Early local neutralization of CC16 in sepsis-induced ALI following blunt chest trauma leads to delayed mortality without benefitting overall survival

JAN TILMANN VOLLRATH[1], PHILIPP STOERMANN[1], NILS BECKER[1,2],
SEBASTIAN WUTZLER[3], FRANK HILDEBRAND[4], INGO MARZI[1] and BORNA RELJA[1,2]

[1]Department of Trauma, Hand and Reconstructive Surgery, Goethe University, D-60590 Frankfurt;
[2]Department of Radiology and Nuclear Medicine, Experimental Radiology, Otto-von-Guericke University,
D-39112 Magdeburg; [3]Department of Trauma, Hand and Orthopedic Surgery, Helios Horst Schmidt Clinic,
D-65199 Wiesbaden; [4]Department of Trauma Surgery, RWTH University, D-52062 Aachen, Germany

Abstract. Blunt thoracic trauma (TxT) is a common injury pattern in polytraumatized patients. When combined with a secondary trigger, TxT often results in acute lung injury (ALI), which negatively affects outcomes. Recent findings suggest that ALI is caused by both local and systemic inflammatory reactions. Club cell protein (CC)16 is an anti-inflammatory peptide associated with lung injury following TxT. Recently, the anti-inflammatory properties of endogenous CC16 in a murine model of TxT with subsequent cecal-ligation and puncture (CLP) as the secondary hit were demonstrated by our group. The present study aimed to determine whether CC16 neutralization improves survival following 'double-hit'-induced ALI. For this purpose, a total of 120 C57BL/6N mice were subjected to TxT, followed by CLP after 24 h. Sham-operated animals underwent anesthesia without the induction of TxT + CLP. CC16 neutralization was performed by providing a CC16 antibody intratracheally following TxT (early) or following CLP (late). Survival was assessed in 48 animals for 6 days after CLP. Sacrifice was performed 6 or 24 h post-CLP to evaluate the anti-inflammatory effect of CC16. The results revealed that CC16 neutralization enhanced pro-inflammatory CXCL1 levels, thereby confirming the anti-inflammatory characteristics of CC16 in this model. Early CC16 neutralization immediately following TxT significantly prolonged survival within 60 h; however, the survival rate did not change until 6 days post-trauma. Late CC16 neutralization did not provide any survival benefits. On the whole, the present study demon-strated that neutralizing CC16 confirmed its anti-inflammatory potential in this double-hit ALI model. Early CC16 neutralization prolonged survival within 60 h; however, no survival benefits were observed after 6 days post-CLP in any group.

Introduction

Blunt chest (thoracic) trauma (TxT) is common in polytraumatized patients, and 20-25% of deaths in severely injured patients treated in emergency departments are caused by chest injuries (1). Typical complications in these patients include disseminated intravascular coagulation, pneumonia, acute lung injury (ALI), or its more severe form, acute respiratory distress syndrome (ARDS) (2,3). While the role of mechanical damage in the pathogenesis of post-traumatic complications associated with lung injury is undisputed, current research indicates that lung damage is driven by local and systemic inflammatory reactions (4).

Hoth *et al* demonstrated increased levels of pro-inflammatory CXCL1 in bronchoalveolar lavage fluid at 3 h post-injury in a murine model of pulmonary contusion (5). Furthermore, the recruitment of neutrophils to injured lung tissue, which is primarily responsible for pulmonary dysfunction following lung contusion, has been shown to be dependent on the expression of CXCL1, among other pro-inflammatory factors (6). Recently, the authors demonstrated that a 'double-hit' model of TxT, followed by cecal ligation and puncture (CLP) as the second hit, which was also applied in the underlying study, increased the expression of pro-inflammatory chemokines and cytokines, including CXCL1 (7). Thus, in line with the two-hit hypothesis, a traumatic insult as the first hit, followed by secondary surgeries or complications, may lead to detrimental outcomes. Consistent with the findings of other researchers, it was found that isolated experimental lung trauma induced a profound inflammatory reaction; however, it was insufficient to establish the ongoing pathological pulmonary changes associated with ALI (7,8). However, secondary CLP following TxT, as applied herein, reflected the human etiology of indirect lung damage ending in ALI (7,9-11). Another murine 'double-hit'

Correspondence to: Dr Borna Relja, Department of Radiology and Nuclear Medicine, Experimental Radiology, Otto-von-Guericke University, Leipziger Strasse 44, D-39112 Magdeburg, Germany
E-mail: borna.relja@med.ovgu.de

model that includes CLP, followed by inducing pneumonia via *Pseudomonas aeruginosa* 4-7 days later, significantly improved survival when pneumonia was induced after 7 days. Improved survival was associated with the restoration of interferon (IFN)-γ by stimulated splenocytes (12). These findings underline the importance of timing of the second hit, and thus also for therapeutic interventions. IFN-γ plays a key role in the regulation of both innate and acquired antimicrobial immunity by inter alia, stimulating macrophage functions, such as phagocytosis, respiratory burst activity, antigen presentation and cytokine secretion (13,14). The functional significance of IFN-γ in antimicrobial defense has been demonstrated by the increased susceptibilities of IFN-γ^{-/-} and IFN-γ-R^{-/-} mice to a variety of infections (15). Furthermore, IFN-γ seemed to facilitate systemic inflammation during CLP-induced abdominal sepsis in mice (16).

Club cell protein (CC)16 is an anti-inflammatory protein derived from epithelial club cells in the lungs (17,18). Its systemic concentrations are associated with the extent of lung contusion and with pulmonary complications in traumatized patients, which underscores its biomarker characteristics (19,20). Although its exact functions *in vivo* remain unclear, CC16 plays an important protective role in the respiratory tract against oxidative stress and inflammatory responses due to its anti-inflammatory properties (21). With regard to the two-hit hypothesis, the inflammatory response following trauma is essential for host defense; however, it can cause further tissue damage if triggered by a secondary stimulus (22,23). Since reducing inflammation attenuates pathological injury and the survival of mice with ALI (24), as a pulmonary anti-inflammatory protein, CC16 may play an important regulatory role during the development ALI following trauma. However, CC16 may also play a pathological role in immunoparalysis with concomitant post-traumatic infectious complications (25). This role was addressed in a recent study by the authors, which demonstrated the anti-inflammatory effects of CC16 on CLP-induced ALI following TxT in mice (9). While the early intrapulmonary inhibition of CC16 following TxT reduced lung damage, the later inhibition of CC16 deteriorated lung damage after 24 h. However, it remains questionable whether the latter may have been followed by reduced lung damage in a prolonged observational period. Since this question and the one regarding the pathophysiological relevance of these observations to survival remain unanswered, the present study evaluated whether the local neutralization of CC16 affects mortality after sepsis-induced ALI following blunt chest trauma in a murine model.

Materials and methods

Animals and experimental model. All experiments were conducted in accordance with German federal laws regarding the protection of animals. The experiments were performed at Goethe University Hospital in Frankfurt, Germany. The present study was approved by the responsible government authority, the Veterinary Department of the Regional Council in Darmstadt, Germany (Regierungspräsidium Darmstadt; AZ: FK 1068), and the study was performed under ARRIVE guidelines (26).

For the survival analysis, 48 male CL57BL/6N mice (weighing 25±5 g at 6-8 weeks of age) were included, and 72 male CL57BL/6N mice (6-8 weeks old, weighing 25±5 g) were included for experiments with sacrifice after 6 or 24 h post-CLP. All animals were purchased from Janvier Labs. The animals were provided with free access to water and food *ad libidum* before and after the experimental procedures. At 30 min prior to the experiment, buprenorphine (Indivior Eu Ltd., 0.1 mg/kg body weight) was administered to all mice subcutaneously. This process was repeated every 12 h. For the survival analysis, buprenorphine (0.1 mg/kg body weight) was administered every 6 h for 48 h after CLP. From 48-96 h post-CLP, buprenorphine (0.1 mg/kg body weight) was administered every 12 h. Drinking water provided to the mice was enriched with metamizole (Ratiopharm) to maintain adequate analgesia during the experiment.

For TxT, mice underwent general mask anesthesia with 3% isoflurane (Baxter Deutschland GmbH), according to partly-modified standardized protocols described previously (7,9,10,27). Briefly, the mice were placed in the supine position under a cylinder. The cylinder was separated by a Mylar polyester film with a thickness of 0.05 mm [DuPont de Nemours (Deutschland) GmbH] and placed 2.5 cm above the sternum. Delivering compressed air to the upper part of the cylinder ruptured the membrane and led to a standardized pressure wave. This resulted in standardized, bilateral, blunt chest trauma. According to their group allocation, mice received an intratracheal application of either a CC16 antibody or a control antibody immediately following TxT. Uteroglobin/SCGB1A1 (CC16 Ab; LS Biosciences) or IgG control (IgG) antibody (10 µg/ml, R&D Systems) were administered either immediately following the induction of thoracic trauma (early application) or following CLP (late application). For antibody administration, mice were placed in the supine position, and the tongue was kept aside. A buttoned cannula was placed at the beginning of the trachea, and 50 µl were carefully administered. Mice were then kept in the reverse Trendelenburg position for 30 sec to ensure proper antibody distribution inside the lungs. After 24 h, mice underwent intraperitoneal anesthesia with ketamine (100 mg/kg body weight, Zoetis Deutschland GmbH) and xylazine (10 mg/kg body weight, Bayer Leverkusen), and depending on group allocation, the following procedures were performed. Either median laparotomy and CLP were performed as previously described (7,28) or median laparotomy and eversion of the caecum without any further manipulation was performed. Briefly, the abdominal cavity was opened by midline laparotomy (approximately 1 cm), and the cecum was carefully exposed. Subsequently, 7 mm of the distal caecum were ligated using Premilene 5-0 suture (B. Braun Melsungen AG) and perforated with a single through-and-through puncture using a 25G cannula (BD Biosciences).

For survival experiments, a 18G cannula (BD Biosciences) was used. After this procedure, a small amount of feces was extruded, and the cecum was restored to the abdominal cavity. Abdominal closure was performed by a two-layer suture. According to group allocation, mice received an intratracheal application of either a CC16 antibody or control IgG antibody, as described above, immediately following thoracic trauma or following CLP. The sham-operated (sham) control group
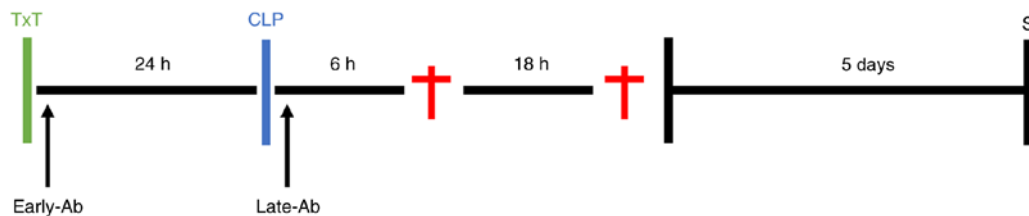
Figure 1. Experimental design. Mice underwent TxT and CLP and were assigned to the ALI group. According to group allocation, a CC16 antibody (Ab) or IgG antibody was administered immediately after either TxT (early Ab) or CLP (late Ab). Animals were then euthanized for sampling (n=6) after 6 or 24 h following CLP. Survival (S) was assessed for 6 days following CLP (n=8). TxT, thoracic trauma; CLP, cecal ligation and puncture; ALI, acute lung injury.

underwent the identical anesthesia procedures described above, but without TxT, as well as with median laparotomy and eversion of the caecum, but without CLP. Immediately following CLP, 1 ml of prewarmed G5% solution (B. Braun Melsungen AG) was administered intraperitoneally. Depending on group allocation, either 6 or 24 h post-CLP, mice received anesthesia as described above and sacrifice was performed, or mice were observed for 6 days after CLP and euthanized via blood withdrawal by a puncture of the heart under general anesthesia with 3% isoflurane (Baxter Deutschland GmbH). During the survival analysis, mice were euthanized when they reached one of the following conditions: Permanently closed eyes, lateral position, severe dyspnea with mouth breathing, cyanosis, body weight loss >20% or apathy. An overview of the experimental design is presented in Fig. 1.

Group allocation. The animals were randomly allocated to experimental groups, as described below. For survival analyses, all mice, apart from those in the sham group received TxT followed by CLP (ALI) after 24 h. In total, 8 mice received an intratracheal application of CC16 antibody, and 8 mice received an intratracheal application of the control antibody (IgG), as described above, immediately following TxT. A total of 16 mice received an intratracheal an application of a CC16 or control antibody (i.e., 8 mice received CC16 Ab, and 8 mice received IgG) immediately following CLP. In total, 8 mice were allocated to the control group without any application of antibodies, while 8 mice were allocated to the sham group, which received median laparotomy and eversion of the caecum, but not CLP or TxT. The same group allocation was used for the groups of mice sacrificed at 6 and 24 h (n=6 in each group). Mice received a CC16 or control antibody (IgG) immediately after TxT (early Ab) and were sacrificed after 6 h (6 h_early Ab) or 24 h (24 h_early Ab) after CLP or received a CC16 or control antibody (IgG) immediately after CLP (late Ab), followed by sacrifice after 6 h (6 h_late Ab) or 24 h (24 h_late Ab).

Systemic IFN-γ determination. The caval vein was punctured by a heparinized syringe for blood withdrawal at 6 or 24 h after CLP. Subsequently, plasma was isolated from blood samples by centrifugation (1,164 x g for 15 min at 4°C) and stored at -80°C for further IFN-γ measurements. IFN-γ was measured with a BD CBA Mouse Inflammation kit (BD Biosciences) according to the manufacturer's instructions. Briefly, 50 µl of capture beads were added to polystyrene FACS tubes (BD Pharmingen™) to 50 µl serum. Subsequently, 50 µl of Mouse Inflammation PE Detection

Reagent were added followed by incubating for 2 h at room temperature. Subsequently, samples were washed with 1 ml of the wash buffer and centrifuged at 200 x g for 5 min at room temperature. The supernatant was discarded, and the pellets were resuspended in 300 µl of the wash buffer. Analyses were performed using a BD FACSCanto 2™ and FCAP Array™ Software (BD Biosciences). Cytometric Bead Array (CBA) was performed as previously described (29).

Quantification of CXCL1 expression levels in the lungs. Following blood withdrawal, the animals were perfused with 20 ml PBS via the inferior caval vein. The lungs were then removed and snap-frozen in liquid nitrogen. Lung tissue was homogenized in a protein lysis buffer at 4°C, followed by centrifugation for 30 min at 4°C and 20,000 x g. Subsequently, supernatants were stored at -80°C. CXCL1 concentrations in the lungs were determined using a Mouse CXCL1/KC ELISA kit from R&D Systems according to the manufacturer's instructions (Wiesbaden-Nordenstadt). ELISA was performed using an Infinite M200 microplate reader (Tecan Deutschland GmbH).

RNA isolation and reverse transcription (RT) semi-quantitative polymerase chain reaction (RT-qPCR). Following sacrifice, snap-frozen specimens from the lungs were stored at -80°C, and RNA isolation was applied using an RNeasy-system (Qiagen GmbH), as previously described (30). Residual DNA was removed using an RNase-free DNase kit (Qiagen GmbH). The amount and quality of RNA were measured photometrically using a NanoDrop ND-1000 device (NanoDrop Technologies, Inc.). Subsequently, RNA was reverse transcribed into cDNA using an AffinityScript QPCR cDNA Synthesis kit (Stratagene) according to the manufacturer's instructions. A specific primer for mouse CXCL1 was used to evaluate CXCL1 gene expression (NM_008176, UniGene no. Mm.21013; cat no. PPM03058A), and GAPDH (NM_008084, UniGene no. Mm.343110; cat no. PPM02946E) (both from SA Biosciences) gene expression measurements served as a reference. According to the manufacturer's instructions, the PCR reaction was set up in a 25 µl volume with 1X RT2 SYBR-Green/Rox qPCR Master Mix (SA Biosciences) and measured on a Stratagene Mx3005p QPCR system (Stratagene). The two-step amplification protocol was as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C with 15 sec of denaturation and 60 sec of annealing/extension at 60°C. The comparative threshold-cycle (CT) method ($2^{-\Delta\Delta Cq}$ method) was used to calculate the target gene's relative expression (31). Sham-operated animals served as 100% expression reference
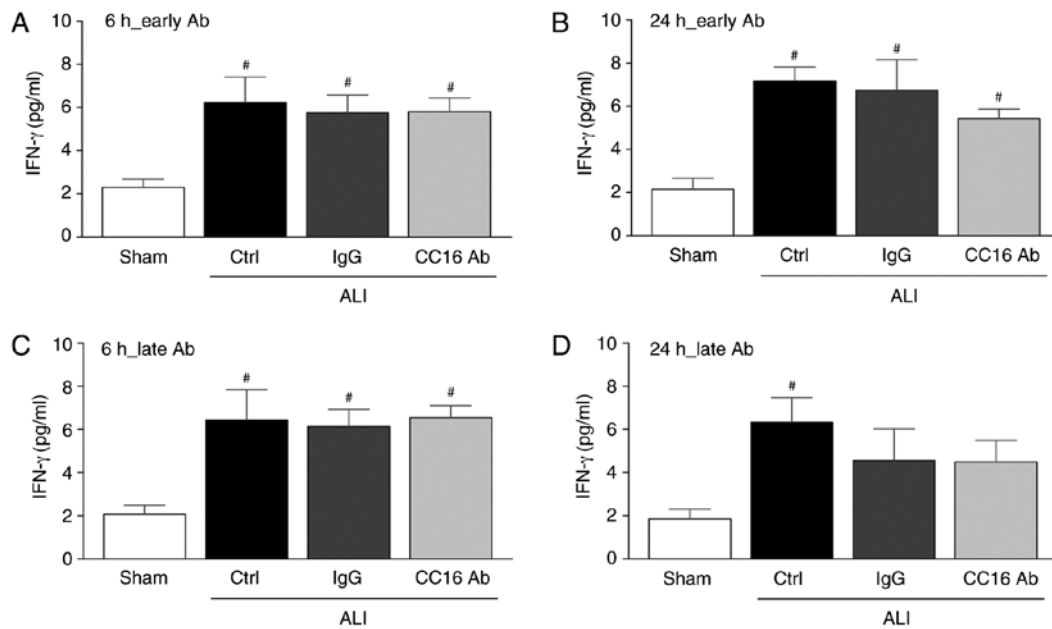
Figure 2. Systemic levels of IFN-γ are shown. A CC16 antibody (Ab) or IgG antibody was administered immediately following either thoracic trauma (early Ab, A and B) or CLP (late Ab, C and D). Animals were euthanized for sampling after 6 or 24 h. Data are presented as the means ± standard error of the mean; [#]P<0.05 vs. sham (n=6). IFN-γ, interferon-γ; CC16, club cell protein 16; CLP, cecal ligation and puncture; ALI, acute lung injury.

following normalization to GAPDH. The method was applied using a previously described protocol (30).

Statistical analyses. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc.). Based on the D'Agostino-Pearson normality test, differences between the groups were analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's post hoc test for the correction of multiple comparisons. The data are presented as the means ± standard error of the mean. Survival was analyzed via the log-rank test, and the Bonferroni correction was applied to correct for multiple comparisons. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

IFN-γ concentrations in the blood. At 6 or 24 h following thoracic trauma and CLP, a significant increase in the levels of pro-inflammatory IFN-γ compared to the sham group was detected (P<0.05; Fig. 2). Early antibody application did not significantly alter the IFN-γ levels between the ALI groups after 6 or 24 h (Fig. 2). Whereas late antibody application led to comparable IFN-γ levels among all the ALI groups after 6 h, a significant increase in the IFN-γ levels vs. the sham group was observed only in the control group after ALI was compared with the sham group (P<0.05; Fig. 2C and D).

CXCL1 gene expression and protein concentration. Although CXCL1 gene expression was significantly elevated in the control and IgG groups at 6 h following thoracic trauma and CLP compared to the sham group (P<0.05; Fig. 3A and C), no significant differences were observed after 24 h among these groups (Fig. 3B and D). The early application of the CC16 antibody significantly increased CXCL1 gene expression after 6 h compared to all other groups (P<0.05; Fig. 3A).

However, after 24 h, no significant changes among the groups were observed (Fig. 3B). Whereas late CC16 antibody application led to levels comparable to those of the other groups undergoing thoracic trauma and CLP after 6 h, a significant increase in CXCL1 gene expression following late CC16 antibody application vs. all other groups was observed after 24 h (P<0.05; Fig. 2D).

CXCL1 protein concentration was significantly elevated in all 3 ALI groups at 6 and 24 h following thoracic trauma and CLP compared to the sham group (P<0.05; Fig. 3E-H). Whereas early CC16 antibody application led to levels comparable to those of the other groups undergoing thoracic trauma and CLP after 6 h, a significant increase in CXCL1 protein concentration following early CC16 antibody application vs. all the other groups was observed after 24 h (P<0.05; Fig. 3F). The late application of the CC16 antibody following thoracic trauma and CLP led to a slight increase in the CXLC1 protein concentrations compared to the control and IgG groups upon ALI after 6 and 24 h; however, this difference was insignificant (Fig. 3G and H).

Body weight changes during the 7-day observation period. Every 24 h for 7 days, the weight of the mice was documented. During the first 48 h, the weight of the animals was stable in all groups. After 48 h, all groups undergoing thoracic trauma and CLP exhibited a decrease in weight compared with the sham group (Fig. 4).

Survival rates after sepsis-induced ALI following TxT. All animals in the sham group survived during the observation period (Fig. 5). Within 48 h, the survival rate decreased among the groups subjected to TxT and CLP compared to the sham group (Fig. 5A). Within 60 h, the survival rate in the ALI control group significantly decreased compared to the sham group (P<0.05; Fig. 5B). The group receiving early
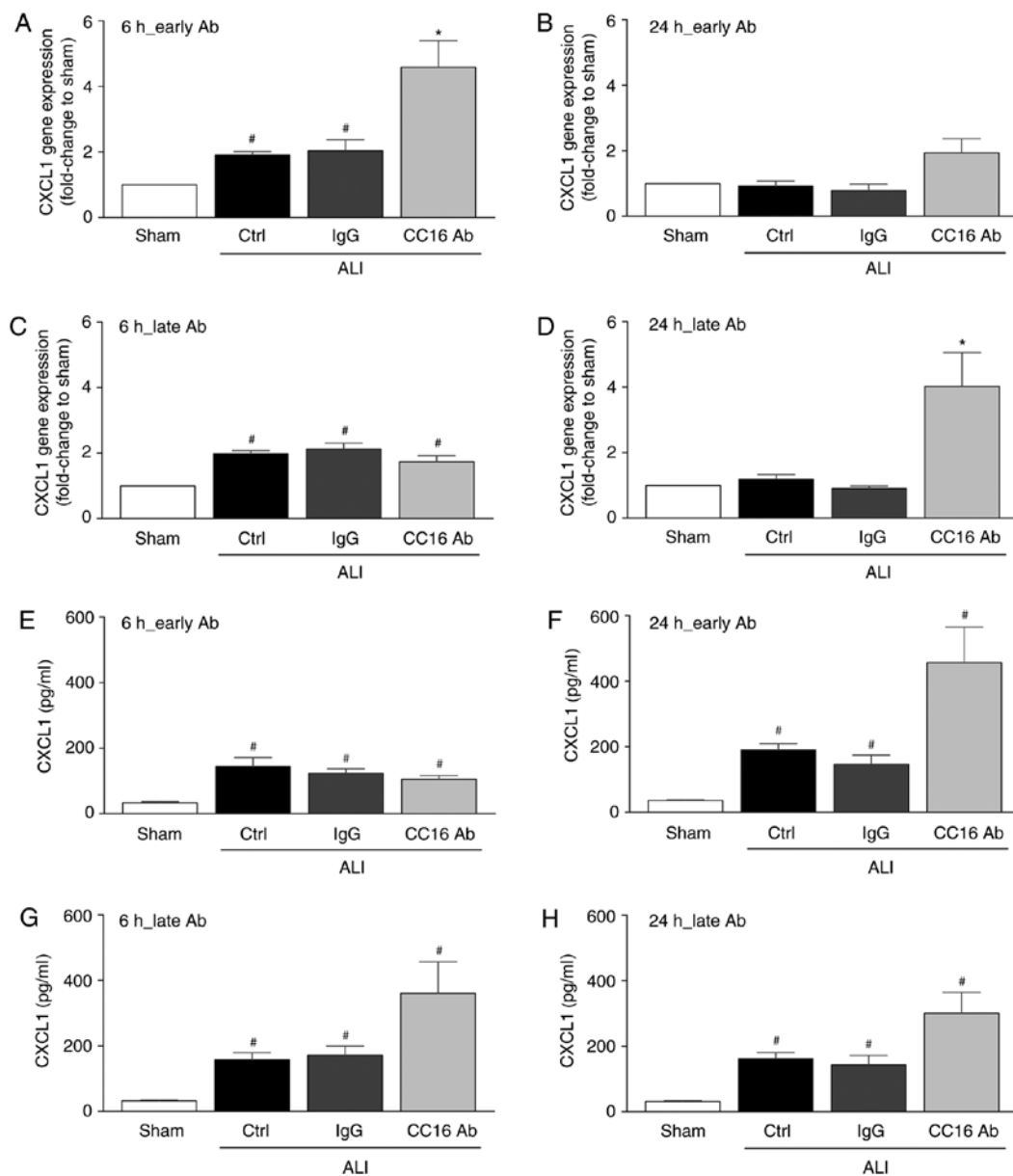
Figure 3. Pulmonary (A-D) CXCL1 gene expression and (E-F) CXCL1 protein expression levels are shown. A CC16 antibody (Ab) or IgG antibody was administered to the lungs immediately following either thoracic trauma (early Ab) (A and B, and E and F) or CLP (late Ab) (C and D, and G and H). After 6 or 24 h, the animals were euthanized for sampling. Data are presented as the means ± standard error of the mean; #P<0.05, vs. sham group; *P<0.05, CC16 Ab ALI group vs. all other groups (n=6). CC16, club cell protein 16; CLP, cecal ligation and puncture; ALI, acute lung injury.
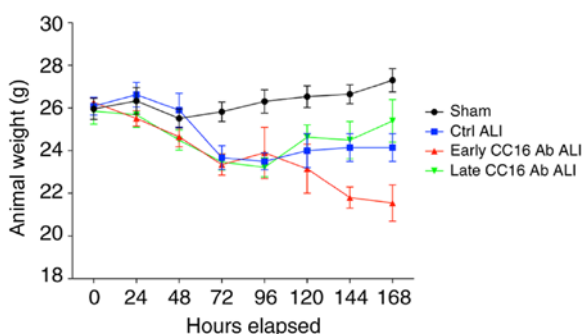

Figure 4. Bodyweight during a 6-day observational period following sepsis induction. According to group allocation, a CC16 antibody (Ab) or IgG antibody was administered immediately after either thoracic trauma (early Ab) or CLP (late Ab). Data are presented as the means ± standard error of the mean. CC16, club cell protein 16; TxT, thoracic trauma; CLP, cecal ligation and puncture; ALI, acute lung injury.

CC16 Ab treatment exhibited a significantly higher survival rate compared to the control group following TxT and CLP (P<0.05; Fig. 5B). After 72 h, no marked differences in survival were observed between the 3 groups undergoing TxT and CLP (Fig. 5C). However, all ALI groups exhibited significantly decreased survival rates compared to the sham group (P<0.05; Fig. 5C). Comparable data were observed until the end of the observational period of 6 days post-CLP (Fig. 5D).

Discussion

The present study investigated whether the intratracheal neutralization of CC16 affects survival following 'double-hit' trauma consisting of TxT followed by CLP in mice. Recently, the anti-inflammatory potential of CC16 24 h post-CLP in this model was confirmed. The local anti-inflammatory character-
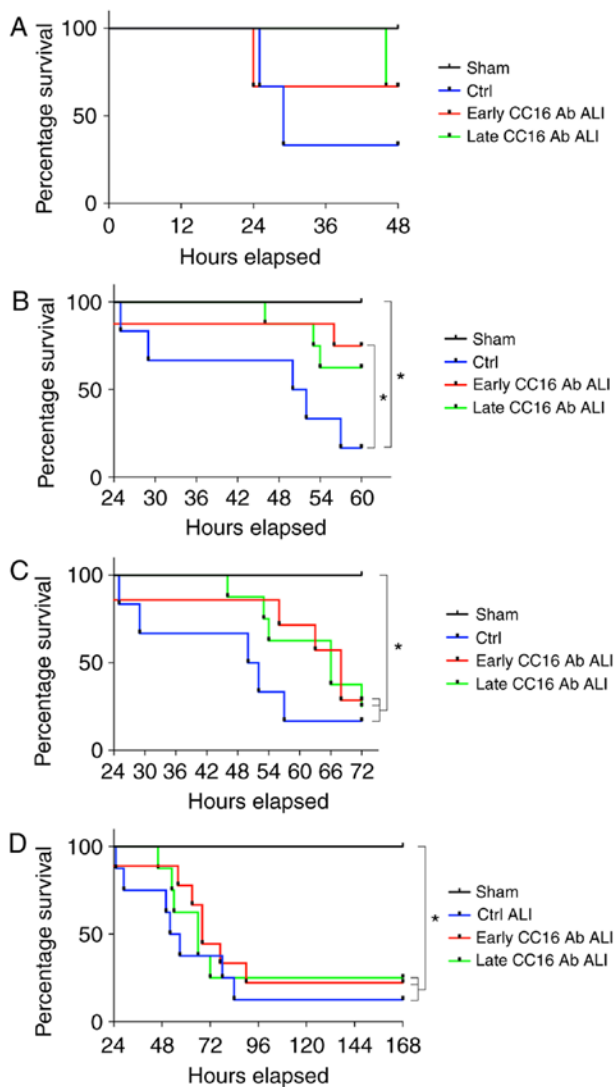
Figure 5. Survival was assessed during a 6-day observational period following sepsis induction. Mice underwent TxT and CLP, and were assigned to the ALI. The sham group underwent identical anesthesia and surgical procedures without performing TxT or CLP. The control group underwent ALI without an antibody application. Interventions with a CC16 antibody (Ab) or IgG antibody were performed immediately after either TxT (early Ab) or CLP (late Ab). Data are presented as Kaplan-Meier estimates for different time periods (A, 0-48 h; B, 24-60 h; C, 24-72 h; D, 24-168 h). *P<0.05 vs. sham group (n=8). CC16, club cell protein 16; TxT, thoracic trauma; CLP, cecal ligation and puncture; ALI, acute lung injury.

istic of CC16 is supported by the present data and by results previously demonstrated in non-traumatic lung injury (32,33). A recent study by the authors demonstrated that upon the early intratracheal neutralization of CC16 following chest trauma, reduced lung injury at 24 h post-CLP was observed (9). Notably, the late neutralization of CC16 following CLP aggravated lung injury during the same observational period (9). Thus, although the anti-inflammatory potential of CC16 in the underlying model was clearly confirmed, previous studies did not determine whether the results are caused by the timing of antibody application or disease kinetics or whether the observed effects affect survival in this model. The present study demonstrated that although the overall mortality rate was not significantly altered upon early CC16 neutralization, delayed mortality within 60 h after the second hit was present in this group.

Blunt chest trauma triggers a potent inflammatory response in lung tissue and the airways, implying the (intrapulmonary) release of pro-inflammatory chemokines and cytokines, which promote the activation of alveolar macrophages, chemotaxis, and pulmonary neutrophil infiltration (34,35). Since several studies have suggested that the pro-inflammatory response is a key driver of lung damage during ALI, a pathologically-relevant role of the anti-inflammatory CC16 has been postulated. Miller *et al* confirmed that increased pulmonary IL-8 (CXCL8) levels in patients suffering from ARDS were associated with an increased influx of neutrophils, key drivers of organ damage (36). Similar findings were confirmed *in vivo* via interleukin (IL)-8 neutralization, which significantly reduced lung damage in a rabbit model of lung injury induced by acid aspiration (37). The recruitment of neutrophils to the lungs is a key event in the early development of ALI and is mediated by CXCL1 (38). Reutershan *et al* reported significantly reduced neutrophilic migration to the lungs in CXCL1 receptor-deficient CXCR2$^{-/-}$ mice in a murine model of LPS-induced ALI (38). Similarly, CXCL1 receptor neutralization reduced neutrophilic infiltration and, moreover, attenuated lung damage in a mouse model of ventilator-induced lung injury (39). Consistent with these findings, the important role of CXCL1 in local tissue inflammation was confirmed by its suppressed expression, which was concurrent with reduced LPS-triggered pulmonary inflammation in mice (40). Furthermore, in sepsis-induced lung injury, the reduced extravasation of neutrophils into alveolar cavities was associated with reduced pulmonary CXCL1 levels (41). Similarly, the pathophysiologically relevant role of CXCL1 was confirmed in other models of lung injury (42,43). The data of the present study indicate that CC16 may have a direct effect on the pulmonary level of CXCL1 in a 'double-hit' model of sepsis-induced ALI following thoracic trauma. Since the early neutralization of CC16 immediately following thoracic trauma increased CXCL1 concentrations after 24 h, this finding confirms the anti-inflammatory effect of CC16. However, the results prompt a conflictive discussion. The above-mentioned studies suggest that CXCL1 plays a detrimental role in such a model, yet the early increase in CXCL1 in our study was associated with delayed mortality. Thus, other factors must also be pathophysiologically relevant.

It is well known that the development of post-traumatic lung injury depends on several factors, including damage- or pathogen-associated molecular patterns (9,22). During post-traumatic ALI/ARDS development, numerous mediators released from tissue-resident cells act as chemoattractants for invading immune cells and further stimulate local cells to build a pro-inflammatory micromilieu (44). While it is widely assumed that this type of hyperinflammatory reaction contributes to subsequent lung damage, Störmann *et al* observed less lung damage following the early neutralization of CC16 immediately after trauma, which was associated with an enhanced influx of neutrophilic granulocytes (9). This group demonstrated prolonged survival following CC16 neutralization within the early post-traumatic phase of post-blunt chest trauma and CLP. However, survival rates will be discussed below. Of note, while local effects have been observed, in line with the previous study by the authors, intrapulmonary CC16 neutralization did not influence systemic inflammation, as assessed via IFN-γ

levels (9). CC16 can function as an anti-cytokine and a natural immunosuppressor by inhibiting the production and biological activity of IFN-γ (45). However, the interplay between IFN-γ and CC16 was demonstrated in the lungs, and no systemic data were provided (46). The authors concluded that IFN-γ may act as a potent regulator of CC16/CC10 gene expression and that increased IFN-γ in lung epithelial cells may be modulated by increasing CC16 production, stimulated by a regulatory feedback loop (46,47), which potentially maintains the balance between the local pro- and anti-inflammatory processes. This remains to be determined in future studies.

Adjacent to its role as a biomarker for pulmonary injury and lung complications following trauma, several studies have demonstrated that CC16 has significant pathophysiological effects on the development of pulmonary complications (9,20,48). Recently, the authors demonstrated that CC16 exerts anti-inflammatory effects in sepsis-induced ALI following thoracic trauma *in vivo* (9). Furthermore, early and local CC16 inhibition decreased pulmonary injury after 24 h, indicating that CC16 may exert protective effects in early inflammation during ALI (9). However, CC16 inhibition after sepsis-induction aggravated lung damage after 24 h (9). Thus, although CC16 neutralization increased early inflammation and was associated with reduced pulmonary damage, the long-term outcome remains unclear. Therefore, the pathological relevance of CC16 to survival in our sepsis-induced trauma model was evaluated. In general, the approach of influencing inflammation after traumatic insult with the aim of preventing infectious and organ complications is highly challenging since the post-traumatic inflammatory response, with its numerous associated mechanisms, is very complex. Although the underlying pathomechanisms of trauma-induced ALI are unclear, in the present study, it was confirmed that CC16 exerts significant local anti-inflammatory effects. Furthermore, lung inflammation induced by cigarette smoke and adverse outcomes were ameliorated by CC16 in a murine COPD model (32,33). Of note, in patients with pulmonary morbidities, including COPD and asthma, CC16 plays an important role in pathology and performs protective functions during exposure to cigarette smoke (33,49). Although reduced lung damage following CC16 neutralization was observed, the general mortality rate did not change in the current model. The present study demonstrates that early CC16 inhibition may delay mortality within a defined timeframe after sepsis-induction; however, no evidence of it benefitting long-term survival was observed. The highest mortality rates were observed between 48 and 72 h in the present model. Similar data from other studies reveal that the majority of deaths following CLP-induced polymicrobial sepsis occur within 24-48 h after CLP or within 24-96 h (50,51). In contrast to later therapeutic interventions, early antibody application improves survival rates (52). Thus, the relevance of delayed mortality to CC16 neutralization within a defined timeframe, as shown in the present study, remains to be further elucidated.

Considering that the timeline and protracted inflammation following sepsis favor hospital-acquired infections and worsen patient outcomes through immunosuppression (53), the data of this and our recent study remain intriguing (9). Throughout their medical care, which may include surgery, severely injured patients are subjected to damaging endogenous or exogenous molecules, which mediate systemic inflammation or immuno-

suppression and have clinical consequences, such as infections and organ failure (53). In a recent study by the authors, it was discussed how, in post-traumatic immunosuppression, it appears reasonable that the anti-inflammatory effects of CC16 may be detrimental in the underlying model (9). However, considering hyperinflammation and the second-hit hypothesis, which suggests the presence of a detrimental hyperinflammatory state following trauma, CC16 may be expected to exert beneficial effects. The lack of significant changes in survival rates following CC16 neutralization for 6 days post-sepsis indicates that there are other, possibly more decisive, factors affecting final outcomes in this model. However, it remains to be further elaborated whether delayed mortality observed following early anti-inflammatory therapy can be improved in long-term outcomes. This effect could be evaluated in future studies by alternatively supplementing the original therapy with a second dose of either anti-CC16 Ab or recombinant CC16 at a later timepoint.

A significant limitation of the present study remains the lack of mechanical ventilation, as all animals were consistently spontaneously breathing. Furthermore, fluid management comparable to the clinical setting was not included. These two major contributors to ALI/ARDS were not taken into account in the present study. Moreover, by means of animal welfare, the number of animals in each group was limited. Interspecies variability in the innate immune response must be considered when *in vivo* experimental results are applied to humans (54). Furthermore, some interesting phenomena, such as decreased bodyweight in the early Ab treatment group, were observed, although this decrease was insignificant between the groups. In the present study, a sufficient explanation cannot be provided, and it remains undetermined whether a longer observational period may have provided evidence of further alterations or significant differences in survival benefits between the groups.

The present study confirmed the anti-inflammatory effects of CC16 following CLP-induced ALI after TxT in mice. As reported in a recent study by the authors (9), the early therapeutic strategy of CC16 inhibition (after TxT) increased inflammation, and it was further concomitant with delayed mortality within a defined time window after CLP; however, no long-term survival benefits were observed. In the previous study by the authors (9), later-stage CC16 inhibition resulted in poor pulmonary outcomes, and no effects on short- or long-term survival were observed. Thus, it remains to be determined whether, in post-traumatic ALI cases, a rapid local pro-inflammatory reaction may be necessary, which should be followed by another therapeutic inflammation-modulating step at a later timepoint.

Acknowledgements

Funding

in the design of the study, in the collection, analyses, or interpretation of data, in writing the manuscript, or in the decision to publish the results.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

BR, SW, FH and IM were involved in the conceptualization of the study. JTV, PS, NB and BR were involved in the study methodology. JTV was involved in data validation. BR and JTV were involved in the formal analysis. JTV, PS and NB were involved in the investigative aspects of the study. JTV and PS were involved in data curation. JTV was involved in the writing and preparation of the original draft. JTV, SW, FH, IM and BR were involved in the writing, reviewing and editing of the manuscript. JTV and BR were involved in visualization of the results. BR supervised the study. JTV and BR were involved in project administration. BR, FH and SW were involved in funding acquisition. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were conducted in accordance with German federal laws regarding the protection of animals. The experiments were performed at Goethe University Hospital in Frankfurt, Germany. The present study was approved by the responsible government authority, the Veterinary Department of the Regional Council in Darmstadt, Germany (Regierungspräsidium Darmstadt, Hessen, Germany; AZ: FK 1068), and the study was performed under ARRIVE guidelines (26).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hildebrand F, Giannoudis PV, Griensven M, Zelle B, Ulmer B, Krettek C, Bellamy MC and Pape HC: Management of polytraumatized patients with associated blunt chest trauma: A comparison of two European countries. Injury 36: 293-302, 2005.
2. Matthay MA, Zemans RL, Zimmerman GA, Arabi YM, Beitler JR, Mercat A, Herridge M, Randolph AG and Calfee CS: Acute respiratory distress syndrome. Nat Rev Dis Primers 5: 18, 2019.
3. Rendeki S and Molnár TF: Pulmonary contusion. J Thorac Dis 11 (Suppl 2): S141-S151, 2019.
4. Hoth JJ, Wells JD, Yoza BK and McCall CE: Innate immune response to pulmonary contusion: Identification of cell type-specific inflammatory responses. Shock 37: 385-391, 2012.
5. Hoth JJ, Wells JD, Brownlee NA, Hiltbold EM, Meredith JW, McCall CE and Yoza BK: Toll-like receptor 4-dependent responses to lung injury in a murine model of pulmonary contusion. Shock 31: 376-31, 2009.
6. Hoth JJ, Wells JD, Hiltbold EM, McCall CE and Yoza BK: Mechanism of neutrophil recruitment to the lung after pulmonary contusion. Shock 35: 604-609, 2011.
7. Störmann P, Becker N, Kunnemeyer L, Wutzler S, Vollrath JT, Lustenberger T, Hildebrand F, Marzi I and Relja B: Contributing factors in the development of acute lung injury in a murine double hit model. Eur J Trauma Emerg Surg 46: 21-30, 2020.
8. Proudfoot AG, McAuley DF, Griffiths MJ and Hind M: Human models of acute lung injury. Dis Model Mech 4: 145-153, 2011.
9. Störmann P, Becker N, Vollrath JT, Köhler K, Janicova A, Wutzler S, Hildebrand F, Marzi I and Relja B: Early local inhibition of club cell protein 16 following chest trauma reduces late sepsis-induced acute lung injury. J Clin Med 8: 896, 2019.
10. Perl M, Hohmann C, Denk S, Kellermann P, Lu D, Braumuller S, Bachem MG, Thomas J, Knöferl MW, Ayala A, et al: Role of activated neutrophils in chest trauma-induced septic acute lung injury. Shock 38: 98-106, 2012.
11. Weckbach S, Hohmann C, Denk S, Kellermann P, Huber-Lang MS, Baumann B, Wirth T, Gebhard F, Bachem M and Perl M: Apoptotic and inflammatory signaling via Fas and tumor necrosis factor receptor I contribute to the development of chest trauma-induced septic acute lung injury. J Trauma Acute Care Surg 74: 792-800, 2013.
12. Muenzer JT, Davis CG, Chang K, Schmidt RE, Dunne WM, Coopersmith CM and Hotchkiss RS: Characterization and modulation of the immunosuppressive phase of sepsis. Infect Immun 78: 1582-1592, 2010.
13. Boehm U, Klamp T, Groot M and Howard JC: Cellular responses to interferon-gamma. Annu Rev Immunol 15: 749-795, 1997.
14. Varma TK, Lin CY, Toliver-Kinsky TE and Sherwood ER: Endotoxin-induced gamma interferon production: Contributing cell types and key regulatory factors. Clin Diagn Lab Immunol 9: 530-543, 2002.
15. Shtrichman R and Samuel CE: The role of gamma interferon in antimicrobial immunity. Curr Opin Microbiol 4: 251-259, 2001.
16. Romero CR, Herzig DS, Etogo A, Nunez J, Mahmoudizad R, Fang G, Murphey ED, Toliver-Kinsky T and Sherwood ER: The role of interferon-γ in the pathogenesis of acute intra-abdominal sepsis. J Leukoc Biol 88: 725-735, 2010.
17. Kropski JA, Fremont RD, Calfee CS and Ware LB: Clara cell protein (CC16), a marker of lung epithelial injury, is decreased in plasma and pulmonary edema fluid from patients with acute lung injury. Chest 135: 1440-1447, 2009.
18. Miele L, Cordella-Miele E, Mantile G, Peri A and Mukherjee AB: Uteroglobin and uteroglobin-like proteins: The uteroglobin family of proteins. J Endocrinol Invest 17: 679-692, 1994.
19. Negrin LL, Halat G, Kettner S, Gregori M, Ristl R, Hajdu S and Heinz T: Club cell protein 16 and cytokeratin fragment 21-1 as early predictors of pulmonary complications in polytraumatized patients with severe chest trauma. PLoS One 12: e0175303, 2017.
20. Wutzler S, Lehnert T, Laurer H, Lehnert M, Becker M, Henrich D, Vogl T and Marzi I: Circulating levels of Clara cell protein 16 but not surfactant protein D identify and quantify lung damage in patients with multiple injuries. J Trauma 71: E31-E36, 2011.
21. Broeckaert F, Clippe A, Knoops B, Hermans C and Bernard A: Clara cell secretory protein (CC16): Features as a peripheral lung biomarker. Ann NY Acad Sci 923: 68-77, 2000.
22. Relja B, Mörs K and Marzi I: Danger signals in trauma. Eur J Trauma Emerg Surg 44: 301-316, 2018.
23. Wutzler S, Lustenberger T, Relja B, Lehnert M and Marzi I: Pathophysiology of multiple trauma: Intensive care medicine and timing of treatment. Chirurg 84: 753-758, 2013 (In German).
24. Dong L, Zhu YH, Liu DX, Li J, Zhao PC, Zhong YP, Chen YQ, Xu W and Zhu ZQ: Intranasal application of budesonide attenuates lipopolysaccharide-induced acute lung injury by suppressing nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 inflammasome activation in mice. J Immunol Res 2019: 7264383, 2019.
25. Horiguchi H, Loftus TJ, Hawkins RB, Raymond SL, Stortz JA, Hollen MK, Weiss BP, Miller ES, Bihorac A, Larson SD, et al: Innate immunity in the persistent inflammation, immunosuppression, and catabolism syndrome and its implications for therapy. Front Immunol 9: 595, 2018.
26. Kilkenny C, Browne WJ, Cuthill IC, Emerson M and Altman DG: Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. PLoS Biol 8: e1000412, 2010.
27. Knoferl MW, Liener UC, Seitz DH, Perl M, Bruckner UB, Kinzl L and Gebhard F: Cardiopulmonary, histological, and inflammatory alterations after lung contusion in a novel mouse model of blunt chest trauma. Shock 19: 519-525, 2003.

28. Rittirsch D, Huber-Lang MS, Flierl MA and Ward PA: Immunodesign of experimental sepsis by cecal ligation and puncture. Nat Protoc 4: 31-36, 2009.

29. Janicova A, Becker N, Xu B, Wutzler S, Vollrath JT, Hildebrand F, Ehnert S, Marzi I, Störmann P and Relja B: Endogenous utero-globin as intrinsic anti-inflammatory signal modulates monocyte and macrophage subsets distribution upon sepsis induced lung injury. Front Immunol 10: 2276, 2019.

30. Relja B, Horstmann JP, Kontradowitz K, Jurida K, Schaible A, Neunaber C, Oppermann E and Marzi I: Nlrp1 inflammasome is downregulated in trauma patients. J Mol Med (Berl) 93: 1391-1400, 2015.

31. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

32. Pang M, Liu HY, Li T, Wang D, Hu XY, Zhang XR, Yu BF, Guo R and Wang HL: Recombinant club cell protein 16 (CC16) ameliorates cigarette smoke-induced lung inflammation in a murine disease model of COPD. Mol Med Rep 18: 2198-2206, 2018.

33. Laucho-Contreras ME, Polverino F, Gupta K, Taylor KL, Kelly E, Pinto-Plata V, Divo M, Ashfaq N, Petersen H, Stripp B, et al: Protective role for club cell secretory protein-16 (CC16) in the development of COPD. Eur Respir J 45: 1544-1556, 2015.

34. Seitz DH, Perl M, Liener UC, Tauchmann B, Braumüller ST, Brückner UB, Gebhard F and Knöferl MW: Inflammatory alterations in a novel combination model of blunt chest trauma and hemorrhagic shock. J Trauma 70: 189-196, 2011.

35. Reutershan J and Ley K: Bench-to-bedside review: Acute respi-ratory distress syndrome-how neutrophils migrate into the lung. Crit Care 8: 453-461, 2004.

36. Miller EJ, Cohen AB, Nagao S, Griffith D, Maunder RJ, Martin TR, Weiner-Kronish JP, Sticherling M, Christophers E and Matthay MA: Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. Am Rev Respir Dis 146: 427-432, 1992.

37. Folkesson HG, Matthay MA, Hebert CA, Broaddus VC: Acid aspiration-induced lung injury in rabbits is mediated by inter-leukin-8-dependent mechanisms. J Clin Invest 96: 107-116, 1995.

38. Reutershan J, Morris MA, Burcin TL, Smith DF, Chang D, Saprito MS and Ley K: Critical role of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. J Clin Invest 116: 695-702, 2006.

39. Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ and Strieter RM: Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. J Clin Invest 110: 1703-1716, 2002.

40. Meng J, Zou Y, Chen J, Qin F, Chen X, Chen X and Dai S: sTLR4/sMD-2 complex alleviates LPS-induced acute lung injury by inhibiting pro-inflammatory cytokines and chemokine CXCL1 expression. Exp Ther Med 16: 4632-4638, 2018.

41. Wang J, Gong S, Wang F, Niu M, Wei G, He Z, Gu T, Jiang Y, Liu A and Chen P: Granisetron protects polymicrobial sepsis-induced acute lung injury in mice. Biochem Biophys Res Commun 508: 1004-1010, 2019.

42. Zarbock A, Allegretti M and Ley K: Therapeutic inhibition of CXCR2 by Reparixin attenuates acute lung injury in mice. Br J Pharmacol 155: 357-364, 2008.

43. Dunn JLM, Kartchner LB, Stepp WH, Glenn LI, Malfitano MM, Jones SW, Doerschuk CM, Maile R and Cairns BA: Blocking CXCL1-dependent neutrophil recruitment prevents immune damage and reduces pulmonary bacterial infection after inhalation injury. Am J Physiol Lung Cell Mol Physiol 314: L822-L834, 2018.

44. Niesler U, Palmer A, Radermacher P and Huber-Lang MS: Role of alveolar macrophages in the inflammatory response after trauma. Shock 42: 3-10, 2014.

45. Dierynck I, Bernard A, Roels H and De Ley M: Potent inhibition of both human interferon-gamma production and biologic activity by the Clara cell protein CC16. Am J Respir Cell Mol Biol 12: 205-210, 1995.

46. Magdaleno SM, Wang G, Jackson KJ, Ray MK, Welty S, Costa RH and DeMayo FJ: Interferon-gamma regulation of Clara cell gene expression: In vivo and in vitro. Am J Physiol 272: L1142-L1151, 1997.

47. Ramsay PL, Luo Z, Magdaleno SM, Whitbourne SK, Cao X, Park MS, Park MS, Welty SE, Yu-Lee LY and DeMayo FJ: Transcriptional regulation of CCSP by interferon-gamma in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol 284: L108-L118, 2003.

48. Michel O, Murdoch R and Bernard A: Inhaled LPS induces blood release of Clara cell specific protein (CC16) in human beings. J Allergy Clin Immunol 115: 1143-1147, 2005.

49. Lam DC, Kwok HH, Yu WC, Ko FW, Tam CY, Lau AC, Fong DY and Ip MS: CC16 levels correlate with cigarette smoke exposure in bronchial epithelial cells and with lung function decline in smokers. BMC Pulm Med 18: 47, 2018.

50. Xu L, Zhang W, Kwak M, Zhang L, Lee PCW and Jin JO: Protective effect of melatonin against polymicrobial sepsis is mediated by the anti-bacterial effect of neutrophils. Front Immunol 10: 1371, 2019.

51. Chen X, Wang T, Song L and Liu X: Activation of multiple Toll-like receptors serves different roles in sepsis-induced acute lung injury. Exp Ther Med 18: 443-450, 2019.

52. Matsuo S, Sharma A, Wang P and Yang WL: PYR-41, a ubiq-uitin-activating enzyme E1 inhibitor, attenuates lung injury in sepsis. Shock 49: 442-450, 2018.

53. Vourc'h M, Roquilly A and Asehnoune K: Trauma-induced damage-associated molecular patterns-mediated remote organ injury and immunosuppression in the acutely ill patient. Front Immunol 9: 1330, 2018.

54. Matute-Bello G, Frevert CW and Martin TR: Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol 295: L379-L399, 2008.