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EFFECTS OF A TREM-LIKE TRANSCRIPT 1–DERIVED PEPTIDE DURING HYPODYNAMIC SEPTIC SHOCK IN PIGS

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ABSTRACT—The objective of this study was to determine the effects of a TREM (triggering receptor expressed on myeloid cells 1)–like transcript 1–derived peptide (LR12) administration during septic shock in pigs. Two hours after induction of a fecal peritonitis, anesthetized and mechanically ventilated adult male minipigs were randomized to receive LR12 (n = 6) or its vehicle alone (normal saline, n = 5). Two animals were operated and instrumented without the induction of peritonitis and served as controls (sham). Resuscitation was achieved using hydroxyethyl starch (up to 20 mL/kg) and norepinephrine infusion (up to 10 μg/kg per minute). Hemodynamic parameters were continuously recorded. Gas exchange, acid-base status, organ function, and plasma cytokines concentrations were evaluated at regular intervals until 24 h after the onset of peritonitis when animals were killed under anesthesia. Peritonitis induced profound hypotension, myocardial dysfunction, lactic acidosis, coagulation abnormalities, and multiple organ failure. These disorders were largely attenuated by LR12. In particular, cardiovascular failure was dampened as attested by a better mean arterial pressure, cardiac index, cardiac power index, and S_vO_2, despite lower norepinephrine requirements. LR12, a TREM-like transcript 1–derived peptide, exhibits salutary properties during septic shock in adult minipigs.

KEYWORDS—Experimental septic shock, minipigs, TLT-1, TREM-1

ABBREVIATIONS—CO—cardiac output; DO_2—oxygen delivery; IL-6-1β—interleukin 6, 1β; LPS—lipopolysaccharide; MAP—mean arterial pressure; TLR—Toll-like receptor; TLT-1—TREM-like transcript 1; TREM-1—triggering receptor expressed on myeloid cells 1; TNF-α—tumor necrosis factor α

Septic shock develops when the initial appropriate host response to systemic infection becomes dysregulated and overamplified with an intimate cross talk between inflammation and coagulation (1). This dysregulation may also exist during severe trauma and possibly other forms of shock (2). Indeed, a recently published article demonstrates that the early leukocyte genomic response consists of a simultaneous increase expression of genes involved in the systemic inflammatory and compensatory anti-inflammatory responses. Importantly, occurrence of bad outcome (organ failure development, nosocomial infections) depends on the magnitude and duration of this genomic reprioritization (3). Some of the potential candidates acting as amplifiers of the innate immune response belong to the TREM (triggering receptor expressed on myeloid cells) family (4). TREM-1 is expressed by neutrophils, macrophages, and mature monocytes ($CD14^{high}$). Its expression by effector cells is dramatically increased in skin, biological fluids, and tissues infected by gram-positive or gram-negative bacteria, as well as fungi (4). In mouse, the engagement of TREM-1 with agonist monoclonal antibodies has been shown to stimulate the production of proinflammatory cytokines and chemokines such as interleukin 8 (IL-8), monocyte chemoattractant proteins 1 and 3, and macrophage inflammatory protein 1α, along with rapid neutrophil degranulation and oxidative burst. The activation of TREM-1 in the presence of Toll-like receptor 2 (TLR2) or TLR4 ligands amplifies the production of proinflammatory cytokines (tumor necrosis factor α [TNF-α], IL-1β, granulocyte-macrophage colony-stimulating factor), together with the inhibition of IL-10 release (4–6). Of note, TREM-1 pathway was among the most upregulated ones in the study of Xiao et al. (3). TREM-1 is known to cooperate with several TLRs in a synergistic manner (4–6), whereas TREM-1 silencing down-modulates lipopolysaccharide (LPS)–induced inflammatory gene activation in myeloid cells (7). The TREM-1 blockade by the use of a fusion protein or LP17, a short inhibitory peptide that mimics an extracellular part of TREM-1, was associated with a survival improvement in experimental sepsis (8–10). These protective effects are also evident in other models of acute or chronic inflammatory disorders (11–17).

In addition to TREM-1, the TREM gene cluster includes TREM-like transcript 1 (TLT-1). TREM-like transcript 1 is abundant and specific to the platelet and megakaryocyte lineage. Upon platelet activation with thrombin or LPS, TLT-1 is translocated to the platelet surface (18). Unlike other TREM family members, TLT-1 does not couple to the DAP 12 activating chain, although it has been shown to enhance Ca^{++} signaling in rat basophilic leukemia cells, suggesting that TLT-1 is a coactivating receptor (19). The specificity of TLT-1

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expression suggests that it plays a unique role in hemostasis and/or thrombosis. We have shown that a soluble fragment of TLT-1 is identifiable in human serum and plasma, the level of which is highly correlated to disseminated intravascular coagulation scores during sepsis (20). sTLT-1 binds to fibrinogen and augments platelet aggregation *in vitro*. Interestingly, crystallographic studies reveal structural similarities between TLT-1 and TREM-1 that suggest the existence of interactions between TLT-1 and TREM-1 (21).

Indeed, we recently showed that TLT-1 and a TLT-1–derived peptide (LR12) exhibit anti-inflammatory properties by dampening TREM-1 signaling and thus behave as naturally occurring TREM-1 inhibitors. The mechanism by which LR12 inhibits TREM-1 signaling derives from its ability to bind to the TREM-1 ligand (22). We further demonstrated that these same peptide also modulates *in vivo* the inflammatory cascade triggered by infection, thus inhibiting hyperresponsiveness, organ damage, and death during sepsis in mice (22).

As mouse models of septic shock are far from recapitulating the human physiology, we investigated the effects of LR12 during peritonitis in adult minipigs to better characterize its beneficial properties. We show that sepsis-induced cardiovascular dysfunction and organ failure were prevented by LR12 administration.

MATERIALS AND METHODS

The experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the University Animal Care Committee. This study protocol was adapted from the one developed and published by Peter Radermacher's group (23).

Animal preparation

Adult male minipigs (*Sus scrofa domestica*, Vietnamese pot-belied minipigs, 30–40 kg) were purchased from Elevage Ferry (Vosges, France). Before surgery, animals were fasted overnight with free access to water. Preanesthesia was performed through intramuscular administration of ketamine (10 mg/kg) and midazolam (0.1 mg/kg). Anesthesia was induced and maintained with intravenously administered pentobarbital (initial bolus: 10 mg/kg, and continuous administration 6–8 mg/kg per hour), intermittent sufentanil (10 μg), and pancuronium (4 mg) if necessary. Animals were mechanically ventilated (tidal volume 8 mL/kg, positive end-expiratory pressure 5 cm H_2O, F_{IO_2} 0.21, respiratory rate 14–16 breaths/min adjusted to maintain normocapnia). Left jugular vein was exposed, and a triple-lumen line was inserted. Right jugular vein was also catheterized, and a Swan-Ganz catheter was positioned, allowing the continuous recording of cardiac output (CO), S_vO_2, and right atria and pulmonary arterial pressures. A right carotid arterial catheter was inserted for continuous measurement of arterial pressure. A catheter in the bladder allowed urine collection.

After instrumentation, a midline laparotomy was performed to collect feces from the left colon: 1.5 g/kg was suspended in 200 mL of 0.9% NaCl and incubated at 38°C for 2 h. After surgery, a tube was left in place for the peritonitis induction and ascites drainage.

After surgery, animals were allowed to recover for 2 h before baseline measurements (defined as "H0"). Normal saline was continuously administered (10 mL/kg per hour) throughout the study. Body temperature was kept constant (±1°C) using heating pads or cooling.

Experimental protocol

The timeline is presented in Figure 1. After baseline data collection (H0), peritonitis was induced by administration of autologous feces through the abdominal tube, which was subsequently maintained clamped. After 2 h (H2), animals were randomized to receive LR12 (LR12 group, n = 6) or the vehicle (normal saline) alone (control group, n = 5). LR12 consists of a 12-amino-acid (aa) part of the extracellular domain of TLT-1 (LQEEDAGEYGCM) and was chemically synthesized (Pepscan Presto BV, Lelystad, the Netherlands) as COOH terminally amidated peptide. The correct compound was obtained with greater than 99% yields and was homogeneous after preparative purification, as confirmed by mass spectrometry and analytic reversed-phase high-performance liquid chromatography. This peptide was free of endotoxin. A bolus of 5 mg/kg (in 60 mL) was intravenously delivered over 30 min, then a 1 mg/kg per hour (15 mL/h) infusion was started and lasted throughout the study period. This dosage was derived from previous experiments performed in rodents (22). Importantly, the intensivist in charge of the animal was blinded for the given treatment, which was prepared by an independent investigator.

Animal care was then provided by an experienced intensive care physician with strict adhesion to the following guidelines throughout the study period:

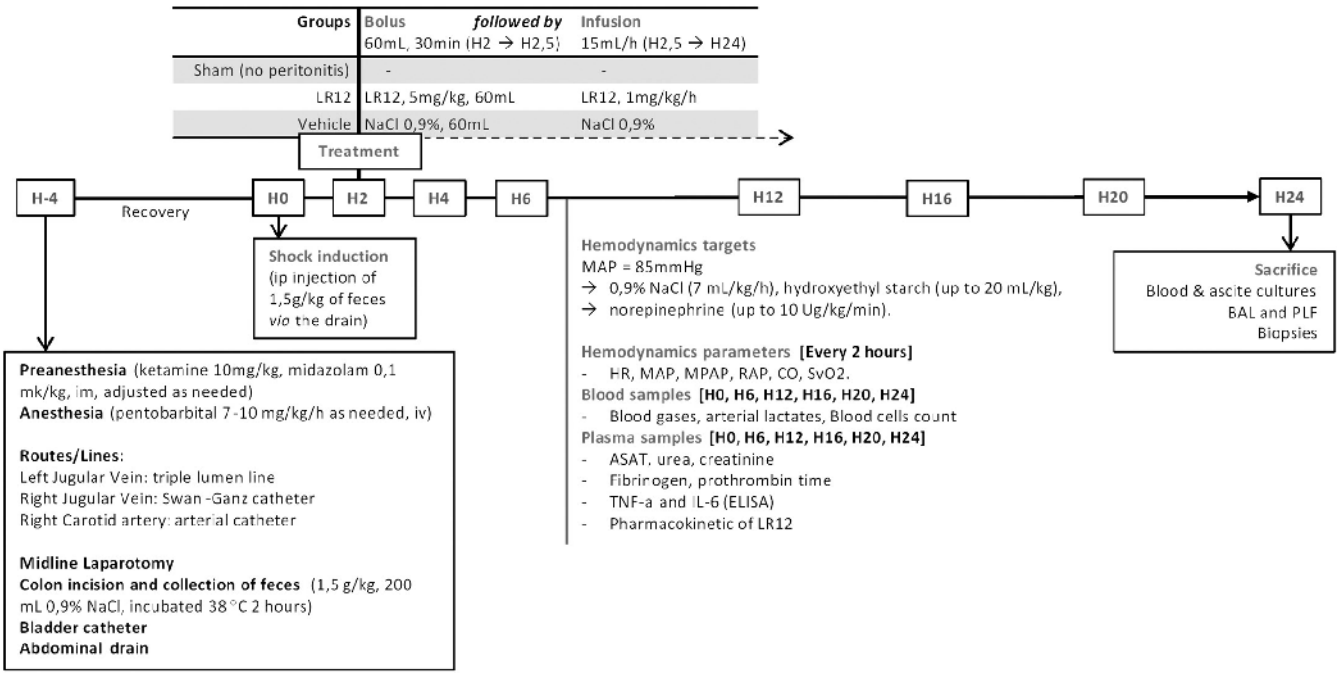

FIG. 1. **Experimental timeline.**

Hemodynamic targets: the main objective was to maintain mean arterial pressure (MAP) above 85 mmHg. To achieve this goal and in addition to the maintenance 0.9% NaCl administration (7 mL/kg per hour), hydroxyethyl starch (up to 20 mL/kg for the entire study period) (HES 130/0.4, Voluven; Fresenius, Fresnes, France) was allowed provided that central venous pressure and pulmonary artery occlusion pressure was less than 18 mmHg. When hydroxyethyl starch maximal volume was reached, a continuous infusion of norepinephrine was started up to 10 μg/kg per minute.

Respiratory targets: the main objective was to maintain a Pao_2/Fio_2 ratio greater than 300 and an arterial $Paco_2$ at 35 to 45 mmHg. Ventilator settings could thus be modified by increasing inspiratory/expiratory ratio close to 1:1, positive end-expiratory pressure up to 15 cm H_2O, and respiratory rate up to 30 breaths/min.

Body temperature should be kept constant (±1°C) using heating pads or cooling.

Intravenous glucose infusion should be administered when necessary to maintain glycemia at 5 to 7 mmol/L.

Animals were then killed under deep anesthesia by KCl infusion 24 h after the induction of peritonitis. Two animals were instrumented, operated, and monitored, but no peritonitis was performed. This additional (sham) group was performed to assess the stability of parameters throughout the study period.

Measurements

Hemodynamic parameters were continuously monitored including MAP, mean pulmonary artery pressure, right atrial pressure, CO, cardiac index, and S_vO_2. Systemic oxygen delivery (DO_2) and systemic oxygen uptake were calculated by the Swan-Ganz monitor. Cardiac Power Index (W/m^2) was calculated as MAP × cardiac index / 451 (24).

Blood was sequentially drawn for the determination of (i) blood gases; (ii) arterial lactate; (iii) plasma concentration of asparte amino transferase (ASAT), urea, creatinine; (iv) fibrinogen, prothrombin time; (v) blood cell count; and (vi) TNF-α, and IL-6 (ELISA; R&D Systems, Minneapolis, Minn).

At the end of the experiment, blood and ascites cultures were performed; bronchoalveolar and peritoneal lavages done for TNF-α and IL-6 determination; and lung, kidney, and liver biopsies obtained for histologic analyses.

Statistical analysis

After testing for their normal distribution (Kolmogorov-Smirnov test), data are presented as means (SD). Between-group differences were tested by two-way analysis of variance for repeated measures with Bonferroni correction or Student *t* test when appropriate. Analyses were performed using GraphPad Prism software (La Jolla, Calif).

RESULTS

Blood cultures could be obtained from 12 of 13 pigs. All septic animals were found bacteremic at the end of the study. Involved microorganisms were *Escherichia coli* (83%), *Klebsiella pneumoniae* (60%), *Streptococcus* spp (50%), and *Enterococcus faecalis* (25%). There were no differences between groups.

All hemodynamic parameters (as well as all other features analyzed) remained constant and stable throughout the 24 h of the study for the sham group. For the sake of clarity, we thus decided not to report these findings in the figures.

For all animals, body temperature was kept constant ($P = 0.56$, not shown).

LR12 attenuates cardiovascular failure

Peritonitis induced a rapid decline of MAP (Fig. 2) despite volume resuscitation (7,750 [SD, 540] mL for controls vs. 6,500 [SD, 800] mL for the LR12 group, $P = 0.137$). Therefore, to maintain MAP at greater than 85 mmHg, norepinephrine was started by H12 in 4/5 and 1/6 control and LR12 animals, respectively. The norepinephrine infusion rate needed to maintain blood pressure was significantly lower in the LR12-treated animals than in controls (Fig. 2).

Associated to hypotension, both cardiac and cardiac power indexes (believed to better describe cardiac performance) became depressed in the control group. This translated into a progressive decline of S_vO_2 and DO_2 (Fig. 3). Again, LR12 showed significant beneficial effects in attenuating cardiac failure. Both groups developed a progressive lactic acidosis (Fig. 4), although largely attenuated by LR12 ($P = 0.0005$). Other selected hemodynamic parameters are shown in Table 1.

These findings thus suggest that LR12 administration was able to attenuate both sepsis-induced vascular and cardiac failure.

LR12 decreases sepsis-induced coagulopathy

Peritonitis was associated in both groups with an initial modest leukopenia by H6 followed by a hyperleukocytosis in LR12-treated animals (Fig. 5). Thrombopenia also appeared, which was significantly less marked in the LR12 group (Fig. 5).

Finally, although fibrinogen plasma concentrations did not vary (not shown), prothrombin ratio progressively decreased, especially in the control group (Fig. 5) ($P = 0.03$ LR12 vs. controls). This suggests that LR12 may attenuate sepsis-induced coagulopathy.

LR12 dampens sepsis-associated organ failure and inflammatory response

A progressive hypoxemia occurred in the control group, whereas this was not observed in LR12-treated pigs (Fig. 6) ($P = 0<001$). We also observed renal and hepatic functions

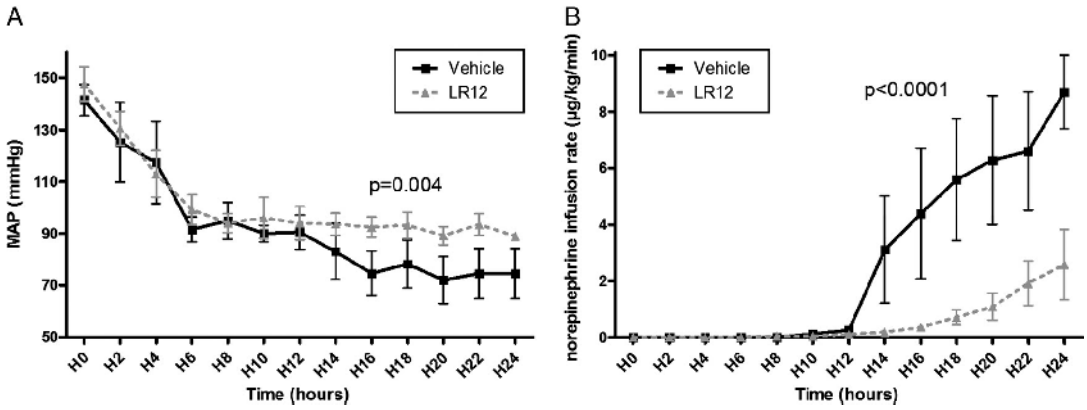

FIG. 2. **LR12 protects from sepsis-induced hypotension**. Evolution of MAP (A) and norepinephrine requirements (B) during the 24-h study period. Mean arterial pressure was constantly higher, and norepinephrine dose lower in LR12-treated animals than in controls.

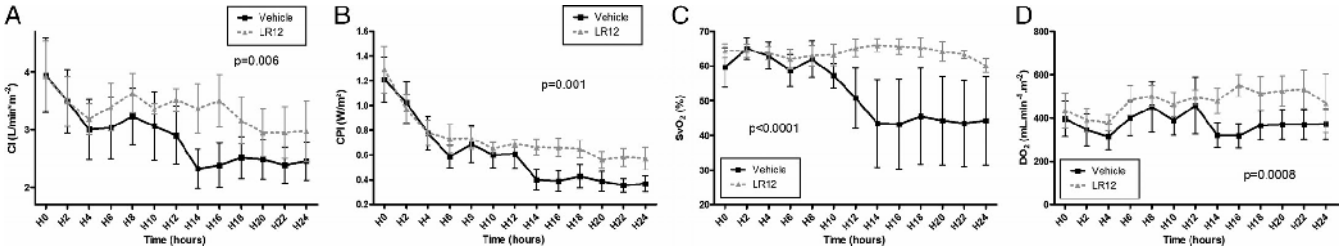

FIG. 3. **LR12 protects from sepsis-induced cardiac dysfunction**. Evolution of cardiac (A) and cardiac power indexes (B), S_vO_2 (C), and DO_2 (D). All these parameters were higher in LR12-treated animals than controls.

impairment that was partially but significantly prevented by LR12 administration (Fig. 6).

Histologic examinations were in line with the biological findings showing disorders (interstitial edema, inflammatory infiltration, tubular damage, etc) that were attenuated by LR12 (Fig. 6; Table 2).

Plasma and alveolar concentrations of TNF-α and IL-6 were significantly lower in LR12-treated pigs than in control animals, whereas there were no differences regarding their peritoneal concentrations (Fig. 7). These data support the existence of a protective effect of LR12 on organ dysfunction and inflammatory response.

LR12 improves survival

Twenty-four-hour mortality rates were, respectively, 60% and 0% for the control and LR12 groups (log-rank test, $P = 0.04$). The survival curve is shown in Figure 8.

DISCUSSION

Based on the current paradigm, complications of severe injuries such as trauma or sepsis are explained by an excessive initial proinflammatory response temporally followed by an immunosuppressive state (25). Although the existence of a late-onset hypoinflammatory state begins to be well documented (26), at least in patients who die in the intensive care unit, it remains unclear whether it results from second-hit episodes of inflammatory events. Very recently, Xiao et al. (3) characterized in a collaborative study (>160 patients) the circulating leukocyte transcriptome after severe trauma, burns, or during endotoxemia (in healthy volunteers). They observed that, as early as 4 h after injury, more than 80% of gene pathways were altered. This phenomenon, they called "genomic storm," consisted of an increased expression of genes involved in innate immunity, systemic inflammatory, and anti-inflammatory responses, concomitant with a decreased expression of genes regulating adaptive immunity. They also observed that complications such as nosocomial infections arose independently of the existence of a second-hit injury but were under the dependence of the magnitude and the duration of the initial leukocytes reprogramming. This new paradigm thus clearly suggests that a targeted therapy aimed at limiting this initial leukocytes' genomic storm may be a valuable approach to improve patients' outcome.

Several proteins are known to amplify the initial inflammatory response, acting as amplification loops. Among them, high mobility group box 1 and TREM-1 have received extensive attention (4, 27). Neutralization of high mobility group box 1 or its signaling has shown promise during acute or chronic inflammatory disorders such as septic shock, pancreatitis, or even myocardial infarction (28–30). The same holds true for TREM-1 pathway modulation that demonstrated encouraging results during sepsis, ischemia-reperfusion, pancreatitis, inflammatory bowel diseases, and chronic arthritis (31).

Although the natural TREM-1 ligand remains unknown, we recently observed that another member of the TREM-1 family, TLT-1, was able to bind this ligand, therefore dampening TREM-1 engagement (22). TREM-like transcript 1 is one of the most abundant proteins released by activated platelets (32) whose role is to promote platelet aggregation through binding to fibrinogen. Large amounts of a soluble form of TLT-1 are released during sepsis (20), and we proposed that TLT-1 may prevent sustained and prolonged inflammation (22).

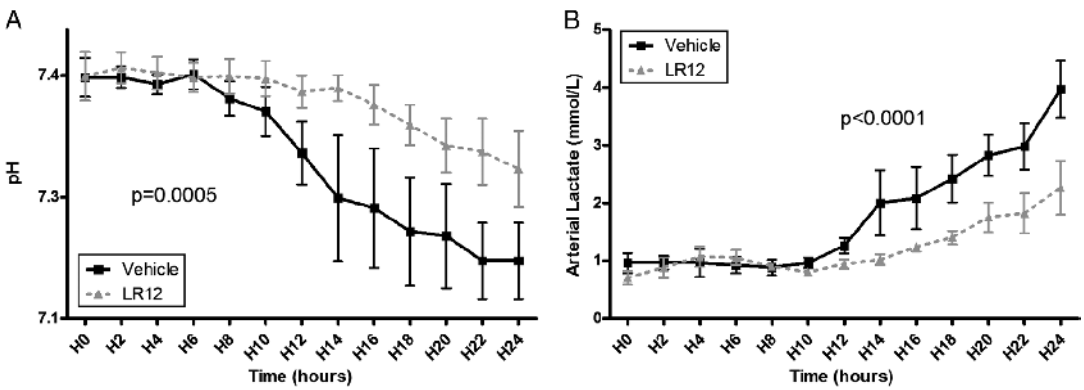

FIG. 4. **LR12 attenuates the development of the lactic acidosis**. Arterial pH (A) and lactate concentration (B) throughout the 24-h study period. Development of acidosis and hyperlactatemia were attenuated by LR12.

TABLE 1. **Selected variables related to hemodynamics and gas exchange**

	H0	H6	H12	H20	H24
HR, control, beats/min	109 (12)	120 (24)	111 (36)	142 (24)	147 (8)
LR12	96 (22)	94 (15)	100 (18)	123 (42)	124 (32)
Central venous pressure, control, mmHg	8 (2)	8 (3)	8 (3)	9 (2)	11 (1)
LR12	7 (1)	8 (2)	8 (2)	7 (1)	8 (3)
Pulmonary artery occlusion pressure, control, mmHg	10 (4)	11 (3)	13 (3)	14 (5)	16 (2)
LR12	11 (1)	10 (2)	12 (3)	11 (2)	12 (3)*
Arterial P_{O_2}, control, mmHg	122 (37)	111 (30)	91 (43)	106 (34)	95 (18)
LR12	126 (42)	128 (34)	133 (43)	128 (45)	119 (46)
VO_2I, control, mL/min per m^2	172 (54)	166 (70)	160 (68)	164 (32)	156 (32)
LR12	179 (60)	176 (51)	170 (34)	182 (52)	188 (70)
Oxygen extraction ratio, control, %	37 (7)	38 (12)	44 (15)	50 (18)	48 (20)
LR12	35 (4)	38 (6)	35 (4)	36 (8)	35 (3)
Hemoglobin control, g/L	89 (19)	104 (36)	121 (16)	121 (35)	129 (24)
LR12	88 (10)	105 (22)	108 (16)	113 (10)	117 (32)

Values are mean (SD).
*P = 0.01 LR12 vs. control.
VO_2I indicates oxygen consumption index.

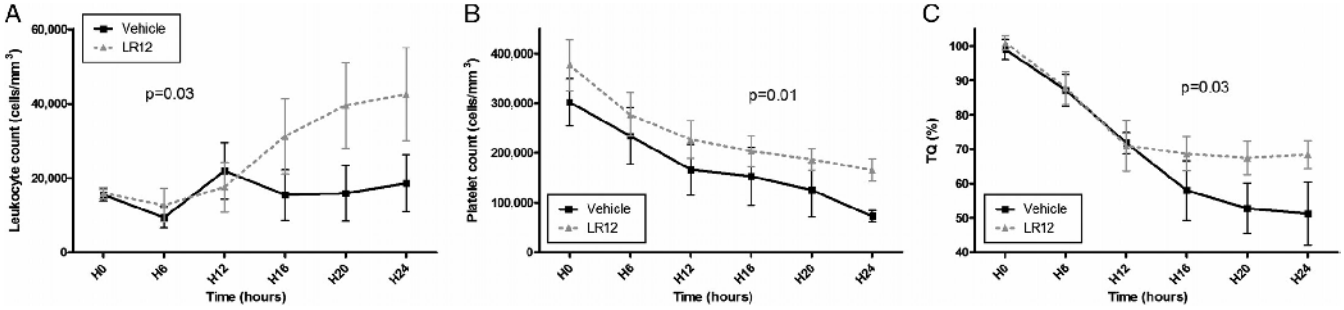

FIG. 5. **Effects of LR12 on leukocytes and platelet counts and prothrombin ratio**. LR12 was associated with progressive hyperleukocytosis (A), reduced thrombopenia (B), and attenuated prothrombin ratio decrease (C).

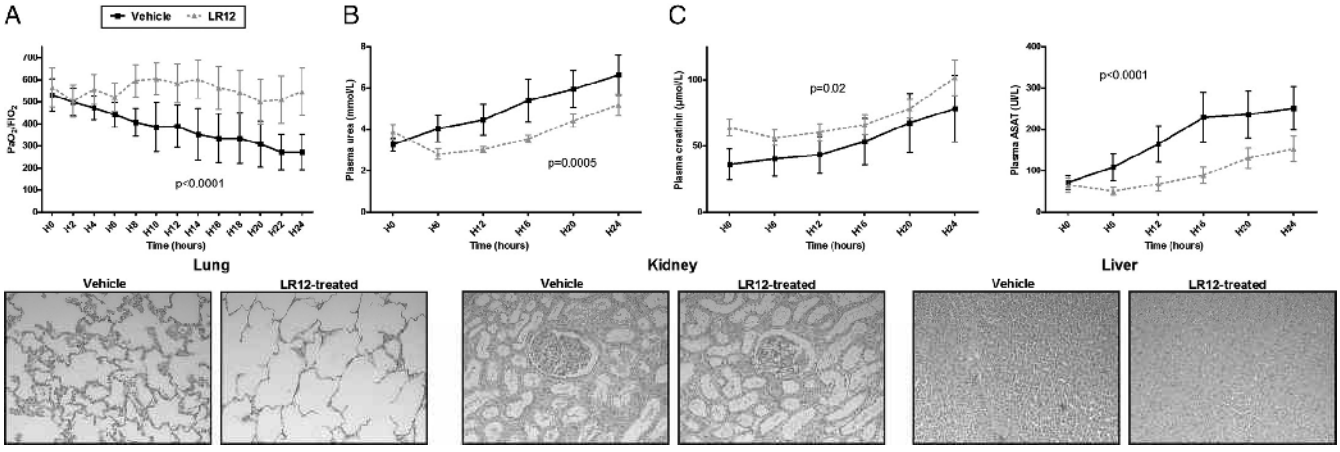

FIG. 6. **LR12 dampens sepsis-induced organ failure**. Pulmonary evaluation was done through Pa_{O_2}/F_{IO_2} ratio assessment (A, top) and histology (A, bottom). Renal function was evaluated by measuring plasma concentration of urea and creatinine (B, top). Kidney histology is also depicted (B, bottom). Liver function was studied by measuring ASAT plasma concentration (C, top); liver histology is also shown (C, bottom). Histologic pictures (hematoxylin-eosin staining, original magnification ×20) are representative of the respective conditions. LR12 constantly dampened the occurrence of organ dysfunction and decreased interstitial edema, inflammatory infiltrate, and architectural alterations in all studied organs.

TABLE 2. **Histopathologic scoring***

	Control	LR12
Lungs		
Vascular congestion and interstitial edema	2	1
Inflammatory infiltrate	3	1
Subtotal	5	2
Liver		
Sinusoidal dilation	2	1
Hepatocyte vacuolization	3	2
Inflammatory infiltrate	1	1
Subtotal	6	4
Kidneys		
Vascular congestion and interstitial edema	1	1
Tubular damage	2	1
Bowman capsule enlargement	1	0
Subtotal	4	2
Total†	15	8

*Each criterion is attributed 0 point when anomaly is absent, 1 point if discrete, 2 points when moderate, and 3 points when severe.
†$P = 0.03$ LR12 vs. control.

To identify which portion of sTLT-1 was involved in this protective effect, we designed several TLT-1 peptides representative of various potential ligand-binding regions (22). Among these, a 12-aa sequence representative of residues 94 to 105, named LR12, was shown to be responsible for the anti-inflammatory effect. Compared with the previously described LR17 compound, LR12 is therefore shorter by 5 aa at the C-terminalpart.

All experiments showing a beneficial effect of the TREM-1 pathway modulation during sepsis have been conducted in rodents. It is nevertheless clearly admitted that these small animal studies are unable to recapitulate the complex human physiology, and most of promising agents tested so far yielded to disappointing results when administered into large animals or humans. Therefore, we studied the effects of the TLT-1–derived peptide (LR12) during septic shock in adult male minipigs.

We show that LR12 administration protects against sepsis-induced cardiovascular dysfunction: the decrease in arterial pressure and CO was partly prevented by LR12, although LR12 animals required less norepinephrine. This translated into a preserved oxygen transport and S_vO_2, and a delayed appearance of lactic acidosis.

The mechanisms by which LR12 improves hemodynamics are not totally elucidated, but we could advance two hypotheses: first, we have already shown that TREM-1 modulation was associated with a decreased production of nitric oxide and of several cytokines (9). Overproduction of nitric oxide and cytokines such as TNF-α or IL-1β is believed to play a role in peripheral vascular and myocardial alterations (33). Second, unpublished observations from our group suggest that, in rodents, both *in vivo* and *in vitro*, LR12 protects from sepsis or LPS-induced endothelial dysfunction and its consequence, vascular hyporeactivity.

Concomitantly, we observed that LR12 dampened organ failure (pulmonary, renal, hepatic). This may be due to hemodynamic improvement, as well as to a modulation of the inflammatory response, as assessed by a decreased local (cytokine concentration, leukocytes infiltration) and systemic (cytokines) inflammation. Coagulation disorders (thrombopenia and prothrombin ratio decrease) were also attenuated: these findings are in line with those observed in rodents (22).

This work has several important strengths. First, we used adult minipigs. Although much more expensive than usual domestic pigs, these animals are physiologically close to adult humans despite their relatively low weight. Second, we designed this study as a randomized and blinded one, therefore limiting experimental biases. Third, resuscitation, following pre-established guidelines, was conducted by an intensive care physician throughout the extended (24 h) study period. Thus, we tried to closely mimic what could be the use of this therapy if administered to humans, at least during the first 24 h.

Nevertheless, several controversial points deserve discussion. Despite an aggressive fluid loading protocol (10 mL/kg per hour of normal saline and up to 20 mL/kg hydroxyethyl starch), all animals developed a hypokinetic state. As we first focused on vascular hyporeactivity and norepinephrine requirements to maintain MAP greater than 85 mmHg, we did not allow the administration of dobutamine. Anyway, as CO decreased less with LR12 infusion, we believe that these treated animals would have required less dobutamine than would the controls. Second, our experimental model is not totally clinically relevant because

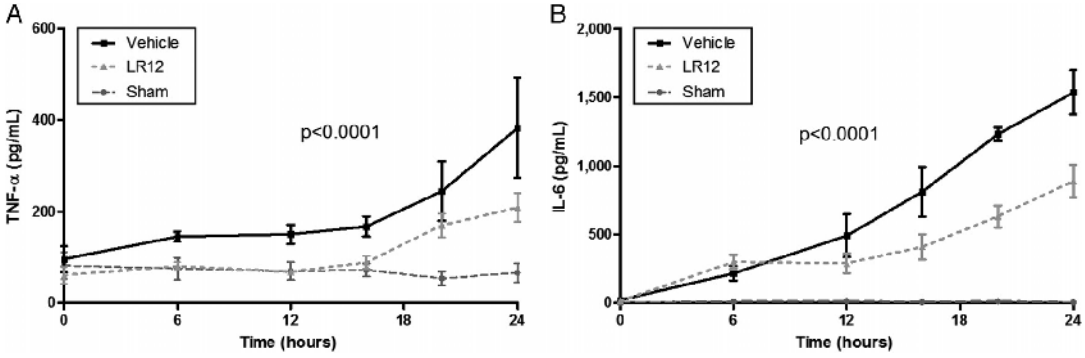

FIG. 7. **LR12 decreases local and systemic cytokine concentrations**. Plasma (A) and alveolar (B) TNF-α and IL-6 concentrations were decreased by LR12 administration.

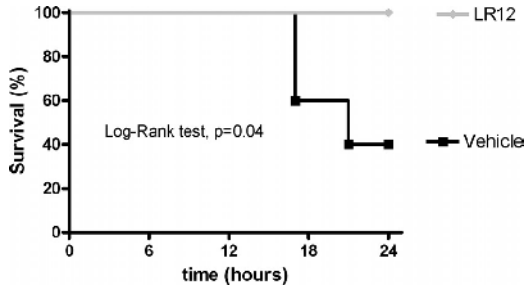

FIG. 8. **LR12 improves survival**. Kaplan-Meier survival curves: LR12 improves 24-h survival (log-rank test, $P = 0.04$).

(i) we did not use antibiotics, (ii) surgery was not performed to treat the peritonitis (i.e., there was no source control), and (iii) obviously our animals were previously healthy without comorbidities. The first two points were deliberate to get a very severe model (that we managed to obtain with a 60% mortality rate in the control group) and thus assess the efficiency of LR12 in its "worst" condition of use. As the mortality rate was very high in our control group, the effect of LR12 treatment may be less impressive if applied to patients with a lower risk of death.

CONCLUSIONS

In this study, we demonstrated that the use of a TLT-1–derived peptide known to modulate the TREM-1 pathway was able to protect against septic shock–induced cardiovascular dysfunction and organ failure in minipigs. We are confident that this promising strategy will be studied in humans in the future.

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