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## Leukotriene B4 indicates lung injury and on-going inflammatory changes after severe trauma in a porcine long-term model

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**Abstract:** **BACKGROUND:** Recognizing patients at risk for pulmonary complications (PC) is of high clinical relevance. Migration of polymorphonuclear leukocytes (PMN) to inflammatory sites plays an important role in PC, and is tightly regulated by specific chemokines including interleukin (IL)-8 and other mediators such as leukotriene (LT)B<sub>4</sub>. Previously, we have reported that LTB<sub>4</sub> indicated early patients at risk for PC after trauma. Here, the relevance of LTB<sub>4</sub> to indicating lung integrity in a newly established long-term porcine severe trauma model (polytrauma, PT) was explored. **METHODS:** Twelve pigs (3 months old, 30 ± 5kg) underwent PT including standardized femur fracture, lung contusion, liver laceration, hemorrhagic shock, subsequent resuscitation and surgical fracture fixation. Six animals served as controls (sham). After 72h lung damage and inflammatory changes were assessed. LTB<sub>4</sub> was determined in plasma before the experiment, immediately after trauma, and after 2, 4, 24 or 72h. Bronchoalveolar lavage (BAL)-fluid was collected prior and after the experiment. **RESULTS:** Lung injury, local gene expression of IL-8, IL-1, IL-10, IL-18 and PMN-infiltration into lungs increased significantly in PT compared with sham. Systemic LTB<sub>4</sub> increased markedly in both groups 4h after trauma. Compared with declined plasma LTB<sub>4</sub> levels in sham, LTB<sub>4</sub> increased further in PT after 72h. Similar increase was observed in BAL-fluid after PT. **CONCLUSIONS:** In a severe trauma model, sustained changes in terms of lung injury and inflammation are determined at day 3 post-trauma. Specifically, increased LTB<sub>4</sub> in this porcine long-term model indicated a rapid inflammatory alteration both locally and systemically. The results support the concept of LTB<sub>4</sub> as a biomarker for PC after severe trauma and lung contusion.

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## Leukotriene B4 indicates lung injury and on-going inflammatory changes after severe trauma in a porcine long-term model<sup>☆</sup>



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### ABSTRACT

**Background:** Recognizing patients at risk for pulmonary complications (PC) is of high clinical relevance. Migration of polymorphonuclear leukocytes (PMN) to inflammatory sites plays an important role in PC, and is tightly regulated by specific chemokines including interleukin (IL)–8 and other mediators such as leukotriene (LT)B4. Previously, we have reported that LTB4 indicated early patients at risk for PC after trauma. Here, the relevance of LTB4 to indicating lung integrity in a newly established long-term porcine severe trauma model (polytrauma, PT) was explored.

**Methods:** Twelve pigs (3 months old, 30 ± 5 kg) underwent PT including standardized femur fracture, lung contusion, liver laceration, hemorrhagic shock, subsequent resuscitation and surgical fracture fixation. Six animals served as controls (sham). After 72 h lung damage and inflammatory changes were assessed. LTB4 was determined in plasma before the experiment, immediately after trauma, and after 2, 4, 24 or 72 h. Bronchoalveolar lavage (BAL)-fluid was collected prior and after the experiment.

**Results:** Lung injury, local gene expression of IL-8, IL-1β, IL-10, IL-18 and PMN-infiltration into lungs increased significantly in PT compared with sham. Systemic LTB4 increased markedly in both groups 4 h after trauma. Compared with declined plasma LTB4 levels in sham, LTB4 increased further in PT after 72 h. Similar increase was observed in BAL-fluid after PT.

**Conclusions:** In a severe trauma model, sustained changes in terms of lung injury and inflammation are determined at day 3 post-trauma. Specifically, increased LTB4 in this porcine long-term model indicated a rapid inflammatory alteration both locally and systemically. The results support the concept of LTB4 as a biomarker for PC after severe trauma and lung contusion.

**Abbreviations:** ARDS, acute respiratory distress syndrome; ATLS, advanced trauma life support; a.tr., after trauma; BAL, bronchoalveolar lavage fluid; BW, body weight; CAE, chloroacetate esterase; COPD, chronic obstructive pulmonary disease; CVP, central venous pressure; CXCR, CXC chemokine receptor; DNA, Deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; FiO<sub>2</sub>, fraction of inspired oxygen; g, earth's gravitational acceleration; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; h, hour; HE, hematoxylin-eosin; Hg, mercury; kg, kilogram; IL, Interleukin; LIS, lung injury score; LTB4, leukotriene B4; MAP, mean arterial pressure; mg, milligram; min, minute; ml, millilitre; mm, millimetre; NaCl, sodium chloride; PC, pulmonary complications; pCO<sub>2</sub>, partial pressure of carbon dioxide; p, p-value; PEEP, positive end expiratory pressure; pg, picogram; PMN, polymorphonuclear leukocytes; PT, polytrauma; qRT-PCR, semi-quantitative real-time polymerase chain reaction; RNA, Ribonucleic acid; RT, room temperature; sem, standard error of the mean; °C, Celsius; µl, microlitre

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## 1. Introduction

Development of pulmonary complications such as pneumonia, embolism, Acute Respiratory Distress Syndrome (ARDS) and other still displays a major risk and remain major causes of long-term morbidity and mortality in trauma patients [1,2]. The mortality in case of acute lung injury in multiply traumatized patients is 10% [3,4]. However, the treatment strategies are still based on clinical preventive procedures such as protective ventilation and kinetic therapy [5,6]. Therefore, the clinical definitions of pulmonary complications e.g. ARDS often remain non-specific and subsequently under-diagnosed or under-treated due to the lack of knowledge concerning the pathophysiology of lung injury, pulmonary complications and repair mechanisms. Kannan et al. have performed very high fidelity 2D and 3D simulations for accurately and efficiently predicting and quantifying local and global injuries (caused by trauma, hemorrhages, blasts) for organs like the brain and the lung [7,8]. They were able to noninvasively "numerically penetrating" the tissues, and reconstruct the optical properties the presence of water, oxygenated and de-oxygenated blood [7,8]. These numerical non-invasive measurements are then used to predict the extent and severity of the organ hemorrhage/injury. With regard to clinical scenario, studies of potential biomarkers as reliable predictive parameters for lung injury and pulmonary complications after trauma would be useful for biologic confirmation of the clinical diagnosis in trauma patients.

In pulmonary complications polymorphonuclear leukocytes (PMN) have been identified as important contributors to the pathogenesis. PMN migrate after their systemic activation by e.g. interleukin (IL)–8 into the pulmonary interstitium, release proteolytic enzymes and induce microvascular damage and harmful airway remodeling in lung tissue, which is associated with poor survival [9–12]. Moreover, the generation of inflammatory mediators including interleukin IL-8 plays an important role in ARDS pathophysiology [13–16].

The proinflammatory lipid mediator Leukotriene (LT)B<sub>4</sub> has been originally discovered as a strong chemotactic factor, which exerts PMN activating abilities and plays a crucial role in neutrophil migration [17–20]. Several studies have reported elevated levels of LTB<sub>4</sub> also in bronchoalveolar lavage (BAL) fluid from patients with ARDS or other inflammatory lung diseases such as COPD [21–24]. Moreover, the evaluation of LTB<sub>4</sub> and IL-8 has been described as useful prognostic indices in patients with early phase ARDS after admission to the intensive care unit [23,25]. With regard to trauma patients, the data is sparse.

Previously, we have shown that high systemic levels of LTB<sub>4</sub> indicated patients at risk for imminent lung complications after polytrauma [26]. But it may be assumed that IL-8 as well as LTB<sub>4</sub> might be directly involved in post-traumatic pulmonary inflammatory processes by attracting PMN to the lung [13,27]. This is considered as one of the main factors of interstitial inflammation in the lungs, extending the transport distance for gas exchange. Recently, *in vivo* imaging has revealed that LTB<sub>4</sub> is required for neutrophil swarming in the extravascular space of a damaged tissue [28]. Moreover, down-regulating the surface expression of CXCR1/2 and BLT1 (receptors for IL-8 and LTB<sub>4</sub>), significantly blocked IL-8-induced and LTB<sub>4</sub>-induced neutrophil migration *in vitro* and *in vivo* as demonstrated in mice by intravital microscopy in a model of airway inflammation [29].

The benefit of early identification of lung injury and pulmonary complications in trauma patients is undoubted, but the prognostic value and relevance of LTB<sub>4</sub> is not fully elucidated, yet. Subsequently, the aim of the present study was evaluating the capability of LTB<sub>4</sub> to indicate these processes in a long-term large animal polytrauma model that simulates a clinically relevant scenario of polytrauma and subsequent harmful pulmonary changes.

## 2. Material and methods

### 2.1. Animals

All experiments were conducted in accordance with the federal German law regarding the protection of animals and were approved by the responsible government authority ("Landesamt für Natur, Umwelt und Verbraucherschutz": LANUV-NRW, Germany: AZ TV-Nr.: 84-02.04.2014. A265). Institutional Guidelines and the criteria in "Guide for the Care and Use of Laboratory Animals" (Eighth Edition The National Academies Press, 2011) were followed [30], and the study was performed in accordance with the use and care of animals as reported in consent with the ARRIVE guidelines [31]). Animal experiments were performed at the Institute for Laboratory Animal Science & Experimental Surgery, RWTH Aachen University, Germany.

Eighteen male German landrace pigs (*Sus scrofa*; 3 months old, 30 ± 5 kg) from a disease-free barrier breeding facility were included in this study. Before experimentation, the animals were fasted for overnight having a free access to water. All animals underwent initial examination by a veterinarian before experimentation and were housed in ventilated rooms and allowed to acclimatize to their surroundings for a minimum of 7 days before surgery.

### 2.2. Experimental model

Twelve animals underwent polytrauma (PT) with standardized femur fracture, unilateral blunt chest injury, liver laceration, hemorrhagic shock (40 mm Hg, 90 min), subsequent resuscitation and surgical fracture fixation. Six non-traumatized animals receiving anesthesia, laparotomy, preparation of arterial, venous and urinary lines served as controls (sham).

Animals were pre-medicated with an intramuscular application of azaperone (Stresni™, Janssen, Germany) in a dose of 1 ml per 15 kg. Anesthesia was induced with an intravenous injection of propofol (3 mg/kg) and orotracheal intubation followed (7.5 ch tube, Hi-Lo Lanz™). During the study period of 72 h, anesthesia was maintained with intravenous injection of propofol. The animals were ventilated on volume control mode (Draeger, Evita, Lübeck, Germany) with room air at a tidal volume setting of 6–8 ml/kg, positive end expiratory pressure (PEEP) of 8 mm Hg (plateau pressure < 28 mm Hg), and pCO<sub>2</sub> of 35 – 45 mm Hg. Catheters were aseptically inserted in the external jugular vein for administration of fluids, anesthesia and continuous monitoring of central venous pressure (CVP, central venous catheter 4-Lumen Catheter, 8.5 Fr., ArrowCatheter, Teleflex Medical, Germany), into the right femoral vein to induce hemorrhage (3-Lumen hemodialysis, 12.0 Fr., ArrowCatheter, Teleflex Medical, Germany) and into the femoral artery for blood pressure monitoring (4.0 Fr. arterial line catheter, Vygon, Germany). A urinary catheter was placed in the bladder (12.0 Fr, Cystofix, Braun, Melsungen, Germany). Crystalloid fluid (Sterofundin ISO®) was used for continuous fluid management (2 ml kg/BW/h). The baseline measurements were acquired after instrumentation and an equilibration period.

The PT was induced as described previously [32]. Initially, prior induction of trauma the inspiratory O<sub>2</sub> (FiO<sub>2</sub>) was defined at 21% and the fluid administration was reduced to 10 ml/h. At this phase, the animals were not prevented from hypothermia for the following hemorrhagic shock period mimicking the pre-clinical scenario. Shortly described, after placing the animal on the right side, femur fracture was induced with a bolt shot on the right hind leg (Blitz-Kerner, turbocut JOBB GmbH, Germany, 9×17, Dynamit Nobel AG, Troisdorf, Germany). Placed back in the dorsal position, blunt thoracic trauma with a bolt shot on the right dorsal lower thorax was induced. Thereafter, a midline-laparotomy and uncontrolled bleeding for 30 s after crosswise incision of the caudal liver lobe (4.5×4.5 cm). Using five sterile gauze-compresses (10×10 cm) the liver was packed. Pressure-controlled hemorrhagic shock using exsanguination from right femoral artery until a

mean arterial blood pressure (MAP) of  $40 \pm 5$  mm Hg was reached and maintained for 90 mins.

Resuscitation starting after hemorrhagic shock by adjusting  $\text{FiO}_2$  to baseline values, and re-infusing the withdrawn blood and additional fluids (Sterofundin ISO®; 2 ml kg/BW/h). Rewarming was performed using forced-air warming systems until normothermia ( $38.7\text{--}39.8^\circ\text{C}$ ).

Thereafter, clinical treatment of open femur fracture was performed according to established trauma guidelines. The intensive care and complications management followed the standardized clinical protocols according to the latest recommendations of the European Resuscitation Council and Advanced Trauma Life Support (ATLS)[33,34]. Antibiotics (Ceftriaxon® 2 g) were given before surgery and after every 24 h until sacrifice. After the observational period the animals were euthanized, and at baseline as well as at 72 h bronchoalveolar lavage (BAL) was collected. After trauma (a.tr), 2 h, 4 h, 24 h and 72 h later blood was collected. At 72 h lung tissue was harvested.

### 2.3. Histological examination of lung injury

After lung perfusion with 0.9% NaCl solution, one piece of both lobes of the lung was removed for the RNA isolation. The remaining lung was flushed and filled with 4% formalin for overnight fixation. After paraffin embedding, lung samples were sectioned to 2–3  $\mu\text{m}$  and stained with hematoxylin-eosin (HE). Determination of histological damage was performed by an independent examiner (K.K.) from the Institute of Veterinary Pathology, Justus Liebig University Giessen, who evaluated the HE-stained lung sections for desquamation, dystelettasis/atelectasis, emphysema, congestion, interstitial thickness/infiltration and bronchial exsudate [35]. Each parameter was assessed according to the degree of severity: 0 = not observed, 1 = mild, 2 = moderate and 3 = marked. Both lung lobes are included in the evaluation. The results are represented as the mean of all scores.

### 2.4. Detection of polymorphonuclear leukocytes

Lung infiltration with PMN was evaluated by the chloroacetate esterase staining (CAE, 4% pararosanilin, 4% sodium nitrite and naphthol solution) for 30 min at room temperature (RT) according to the manufacturer's instructions (Naphthol AS-D Chloroacetate Specific Esterase Kit, Sigma). Sections were counterstained with hematoxylin. Polymorphonuclear leukocytes were determined by counting the number of CAE positive cells in a total of 25 high power (400x) fields per lung section per pig in a blinded manner as described before [36]. Both lung lobes are included in the evaluation. Data from each tissue section were pooled to determine means.

### 2.5. Blood processing and analysis

Blood samples were obtained after trauma (a.tr), 2 h, 4 h, 24 h and 72 h in prechilled ethylenediaminetetraacetid acid tubes (BD Vacutainer, Becton Dickinson Diagnostics, Aalst, Belgium) and kept on ice. Blood was centrifuged at  $2000 \times g$  for 15 min at  $4^\circ\text{C}$ . The supernatant was stored at  $-80^\circ\text{C}$  until the batch sample analysis for LTB4 concentrations using the Leukotriene B4 ELISA Kit from abcam according to manufacturer's instructions (Cambridge, UK). Blinded specimens were used for duplicate measurement of LTB4 levels. LTB4 was determined by the laboratory of the Department of Trauma, Hand and Reconstructive Surgery at the University Hospital of the Goethe University Frankfurt.

### 2.6. LTB4 in the bronchoalveolar lavage fluid

At baseline and at 72 h a cannula was located into the trachea and the lungs were lavaged with 5 ml of saline 4 times (total: 20 ml). The BAL fluid was centrifuged at 1100 g for 10 min at  $4^\circ\text{C}$ . The supernatant was removed and stored at  $-80^\circ\text{C}$  until the batch sample analysis for

LTB4 concentrations using the Leukotriene B4 ELISA Kit according to manufacturer's instructions (abcam) as described above.

### 2.7. Ribonucleic acid (RNA) isolation, semi-quantitative reverse-transcription-polymerase chain reaction (RT-PCR)

Total RNA of snap-frozen lung samples was isolated using the RNeasy-system (Qiagen, Hilden, Germany) according to the manufacturer's instructions and as described previously [37]. Both lung lobes are included in the evaluation. For DNA removal the RNase-Free DNase Set was applied according to the manufacturer's instructions (Qiagen). Both, quality and amount of the isolated RNA were determined photometrically using the NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE, USA). RNA was stored immediately after isolation at  $-80^\circ\text{C}$ . For the qRT-PCR, 100 ng of total lung RNA was reversely transcribed using the Affinity script QPCR-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. qRT-PCR was carried out on a Stratagene MX3005p QPCR system (Stratagene) to determine the mRNA expression of IL-8 and as reference gene GAPDH using gene-specific primers for pig IL-8, IL-1 $\beta$ , IL-10 and IL-18 (IL-8: NM\_213867, UniGene#: Ssc.658, Cat#: PPS00237A; IL-1 $\beta$ : NM\_001005149, UniGene#: Ssc.28829, Cat#: PPS00015A; IL-10: NM\_214041, UniGene#: Ssc.148, Cat#: PPS00445B and IL-18: NM\_213997, UniGene#: Ssc.20, Cat#: PPS00399A) and pig GAPDH (NM\_001206359, UniGene#: Ssc.16135, Cat#: PPS00192A) purchased from SABiosciences (SuperArray, Frederick, MD, USA). Sequences of these primers are not available. PCR reaction was set up with 1x RT2 SYBR Green/Rox qPCR Master mix (SABiosciences) in a 25  $\mu\text{l}$  volume according to manufacturer's instructions. A two-step amplification protocol consisting of initial denaturation at  $95^\circ\text{C}$  for 10 min followed by 40 cycles with 15 s denaturation at  $95^\circ\text{C}$  and 60 s annealing/extension at  $60^\circ\text{C}$  was chosen. In order to control the specificity of amplification products a melting-curve analysis was applied. The relative gene expression of IL-8 was calculated using the comparative threshold-cycle (CT) method ( $2^{-\Delta\Delta\text{CT}}$  method) as described previously by Schmittgen and Livak [38]. Briefly, the amount of target mRNA in each sample was normalized to the amount of GAPDH mRNA, to give  $\Delta\text{CT}$  and then to a calibrator consisting of samples obtained from the sham\_ctrl group. The relative mRNA expression of target genes is presented as fold increase calculated in relation to sham\_ctrl after normalization to GAPDH in percentage (%).

### 2.8. Statistical analysis

Differences between the groups were compared using the Mann-Whitney-U test. Changes in target gene expression were analyzed by Wilcoxon matched-pair analysis followed by Bonferroni correction. A p value of less than 0.05 was considered significant. Data are given as mean  $\pm$  standard error of the mean (sem). All statistical analyses were performed employing GraphPad Prism 5 (Graphpad Software, Inc., San Diego, CA).

## 3. Results

### 3.1. Systemic and local LTB4 levels

The systemic baseline levels of LTB4 were comparable between the sham and PT group and were at the detection limit (Fig. 1). At 4 h after trauma, plasma LTB4 levels increased markedly in both groups, again to comparable levels (sham:  $380.10 \pm 64.17$  vs. PT:  $341.10 \pm 70.62$  pg/ml, respectively, Fig. 1). While the levels of LTB4 in the sham group declined continuously to the baseline after 72 h, plasma LTB4 levels increased further significantly in the PT group as compared to sham (PT:  $405.50 \pm 66.97$  vs. sham:  $209.40 \pm 56.27$  pg/ml, respectively,  $p < 0.05$ , Fig. 1).

The local LTB4 concentration in the BAL fluid was comparably low

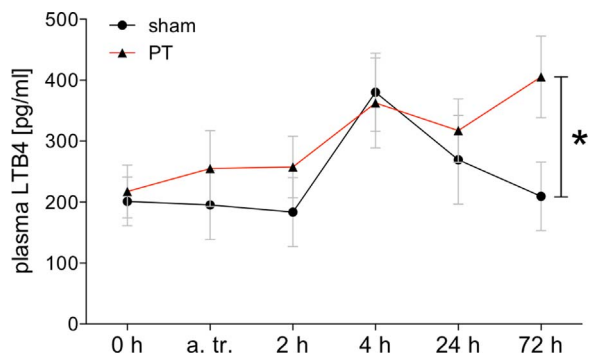


Fig. 1. Time course of mean concentrations of Leukotriene (LT) B 4 levels in plasma samples (pg/ml). Blood samples were obtained before (0 h) trauma, after trauma (a.tr), 2 h, 4 h, 24 h and 72 h; sham: control group, polytrauma (PT): animals undergoing PT. The results are represented as the mean  $\pm$  sem, \* $p$  < 0.05 vs. sham at 72 h.

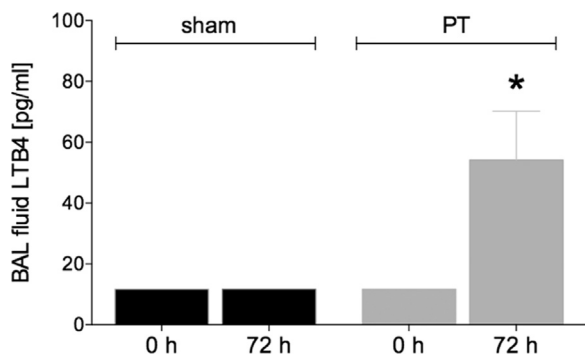


Fig. 2. Summary of mean concentrations of Leukotriene (LT)B4 levels (pg/ml) in bronchoalveolar lavage (BAL) fluid. BAL fluid was collected before (0 h) trauma and 72 h after trauma; sham: control group, polytrauma (PT): animals undergoing PT. The results are represented as the mean  $\pm$  sem, \* $p$  < 0.05 vs. all.

in both, sham and PT group before trauma (Fig. 2). However, at 72 h after trauma, LTB4 levels in BAL fluid increased significantly in the PT group ( $54.12 \pm 16.07$  pg/ml) compared to sham group, where LTB4 levels were at the detection limit of  $11.70$  pg/ml ( $p$  < 0.05, Fig. 2).

### 3.2. Histopathological changes in lung tissue after trauma

The lung injury score was significantly enhanced in the PT group compared with the sham group (PT:  $1.45 \pm 0.13$  vs. sham:  $0.37 \pm 0.18$ , respectively,  $p$  < 0.05, Fig. 3). In general, the sections from PT animals (Fig. 3) revealed areas of increased interstitial thickness compared to sham group. Similarly, dyslectasis/atelectasis and emphysema were more often observed in PT compared with sham (Fig. 3).

### 3.3. Local pro-inflammatory changes after trauma - IL-8 levels and lung neutrophil accumulation

The semi-quantitative real-time PCR showed a significant increase of IL-8 expression at 72 h in lung samples after trauma as compared to the sham group ( $216.10 \pm 53.58$  vs.  $83.31 \pm 15.01$ ,  $p$  < 0.05, Fig. 4A). IL-18 and IL-10 showed a trend to increased levels after trauma, however this difference was not significant (Fig. 4B and C). Gene expression of IL-1 $\beta$  did not change markedly.

Lung neutrophil infiltration increased to  $2.38 \pm 0.22$  cells per high power field at 72 h after trauma as compared to sham controls ( $0.98 \pm 0.08$  cells per high power field,  $p$  < 0.05, Fig. 5).

## 4. Discussion

Thoracic trauma can crucially deteriorate the outcome of trauma

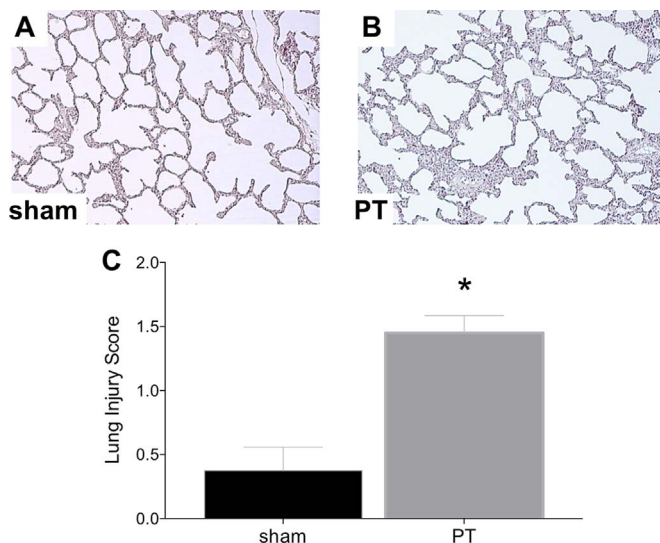


Fig. 3. Histological evaluation of lung damage after 72 h after trauma. Sham operated animals underwent the same surgical procedures but polytrauma (PT) was not carried out. Representative hematoxylin and eosin stained lung sections from sham (A) and PT (B) groups are shown. Lung tissue was evaluated for desquamation, dyslectasis/atelectasis, emphysema, congestion, interstitial thickness/infiltration and bronchial exudate. Each parameter was assessed according to the degree of severity: 0 = not observed, 1 = mild, 2 = moderate and 3 = marked. The results are represented as the mean  $\pm$  sem of all scores (Lung Injury Score, C), \* $p$  < 0.05 vs. sham.

patients [39,40]. Pulmonary complications including pneumonia, ARDS and other can detrimentally influence the outcome after trauma [1,5,41–43]. Due to the lack of reliable clinical biomarkers for predicting these pulmonary complications after trauma, the current treatment strategies are based on clinical preventive procedures such as protective ventilation and kinetic therapy [5,6,44,45]. Therefore, pulmonary complications often remain non-specific and subsequently under-diagnosed. In order to improve the clinical prediction of pulmonary complications in trauma patients, both, understanding the underlying pathophysiology and the biology of the repair mechanisms as well as clinically reliable predictive biomarkers are necessary [46]. Moreover, improvement and development of translational trauma models are essential [47]. In the present study, we have evaluated the reliability of the clinically described biomarker LTB4 to indicate the risk for late lung injury and pulmonary complications in the long-term porcine severe polytrauma model.

The development of pulmonary complications after trauma has been closely associated with an excessive systemic and local immune reaction [13,48,49]. This complex immune response to trauma is characterized by the release of several inflammatory mediators including IL-8, IL-1 $\beta$ , IL-10 and IL-18 but also neutrophil migration into the lung [13,48–53]. Persistent accumulation of neutrophils in the lung has been linked to pulmonary damage and poor survival via an interstitial inflammation, increase of interstitial space and limitation of the oxygen transport [9–12]. In line with these reports, in the present study, we can show for the first time in a porcine animal model, that the trauma-induced lung injury (depicted by increased LIS, Fig. 3) is associated with increased expression of proinflammatory IL-8 but also enhanced infiltration of the lungs with neutrophils (Figs. 4 and 5). As rationale for an increased neutrophil accumulation via IL-8, it has been demonstrated that IL-8 might be directly involved in post-traumatic pulmonary inflammatory processes by attracting PMN to the lung [13]. More recently, it has been shown that down-regulating the surface expression of the IL-8 receptors CXCR1/2 significantly blocked IL-8-induced neutrophil migration *in vitro* and *in vivo* in a model of pulmonary inflammation [29]. Other biomarkers of inflammatory pulmonary complications after trauma as IL-1 $\beta$ , IL-10 and IL-18 did not show significant changes (Fig. 4). However, IL-18 demonstrated a clear

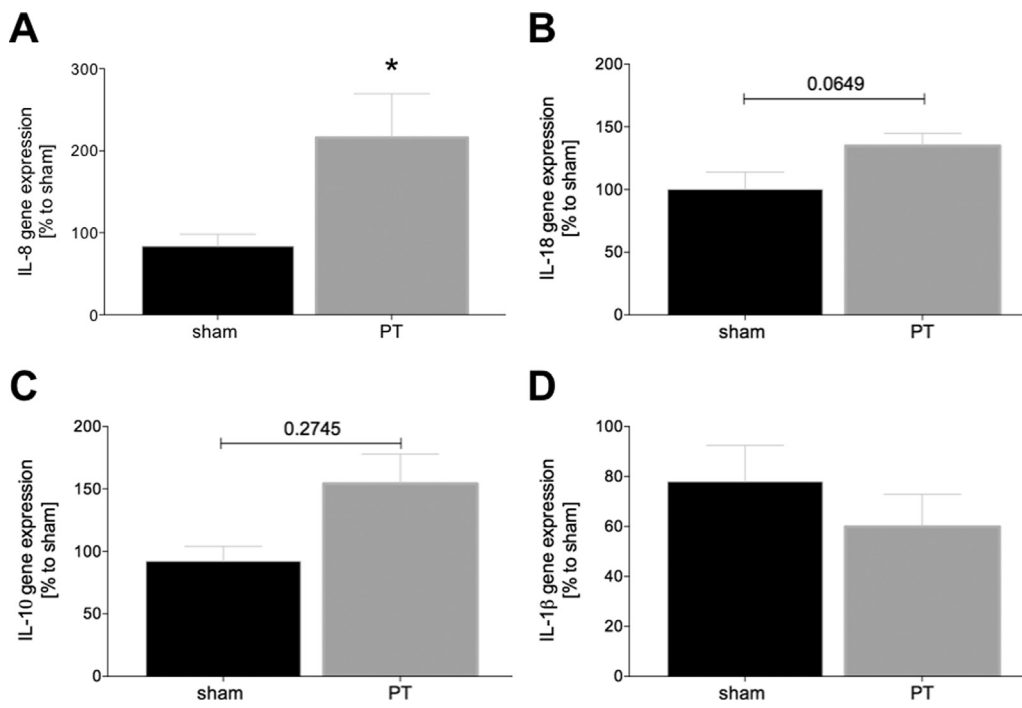


Fig. 4. Lung interleukin (IL)-8 (A) IL-18 (B), IL-10 (C) and IL-1β (D) gene expression at 72 h after trauma. Sham operated animals underwent the same surgical procedures but polytrauma (PT) was not carried out. After normalization to GAPDH expression, gene expression was measured as fold increase compared to the corresponding sham operated ctrl group. The results are represented as the mean ± sem of all scores, \*p < 0.05 vs. sham.

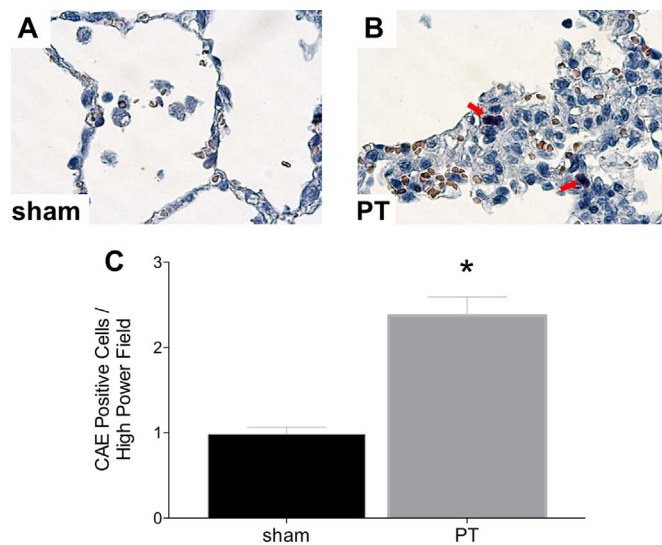


Fig. 5. Lung neutrophil infiltration at 72 h after trauma. Sham operated animals underwent the same surgical procedures but polytrauma (PT) was not carried out. Neutrophils in lung sections were identified by chloroacetate esterase (CAE) cytochemistry. Representative CAE stained lung sections from sham (A) and PT (B) groups are shown. CAE positive cells were counted in 25 high power fields. The results are given as the mean ± sem of CAE positive cells / high power field (C), \*p < 0.05 vs. sham.

trend to increased levels after trauma, indicating at findings from others that have represented the importance of IL-18 as a promising biomarkers for predicting morbidity and mortality of ARDS [52]. Similar findings were reported by Dolinay et al. (2012), showing that patients with trauma- or sepsis-induced ARDS had increased IL-18 levels, which correlated with increased in-hospital mortality [54]. Additional studies to validate these findings are needed.

Moreover, both, local and systemic levels of LTB<sub>4</sub>, one of the strongest chemoattractive agents for neutrophils have been associated with the onset of pulmonary inflammation in the present study [17–19]. LTB<sub>4</sub> levels have been shown to correlate with the number of neutrophils recovered from the BAL fluid of patients with ARDS [55]. Kalsotra et al. have shown that in rats suffering from brain contusion

the amount of inflammatory cells in the lung as well as the intrapulmonary production of LTB<sub>4</sub> have been increased and blamed for pulmonary complications after blunt brain injuries [56]. In line with these findings, lately increased LTB<sub>4</sub> levels in the BAL fluid were associated with enhanced pulmonary infiltration with neutrophils as well as IL-8 expression. Previously, a close association of increased IL-8 and LTB<sub>4</sub> levels has been proposed as useful prognostic indices in patients with early phase ARDS after admission to the intensive care unit [23,25]. Nonetheless, the role of LTB<sub>4</sub> in trauma has not been fully elucidated. Auner et al. have reported a close correlation of early increased circulating levels of LTB<sub>4</sub> with the incidence of pulmonary complications in polytrauma patients [26]. According to this clinical study, not only the local BAL fluid as reported above, but also the plasma levels of LTB<sub>4</sub> have been increased three days after trauma in the established porcine model. This data underlines that plasma LTB<sub>4</sub> levels may be useful for the diagnosis of pulmonary complications after polytrauma. Summarized, locally increased LTB<sub>4</sub> levels in BAL fluid may promote pulmonary neutrophil immigration enhancing the harmful proinflammatory processes underlying pulmonary complications. On the other hand, increased plasma LTB<sub>4</sub> levels after 72 h may be used as indicators for the above mentioned deteriorating pulmonary changes after polytrauma.

Interestingly, a systemic LTB<sub>4</sub> peak emerging four hours after trauma has been detected in both, sham and polytrauma group. However, we assume that this early peak is caused by the intubation and ventilation (barotrauma) procedures.

Our study has several limitations, maybe one of most relevant constitutes the assay for the determination of LTB<sub>4</sub>. Lipid mediators employ various methods for their determination including liquid chromatography-ultraviolet-tandem mass spectrometry (LCUV-MS/MS), gas chromatography-mass spectrometry (GC-MS), computer-based automated systems equipped with databases and novel searching algorithms, and ELISA. In the underlying study, we have chosen the ELISA technique, though the LC-MS/MS is a very powerful and more sensitive and specific technique for highly multiplexed protein quantification and differentiation [57]. ELISA certainly has its specificity limitations due to used detection antibodies. And though both methods are applicable for e.g. LTB<sub>4</sub> determination, the current field of highly interesting oxidative lipidomics mostly rely on LC-MS/MS techniques

[58]. Therefore, for future studies, samples should be evaluated or analyzed by the LC-MS/MS technique, since MS/MS with LC is more reliable and sensitive method to identify the quantity of LTB4.

Furthermore, it is known that post-traumatic response includes not only the inflammatory components but also changes in lipid mediators. Therefore, it is important to gain more detailed information on the involved molecules and mechanisms controlling the post-injury response and its resolution. Several additional lipid mediators as resolvins, lipoxins, maresins or protectins such as prostaglandin F2alpha, resolving E1, D5, and 17R-protectin D1 play an important role during resolution of inflammation and regeneration, and have not been addressed in our study [58–60]. Recent studies indicate at their critical importance not only in health and diseases, but in those conditions that are related to inflammation and resolution occurring after trauma as well [60,61]. Therefore, analyses of additional lipid mediators in the setting of trauma and post-traumatic complications is a daunting but necessary task for future studies.

Our data demonstrate 1. a clinically relevant severe trauma model showing lung injury and inflammatory changes, which are typical for pulmonary complications, and 2. a significant secondary increase of LTB4 in this porcine long-term model, which is a clinically described early biomarker for patients at risk for pulmonary complications after severe trauma. It may be assumed that LTB4 is part of the proinflammatory response after lung contusion in polytrauma, disturbing gas exchange and increasing the risk for subsequent infections. These findings could be used to further monitor and improve the use of preventive therapies for post-traumatic pulmonary complications in future prospective studies.

## 5. Conclusions

LTB4 rises locally and systemically with increasing lung injury after trauma in the porcine polytrauma model.

Local inflammatory markers (IL-8 expression and PMN infiltration) are increased after trauma in the porcine polytrauma model.

LTB4 seems to be a potential biomarker to indicate lung injuries and on-going inflammatory changes after polytrauma not only in human patients but in the porcine model as well.

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## Disclosure of conflicts of interest

The authors state that they have no conflict of interest.

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