Severe gunshot injuries in a porcine model: impact on central markers of innate immunity

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Background: The mechanisms behind lipopolysaccharide (LPS) tolerance remain obscure. LPS signals through Toll-like receptor 4 (TLR4) and severe trauma/haemorrhage may influence binding and signalling through this receptor, e.g. by changing membrane expression or by releasing endogenous ligands like High Mobility Group Box 1 (HMGB1). The aim of this study was to examine these relations further in a porcine model with standardized trauma.

Methods: Nine anaesthetized pigs sustained one gunshot through the femur and one pistol shot through the upper abdomen. Blood was sampled before and 90 min after shooting. The samples were stimulated for 4 h with LPS 10 ng/ml or an equivalent amount of normal saline. The leucocyte response was evaluated by measuring the tumour necrosis factor-α (TNF-α) and CXC ligand 8 (CXCL8) in the supernatant. Flow cytometry was used to measure the surface expression of TLR4 on CD14+ monocytes. HMGB1 concentrations were measured in the plasma.

Results: Trauma and treatment caused a significant decline in the LPS-stimulated concentrations of TNF-α [4.53 ± 0.24 pg/ml (ln) at 0 min, 3.54 ± 0.35 pg/ml (ln) at 90 min, P = 0.026], but did not modify the release of CXCL8. Monocyte TLR4 expression was unchanged. Plasma HMGB1 increased significantly [≤0.92 vs. 3.02 ± 0.19 ng/ml (ln), P < 0.001]. The concentrations of TNF-α and CXCL8 did not correlate with TLR4 expression or HMGB1 concentrations.

Conclusion: The results suggest that trauma-induced LPS tolerance is not primarily regulated by TLR4 expression on circulating CD14+ monocytes or by the release of HMGB1 from damaged tissues.

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TRAUMA is a main cause of mortality, particularly among younger people.1 In addition to the direct injury, it also affects the immune system in several ways, disturbing the normally fine-tuned balance, and leaving the patient more susceptible to infections.2 The ability to resist invading pathogens depends on a rapid and powerful immune response, in physiological situations a localized reaction, to restore homeostasis. However, the response can be overwhelming and can become a threat to the host. In the first period after the trauma, the patient is at risk of developing a systemic inflammatory response syndrome, while infections and sepsis are common later. Furthermore, severely injured patients often develop multiple-organ failure.3 The first line of immune response is carried into effect by the innate branch, with the activation of monocytes/macrophages and neutrophils. These cells recognize structures representing threats, e.g. bacterial components, using genetically highly conserved receptors (pattern recognition receptors). The Toll-like receptors (TLRs) fit into this group, expressed in leucocytes and other cells. They were first described in humans in 1997,4 and so far, 12 members of the TLR family have been identified in mammals.5 Lipopolysaccharide (LPS), an integral component of the cell wall of Gram-negative bacteria, signals by means of a cell surface complex involving TLR4, myeloid differentiation protein-2 (MD-2) and CD14. Subsequently, the recruitment of intracellular adaptor molecules and downstream activation of transcription factors, as nuclear factor κ B, induces the production of pro-inflammatory cytokines.5 Tumour necrosis factor-α (TNF-α) occupies a key position in the development of inflammation, but other cytokines, like the chemokine CXC ligand 8 (CXCL8), are also involved. Endo-
toxin tolerance, first described by Beeson,\(^6\) implies reduced LPS activation after previous exposure to LPS. In addition, it is well documented that sterile tissue damage can induce tolerance to endotoxin.\(^7\) In gunshot injury in pigs, our group has described an almost instantaneous reprogramming of circulating leucocytes, including a profound reduction of LPS-stimulated TNF-\(\alpha\) production,\(^8\) the hallmark of endotoxin tolerance. In connection with tissue damage, endogenous molecules signalling danger, damage-associated molecular patterns, are released into circulation, i.a. fibrinogen, Heat Shock Proteins (HSPs) and High Mobility Group Box 1 (HMGB1). These molecules are able to induce an immune response via TLR4,\(^9\) which thus represents a common pathway of innate immune activation by infection and injury. Interestingly, HSP-70 and HMGB1 have been linked to the development of endotoxin tolerance.\(^10,11\)

The aim of the present study was to investigate the possible molecular explanations for the development of endotoxin tolerance related to tissue damage. By means of a standardized porcine gunshot injury model, we tested our hypothesis that trauma might induce a reduced expression of TLR4 on CD14\(^+\) monocytes and thus a reduced production of cytokines in response to LPS. In addition, we investigated whether HMGB1 released from injured tissues could influence post-traumatic LPS tolerance.

Materials and methods

Animals

The study was conducted in conjunction with a course in traumatology and war surgery, arranged by the Norwegian Army Medical Service and the University of Oslo, as described previously.\(^12,13\) Based on previous results with this model,\(^8,12\) nine Norwegian landrace pigs, weighing 52.4 ± 2.5 kg, were used. The animals were handled according to the Animal Welfare Act and statutes from the Norwegian Ministry of Agriculture. They were fully anaesthetized before the start of the experiment and remained so until they were euthanized at the end of each surgical training session. For premedication, the animals were given medetomidin hydrochloride (CliniPharm, Zurich, Switzerland) 0.06 mg/kg and tiletamin/zolazepam (Boehringer Ingelheim, Ingelheim, Germany) 3 mg/kg i.m. Thereafter, they were given butorphanol tartrate (Pfizer Animal Health, New York, NY, USA) 0.2 mg/kg as an analgesic. After the induction of anaesthesia, the pigs were orally intubated and ventilated with room air. Intravenous cannulas in the ears were used for medication and fluid administration. To ensure absence of pain, the animals also received lidocain 2% epidurally in the lumbosacral region, the dose adjusted to body length. Anaesthesia was continued with a mixture of isoflurane, oxygen and air. Analgesia was tested repeatedly with forceps pressure interdigitally. The femoral artery was cannulated using a Secalon-T subclavian catheter (BD Critical Care Systems, Singapore), used for haemodynamic monitoring and blood sampling.

Experimental procedure

In general anaesthesia, the pigs were transported to a nearby firing range. According to the protocol, they received one rifle shot from a distance of 25 m in the right thigh, and one superficial pistol shot from a short distance against the left upper abdomen. Both entrance points, and also wanted exit point for the abdominal shot, were marked beforehand to ensure a standardized injury and not to injure the liver and large abdominal vessels. All the animals were hit at the marked points. After shooting, first-aid treatment was started immediately, including dressing and compression of wounds and control of ventilation. The animals were then taken to a field hospital 300 m away, where surgeons first controlled the thigh for ongoing bleeding and, thereafter, within 10–15 min after shooting, proceeded to damage control laparotomy. All the animals had intestinal perforations, caused directly by the bullet or indirectly by the pressure wave, which were immediately closed. The peritoneal cavity was rinsed with a saline solution. NaCl 0.9% and dextran 70/NaCl were infused as needed to compensate for blood loss and to maintain systolic blood pressure above 90 mmHg. Transfusions of blood or blood substitutes were not given. The experimental period was terminated after 90 min.

The whole blood model

To elucidate the ability of leucocytes to respond with cytokine production to LPS stimulation, we used a previously described whole blood model.\(^14\) Heparinized whole blood drawn at time points 0 and 90 min was stimulated with 10 ng/ml LPS (Escherichia coli serotype 0111:B4; Sigma-Aldrich,
St. Louis, MO) or an equivalent volume of normal saline. The tubes were incubated in room air at 38 °C for 4 h and gently rotated × 6/min to avoid cell sedimentation. Thereafter, the samples were centrifuged, the supernatant was removed and immediately frozen at −20 °C.

**Measurements**
Heart rate and oxygen saturation were monitored continually. Invasive blood pressure monitoring was established after arrival to the hospital. Sixty millilitre of arterial blood was sampled at 0 and 90 min. Analyses performed immediately included arterial blood gases and a complete blood cell count. Standard kits were used to measure the concentrations of the cytokines TNF-α (R&D Systems Inc., Minneapolis, MN) and CXCL8 (Invitrogen Corporation, Camarillo, CA). Cytokine concentrations were corrected according to the white blood cell (WBC) count in the samples. HMGB1 was measured using a standard kit (IBL International, Hamburg, Germany) and corrected according to plasma volume change.

**Isolation of peripheral blood mononuclear cells (PBMCs)**
PBMCs were isolated by centrifugation on a Histopaque-1077 (Sigma-Aldrich) gradient, described previously in pigs by Zelnickova et al.,15 consistent with the manufacturer’s recommendations. Unstimulated heparinized blood was diluted in a phosphate-buffered saline (PBS) solution (Sigma-Aldrich) to avoid overloading the gradient, 1:4 in the pre-traumatic (0 min) sample and 1:2 in the post-traumatic (90 min) sample, according to the lower cell count. Histopaque-1077 was carefully laid onto the pre-diluted blood, and the tubes were then centrifuged for 30 min at 700 g at room temperature. Cells were collected from the interface between the Histopaque medium and plasma and then washed three times in PBS solution. Finally, the cells were resuspended in 0.5 ml PBS solution supplemented with 10% foetal bovine serum (Invitrogen Corporation, Grand Island, NY, USA).

**Cell surface staining**
One hundred microlitre cell suspension was incubated with 10 μl mouse serum (Sigma-Aldrich) on ice for 10 min in the dark to block unspecific receptors. Then, 2 ml of PBS solution was added for washing, the tubes were centrifuged at 300 g for 5 min at 4 °C and the supernatant was discarded. The cells were then stained according to the manufacturer’s recommendations with either 10 μl fluorescein isothiocyanate (FITC)-conjugated mouse anti-pig CD14 monoclonal antibody (mAb) (clone MIL-2) or FITC-conjugated anti-mouse IgG2b isotype control (both AbD Serotec, MorphoSys UK, Oxford, UK) combined with 20 μl phycoerythrin (PE)-conjugated anti-human TLR4 mAb (clone HTA 125) or PE-conjugated anti-mouse IgG2a isotype control (both eBioscience, San Diego, CA). The HTA 125 clone was tested to be effective on porcine CD14+ monocytes (Fig. 1), previously also shown for piglet leucocytes.16 The stained cells were incubated on ice in the dark for 30 min; thereafter, 2 ml of FACS Lysing solution (BD Biosciences, San Jose, CA) was added to each tube and they were incubated in the dark for 5 min. Subsequently, the cells were centrifuged at 300 g for 5 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 2 ml cold PBS with 10% FCS. After centrifuging at 300 g for 5 min at 4 °C, the supernatant was discarded and the pellet was finally resuspended in 0.25 ml 1% paraformaldehyde (Sigma-Aldrich) in PBS with 4% bovine serum albumin (Sigma-Aldrich) and 0.1% NaN3 and stored in the dark at 4 °C until flow cytometry could be performed.

**Flow cytometric analysis**
The samples were analysed within 24 h in a FACS-Canto II flow cytometer (BD Biosciences) equipped with FACS Diva Software version 6.1.1 (BD Biosciences). The monocytes were defined according to

![Fig. 1. Expression of phycoerythrin (PE)-conjugated isotype control (grey histogram) and PE-conjugated anti-TLR4 mAb (open histogram) on CD14+ cells from one representative animal.](image-url)
their CD14-staining and side scatter light characteristics. For each sample, 5000 monocytes were acquired with the help of an electronic gate. The median fluorescence intensity (MFI) was recorded and corrected for nonspecific antibody binding by subtracting the MFI measured for the matched isotype control sample (Fig. 2). Cyflogic version 1.2.1 (CyFlo Ltd., Turku, Finland) was used for an overlay histogram.

Statistical analysis
SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) was used for statistical calculations. Data are presented as mean ± standard error of the mean (SEM). Cytokine and HMGB1 concentrations and TLR4 MFI-values were ln-transformed to achieve normality. A paired t-test was used to evaluate the differences between baseline and end values. For analysis of correlation, Pearson’s product–moment correlation was used. P-values <0.05 were considered statistically significant.

Results
Survival
The injuries after shooting were evaluated as uniform and all animals survived the study period.

Physiological variables
The bleeding was extensive, with a significant decline in haemoglobin from initially 10.8 ± 0.2 to 7.5 ± 0.5 g/l at the end of the study. There were also significant reductions in platelets from 463 ± 28 to 313 ± 28 cells × 10⁹/l and WBC counts from 19.5 ± 1.3 to 7.7 ± 1.1 cells × 10⁹/l (Table 1). During the 75- to 80-min in-hospital time, the animals received 2606 ± 264 ml 0.9% NaCl plus 628 ± 87 ml dextran 70/NaCl, a total fluid amount of 3233 ± 301 ml. There were no significant changes in the mean arterial pressure, heart rate or saturation of peripheral oxygen (SpO₂) between the two time points (Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Blood biochemistry (mean ± SEM)</th>
<th>0 (min)</th>
<th>90 (min)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/l)</td>
<td>10.8 ± 0.2</td>
<td>7.5 ± 0.5</td>
<td>0.009</td>
</tr>
<tr>
<td>Platelets (× 10⁹/l)</td>
<td>463 ± 28</td>
<td>313 ± 28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>White blood cells (× 10⁹/l)</td>
<td>19.5 ± 1.3</td>
<td>7.7 ± 1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>11 ± 1</td>
<td>13 ± 1</td>
<td>0.21</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>38 ± 4</td>
<td>24 ± 3</td>
<td>0.004</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>51 ± 4</td>
<td>64 ± 4</td>
<td>0.016</td>
</tr>
<tr>
<td>pH</td>
<td>7.34 ± 0.01</td>
<td>7.26 ± 0.04</td>
<td>0.042</td>
</tr>
<tr>
<td>pO₂ (kPa)</td>
<td>9.7 ± 0.4</td>
<td>26.0 ± 4.4</td>
<td>0.005</td>
</tr>
<tr>
<td>pCO₂ (kPa)</td>
<td>9.1 ± 0.2</td>
<td>8.4 ± 0.7</td>
<td>0.39</td>
</tr>
<tr>
<td>Base excess</td>
<td>9.0 ± 0.5</td>
<td>-0.8 ± 1.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values before and 90 min after gunshot injuries in pigs.
Changes in the relative plasma volume were calculated from haemoglobin and haematocrit according to the formula: % plasma volume change = \[ \frac{(Hb_{control}/Hb_{test})}{(100 - Hct_{test})/(100 - Hct_{control})} \times 100 \]. After 90 min, the plasma volume increased to 175 ± 22% (P < 0.009 vs. baseline value).

Cytokine production in ex vivo whole blood
Trauma increased the production of TNF-α from 1.17 ± 0.17 to 3.22 ± 0.51 pg/ml (ln) (P = 0.005, Fig. 3). Stimulation with LPS, as compared with saline, caused a significant fourfold increase in TNF-α production before shooting (P < 0.001) but caused no change at 90 min (P = 0.46). LPS-stimulated production of TNF-α was reduced from 4.53 ± 0.24 before to 3.54 ± 0.35 pg/ml (ln) after the trauma (P = 0.026). In ex vivo whole blood stimulated with LPS, the concentrations of CXCL8 were 1.75 ± 0.34 pg/ml (ln) before shooting and 1.63 ± 0.29 pg/ml (ln) after 90 min (NS, P = 0.82). The concentrations of CXCL8 were below detectable values in ex vivo whole blood without LPS stimulation.

TLR4 expression
The surface expression of TLR4 on circulating CD14+ monocytes was unchanged; MFI (ln) was 6.64 ± 0.14 at 0 min and 6.42 ± 0.22 at 90 min (P = 0.41, Fig. 4). The expression values increased in four and decreased in five animals after trauma. No correlation was found between TLR4 expression and LPS-stimulated concentrations of TNF-α or CXCL8 in ex vivo whole blood.

Plasma HMGB1
HMGB1 increased from undetectable values [<0.92 ng/ml (ln)] before shooting to 3.02 ± 0.19 ng/ml (ln) after 90 min (P < 0.001). No correlations were found between the post-traumatic release of HMGB1 and ex vivo concentrations of TNF-α and CXCL8.

Discussion
In the present study, we observed a significantly increased ex vivo TNF-α concentration after standardized trauma, thus unmasking an activation of the leucocytes. Stimulation with LPS, as compared with saline, induced a significant fourfold increase in the production of TNF-α before the trauma.

Table 2
<table>
<thead>
<tr>
<th>Haemodynamics (mean ± SEM) after gunshot injuries in pigs.</th>
<th>15 (min)</th>
<th>90 (min)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>72 ± 8</td>
<td>59 ± 4</td>
<td>0.14</td>
</tr>
<tr>
<td>Heart rate (min⁻¹)</td>
<td>103 ± 7</td>
<td>89 ± 10</td>
<td>0.30</td>
</tr>
<tr>
<td>SpO₂ (%)</td>
<td>94 ± 1</td>
<td>93 ± 1</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Values at hospital arrival (15 min) and at the end of the study period (90 min).

Fig. 3. TNF-α concentrations in ex vivo whole blood after stimulation with lipopolysaccharide (LPS) 10 ng/ml or normal saline (incubation time 4 h). Vertical bars denote standard error of the mean. *P < 0.05 stimulation with LPS vs. normal saline. #P < 0.05 before vs. after gunshot injuries in pigs.

Fig. 4. Surface expression of TLR4 on circulating CD14+ monocytes. Vertical bars denote standard error of the mean. No significant difference was seen before vs. after gunshot injuries in pigs.
After shooting and peri-traumatic stress, there was no difference in TNF-α release, whether stimulated with LPS or saline. The injury significantly reduced the TNF-α response to LPS stimulation, the hallmark of endotoxin tolerance, whereas CXCL8 production was unaffected. We hypothesized decreased surface expression of TLR4 on circulating CD14+ monocytes as a possible explanation for trauma-induced endotoxin tolerance, but found unchanged TLR4 expression, and no correlation between receptor expression and the corresponding LPS-induced cytokine concentrations. The plasma concentration of HMGB1 increased significantly after the trauma, but no correlations were found with ex vivo cytokine concentrations.

The clinical relevance of endotoxin tolerance remains inconclusive. A powerful cytokine response is a double-edged sword: on the one hand, it is vital to withstand systemic infections caused by invading organisms, but on the other, the result can be increased organ damage. The immune response is regulated by complex interactions between humoral and cellular factors. In a study of murine sepsis, animals pre-conditioned with LPS surprisingly showed improved survival despite an attenuated cytokine response. Augmented bacterial clearance caused by increased phagocytic activity could be a possible explanation.

The complex of TLR4, MD2 and CD14 plays a crucial role in LPS signalling, but when it comes to the fine-tuned regulation, the picture is obscure and the results are partly conflicting. Bruniali et al. found no differences in TLR4 surface expression on CD14+ monocytes between patients with sepsis, severe sepsis, septic shock or healthy controls, whereas others have found increased expression on monocytes from patients with sepsis. In trauma-induced inflammation, TLR4 is also of vital importance, showed in vivo by comparing wild-type and TLR4 mutant mice. Studies have reported lower receptor expression in connection with surgery, e.g. after major gastrointestinal surgery. Ikushima et al. found significantly decreased surface expression of TLR4 and suppressed cytokine production. Reduced monocyte TLR4 expression is also described at the end of cardiopulmonary bypass. In contrast, we found no change in the surface expression of TLR4 on circulating CD14+ monocytes in pigs exposed to standardized trauma, in line with results from patients with blunt trauma. Lendemans et al. found no change in TLR4 expression on monocytes from trauma patients compared with healthy volunteers, even though LPS-induced TNF-α release was significantly reduced.

HMGB1, a nuclear protein that facilitates gene transcription, is able to induce tolerance to LPS. Studies with mutant mice have shown that it is of vital importance for the development of remote organ injury following trauma. In patients exposed to severe trauma, plasma levels of HMGB1 increased within 30 min and correlated with a systemic inflammatory response and tissue hypoperfusion as well as severity of the injury. In our model, trauma induced a highly significant HMGB1 plasma release, but no correlation with ex vivo cytokine production was seen. Recent studies have brought into question whether HMGB1 acts as a pro-inflammatory molecule activating cells directly or rather potentiates inflammatory responses through binding to cytokines (e.g., TNF-α), DNA or LPS. After extensive trauma, as in our model, circulating TNF-α increase, and also LPS will be released into the circulation in connection with the injury and the following laparotomy. Binding of HMGB1 to different molecules could imply that a direct correlation between the concentrations of this alarmin and pro-inflammatory cytokines like TNF-α and CXCL8 may not be expected.

The limitations of the study include the number of animals and the plasma dilution after trauma. Even though we used corrected concentrations of HMGB1 and cytokines, the results may have been affected. The injury induced increased lymphocyte proportion and decreased neutrophil proportion in the circulation; however, the proportion of monocytes, the main producer of LPS-induced pro-inflammatory cytokines, remained unchanged. Thus, the observed post-traumatic endotoxin tolerance cannot be explained by a reduced number of monocytes, but nevertheless, a selection of circulating monocytes caused by bleeding and migration of activated cells to damaged tissue could occur. However, circulating mononuclear cells sampled from trauma patients within 24 h showed unchanged ATP levels and function compared with healthy volunteers.

In conclusion, standardized gunshot injuries and peri-operative stress significantly reduced TNF-α response to LPS in pigs, whereas CD14+ monocyte surface expression of the LPS receptor TLR4 remained unchanged. Trauma induced a highly significant increase in plasma HMGB1 that did not correlate with the cytokine concentrations. Taken together, the findings suggest that trauma-related endotoxin tolerance is not primarily regulated by monocyte expression of TLR4 or by the release of HMGB1 from injured tissues.
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References


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