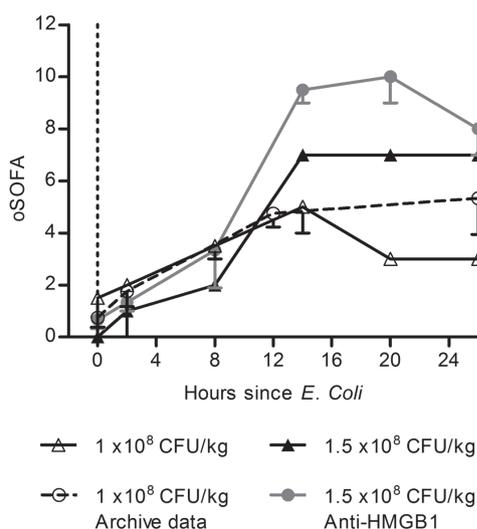


Figure 5. Modified SOFA scores increase over time in the ovine septic shock incorporating intensive care support model. Sheep were administered  $1.5 \times 10^8$  CFU/ml *E. coli* (IV) to induce sepsis and either treated with anti-HMGB1 two hours post challenge ( $n=3$ ) or placebo-treated ( $n=1$ ). A total of two sheep were administered a lower *E. coli* dose ( $1 \times 10^8$  CFU/ml). Data representing the degree of cardiovascular hypotension (mean arterial pressure and noradrenaline requirement), coagulation status (platelet count) and renal damage (creatinine) were compiled into a modified SOFA score using human clinical ranges. HMGB1, high mobility group box 1; SOFA, sequential organ failure assessment.

or consumption within immune complexes (26). All sheep exhibited vasodilation and developed a positive fluid balance as fluids were administered while urinary production slowed, thus loss of administered pAb into edematous tissues and peritoneal fluid accumulations may have occurred. Homology of HMGB1 between humans and sheep is predicted to be over 98% (27) thus a commercial ELISA manufactured for measurement of human HMGB1 was able to measure plasma HMGB1 in septic sheep. The administration of anti-HMGB1 pAb did not reduce the detectable plasma HMGB1 in the timeframe however it is not known if detected HMGB1 was bioactive or functionally neutralized as the antibodies used in the ELISA method may bind different epitopes to therapeutic pAbs.

Analysis of hematological parameters within this study supported previous evidence that progressive anemia develops within the OSSICS model (15). Rapid declines in WBC and

neutrophil counts occurred upon challenge (28), which failed to recover in groups receiving a high *E. coli* dose. While the magnitude of leukocytosis and neutrophilia have been correlated with the dose of endotoxin in human challenge studies (29), leukopenia has been associated with mortality in gram-negative sepsis in humans (30). Thus, in this model, delayed leukocyte responses may indicate failure to control bacteremia resulting in a more severe septic phenotype. Sheep from previous studies exhibited coagulative deficiency and fibrinogen depletion, however similarly dosed sheep in the current study did not exhibit coagulative deficiency of this degree. This variation between experiments is likely to represent alterations in sheep cohorts, as clotting assays and challenge organism were consistent between the studies. For future studies, additional controls or a rolling control group may be employed to minimize the effect of variation between cohorts. In the current study, anti-HMGB1 pAb treated sheep exhibited the most profound clotting deficiencies, which may be a result of marked fibrinogen depletion (apparent in anti-HMGB1 pAb treated sheep 2 h following challenge) or the depletion of other clotting factors. Antibody-induced type III hypersensitivity reactions are associated with platelet activation and the formation of clotting-factor depleting microthrombi (31), however intravenous immunoglobulin has also been used to treat sepsis-associated coagulopathy (32). Anti-HMGB1 pAb treated sheep also exhibited increased acidosis, with creatinine and lactate increased compared to other groups. Comparison of the current study to OSSICS sheep administered non-immune ovine pAbs may provide answers as to the potential relationship between coagulopathy, acidosis and organ dysfunction and anti-HMGB1 pAb therapy. The high degree of variation in the OSSICS model compared to murine models does however allow greater comparison to heterogeneous human sepsis, but as with human studies large numbers must be included for reliable conclusions to be drawn (33).

An ideal preclinical sepsis model would incorporate relevant clinical endpoints to enable clinical translation. Clinical endpoints for sepsis RCT's have been under scrutiny (34), and the failure of multiple sepsis-specific therapies to generate meaningful differences in study endpoints highlights the need for the careful endpoint selection. Measures of morbidity during ICU stay are frequently employed as secondary outcomes

in RCT's (35). In the context of animal models, short-term morbidity endpoints also circumvent ethical and practical issues associated with measuring long-term mortality (2). Clinically, composite morbidity scores such as the SOFA score are used to assess sepsis severity and morbidity (6). Within this study, human ranges for the SOFA score calculation were used to develop a modified SOFA score incorporating measures of cardiovascular, hemostatic and renal dysfunction (Table IV). Modified SOFA scores were calculated for experimental sheep and archive data and septic sheep exhibited increased scores over time, thus a modified SOFA score may provide a useful assessment of sepsis severity. Practical limitations prevent the use of the Glasgow coma scale in the OSSICS model, however future studies could incorporate liver function tests and respiratory measurements for the purpose of developing an ovine SOFA. Inclusion of appropriate reference ranges for physiological criteria and a validated ovine SOFA score equivalent would increase the utility of the model (36). As the highest bacterial dose used in this study caused early mortality in two sheep, a lower bacterial dose or the administration of antibiotics may allow the analysis of morbidity endpoints, an increasingly common outcome in clinical trials (35). Short study periods may only enable evaluation of agents that show marked efficacy in disease, thus a longer septic period may allow the study of more moderate differences between groups. Performing additional tests could provide more insight into the course of OSSICS, such as liver function tests for liver damage and D-dimer assessment for the detection of DIC (37). These foundation studies could help further develop a robust and authentic large animal model for the assessment of immunotherapeutics in sepsis.

The increased focus on organ damage in sepsis monitoring highlights the need for measurement of multiple parameters in preclinical studies<sup>8</sup>. While small animal models are useful for the study of molecular processes and screening potential therapeutics, large animal models remain an important step for the study of physiological processes involving multiple organ systems. While no singular model can possibly recapitulate the complexities encountered in human sepsis, animal models remain a crucial tool for preclinical evaluation of therapeutics. With the refinements, the OSSICS model could become a robust tool in assessing sepsis therapies via multiple organ dysfunction measures and facilitate translation into the clinic by using relevant endpoints. The intelligent and rational optimization of such models may reduce the discrepancy seen in results between preclinical and clinical studies and enable the development of effective host-specific therapeutics targeting sepsis.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

NES acquired and analyzed the data, and wrote the main manuscript text. CHN contributed to the design of the study and data acquisition. CKF contributed to the data analysis and data interpretation, and edited the manuscript. TRK designed the experiments and performed the sheep study. MJM designed the study, interpreted the data and provided archive data. MJC contributed to the design of the study and edited the manuscript. KRD contributed to the design of the study interpreted data, and wrote and edited the main manuscript. JDH contributed to the conception of the study, interpreted the data and edited the manuscript.

### Ethical approval and consent to participate

All sheep studies were approved by The South Australian Health and Medical Research Institute Animal Ethics Committee (Adelaide, Australia) and conducted following institutional and national ethical guidelines according to the 'Australian code for the care and use of animals for scientific purposes (2013)'.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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