ORIGINAL ARTICLE

Isolated blunt chest injury leads to transient activation of circulating neutrophils

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Abstract

Introduction The acute respiratory distress syndrome (ARDS) is a severe and frequently seen complication in multi-trauma patients. ARDS is caused by an excessive innate immune response with a clear role for neutrophils. As ARDS is more frequently seen in trauma patients with chest injury, we investigated the influence of chest injury on the systemic neutrophil response and the development of ARDS.

Materials and methods Thirteen patients with isolated blunt chest injury [abbreviated injury score (AIS) 2–5] were included. To avoid systemic inflammation caused by tissue damage outside the thorax, injuries to other regions than the chest did not exceed an AIS of 2. At 3, 9 and 24 h after injury, the expression of circulating activating molecules on neutrophils and levels of circulating interleukin (IL)-6 were determined. Blood samples from eight healthy volunteers were used as control.

Results Blunt chest injury resulted in the activation of circulating neutrophils, as characterized by a decreased expression of L-selectin (CD62L), CXCR2 (CD182b) and C5aR (CD88) compared to control ($p < 0.05$). Expression of L-selectin, CXCR2 and C5aR was partially restored at 24 h after injury. In addition, the mean expression of FcγRIII (CD16) dropped ($p < 0.001$), indicating the recruitment of young neutrophils into the circulation. IL-6 levels increased to a maximum mean concentration of 86 ± 31 pg/ml at 24 h postinjury. None of the patients developed ARDS.

Conclusion Blunt chest trauma caused a systemic inflammatory reaction with transient activation of neutrophils and mobilization of young neutrophils into the circulation. Isolated chest injury, however, was not abundant enough to cause ARDS, so a second hit appears crucial.

Keywords Acute respiratory distress syndrome · Chest injury · Neutrophil · Innate immune response

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Introduction

In multi-trauma patients, the acute respiratory distress syndrome (ARDS) is a commonly seen complication. Important risk factors for the development of ARDS after trauma are severe injury [injury severity score (ISS) >25] and pulmonary contusion [1, 2]. Although the occurrence of ARDS in multi-trauma patients increases considerably with severity of pulmonary contusion, the incidence is surprisingly low in patients with isolated chest injury [2–4].

An excessive innate immune response to tissue injury is considered to be the cause of ARDS. Due to the heterogeneity of injuries present in multi-trauma patients, the specific role of chest injury in the pathogenesis of ARDS is not well known. In this study we investigated the effect of isolated chest injury on the systemic innate immune response to test whether this increased risk of ARDS is caused by priming of the circulating neutrophils or is solely due to local damage. We focused on differences in the phenotype of circulating neutrophils during the first 24 h after chest injury. Neutrophils are important effector cells

of the final common pathway of the innate immune response. Activated neutrophils migrate out of the circulation into the alveolar compartment. A massive release of radical oxygen species (ROS) and proteases by neutrophils can cause damage to the parenchyma, which can eventually result in organ dysfunction (ARDS) [5].

Systemic inflammation is characterized by the activation of circulating neutrophils [6, 7] and the mobilization of young neutrophils from the bone marrow. Surface receptors of circulating neutrophils have been used to determine the activation of circulating neutrophils in order to discriminate the severity of inflammation and to predict the occurrence of organ failure [6–9]. Systemic neutrophil activation is typically characterized by the shedding of L-selectin (CD62L) and the upregulation of expression of αM integrin (CD11b) [10–13]. Decreased L-selectin and increased αM expression have been shown to correlate with ISS and to be associated with the development of posttraumatic complications such as ARDS [14–20]. A previous study by our group showed a significant decreased responsiveness after in vitro stimulation of active FcγRII (CD32), the main IgG receptor on neutrophils, in multi-trauma patients [20]. Decreased responsiveness of active FcγRII was more pronounced in patients who developed ARDS or acute lung injury (ALI) compared to patients without complications. Expression of active FcγRII is under the control of inside-out signals induced by both chemoattractants and cytokines, and seems to be more sensitive to priming stimuli compared with αM/CD11b [21]. The responsiveness of active FcγRII was found to correlate better with outcome than other neutrophil receptor expressions in preceding observational studies in trauma patients [11, 20].

We investigated whether isolated chest injury leads to a systemic innate immune response quantified by the activation of circulating neutrophils. Furthermore, the release of interleukin (IL)-6 was measured as an additional marker for inflammation.

Methods

Patients

Patients suffering from chest injury with an abbreviated injury score (AIS) of 2 or more who were admitted to the trauma department of the University Medical Centre Utrecht were included. Patients with injuries with an AIS > 2 in other regions than the thorax were excluded to reduce systemic inflammation caused by tissue damage outside the thorax. Other exclusion criteria included age <18 or >70 years, death within 24 h after admission, and patients with altered immunological status (e.g., corticosteroid use or chemotherapy).

At admission, injury severity score (ISS) [22], new injury severity score (NISS) [23], Apache II score [24], and leukocyte count were determined. All patients were followed until discharge. The presence of ARDS was assessed according to their clinical criteria, as determined in the consensus conferences on ARDS [25].

Blood samples ware taken at approximately 3 (2–4), 9 (8–10) and 24 (22–26) h after the accident to investigate the relationship between chest injury and systemic neutrophil activation. In an in vivo human inflammation model, we saw previously that systemic neutrophil activation is most prominent between 2 and 4 h after the induction of inflammation [26]. The first measurement time point was therefore set at 3 h postinjury.

The local ethics committee approved the study, and written informed consent was obtained from all patients or their legal representatives in accordance with the Helsinki Declaration.

Expression of activation markers on neutrophils determined by flowcytometry

The following commercially available mouse–antihuman monoclonal antibodies were purchased for analyzing neutrophil receptor expression by flowcytometry: fluorescein isothiocyanate (FITC)-labeled IgG1 isotype control (clone MOPC-21, BD Pharmingen, USA), Alexa Fluor® 647-labeled IgG1 isotype control (clone MOPC-21, BD Pharmingen, USA), R-phycoerythrin (RPE)-labeled IgG2a isotype control (clone MRC OX-34, Serotec, Germany), RPE-labeled IgG1 anti-αM (CD11b; clone 2LPM19c, DAKO, Denmark), FITC-labeled IgG1 anti-L-selectin (CD62L; clone Dreg56, BD Pharmingen, USA), Alexa Fluor® 647-labeled IgG1 anti-FCγRIII (CD16; clone 3G8, BD Pharmingen, USA), RPE-labeled IgG2b anti-FcγRII (CD32; clone FLI8.26, BD Pharmingen, USA), FITC-labeled IgG2a anti-CXCR1 (CD181a; clone 42705, R&D Systems Europe, UK), RPE-labeled IgG2a anti-CXCR2 (CD182b; clone 48311, R&D Systems Europe, UK), and FITC-labeled IgG2a anti-C5aR (CD88; clone P12/1, Serotec, Germany).

A FITC-labeled monoclonal phage antibody (A27), which recognizes the active configuration of FcγRII (CD32), was manufactured at the Department of Respiratory Medicine at the University Medical Centre Utrecht (MoPhab A27, UMC Utrecht, The Netherlands) [21, 27]. The functionality and configuration of FcγRII (CD32) on granulocytes is regulated by inside-out control [28]. Visualization of this process by the antibody A27 is a very sensitive means of monitoring the subtle activation of innate immune cells such as neutrophils in vivo.

Blood was collected in a Vacutainer® with sodium heparin as anticoagulant and cooled immediately on melting ice. Blood samples of eight healthy volunteers served as a

control values. Red cells were lysed with ice-cold isotonic NH_4Cl [27]. After lysis, white blood cells were washed and resuspended in PBS2+ [phosphate-buffered saline supplemented with sodium citrate (0.4% wt/vol) and pasteurized plasma protein solution (10% vol/vol)]. Resuspended cells were incubated for 45 min on ice with commercially obtained directly labeled antibodies against activation molecules: L-selectin, αM, CXCR1, CXCR2, C5aR, FCγRII and FCγRIII.

After incubation and a final wash, expression was measured on a FACScalibur flow cytometer (Becton, Dickinson & Co., Mountain View, CA, USA). The neutrophils were identified according to their specific side-scatter and forward-scatter signals.

To measure FcγRII* expression, whole blood was incubated with FITC-labeled monoclonal phage antibody A27 for 45 min on ice [11]. Active upregulation of FcγRII* expression was measured after 5 min of stimulation of whole blood at 37°C with N-formyl-methionyl-leucyl-phenylalanine (fMLP 10^{-6} M) to evaluate the responsiveness of the cells for bacterially derived protein products/peptides. After stimulation, the samples were put on ice again and stained with phage antibody A27. After staining, red cells were lysed, and expression was measured on FACScalibur, as described above.

Data from individual experiments are depicted as the median fluorescence intensity (MFI) of at least 10,000 neutrophils.

IL-6

Plasma samples were obtained at 3, 9 and 24 h after injury and stored at −80°C until further analysis. IL-6 levels were measured by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Ebioscience, San Diego, USA).

Statistics

All data are presented as means ± SE, unless described otherwise. To compare differences in admission variables between patients or control values, the Mann–Whitney U test was used as appropriate. A p value of <0.05 was considered significant.

Results

Patient demographics

Seventeen patients from April 2008 until April 2009 were included in the study. Four patients were eventually excluded because considerable additional injury (AIS > 2) was diagnosed within 24 h after admission. All of the patients, nine of whom were male and four female, had blunt chest injuries. Thorax AIS varied from 2 to 5. The injury mechanism and admission characteristics are listed in Table 1. Four patients were under the influence of alcohol at admission. One of them was also hypothermic, with a body temperature of 35.4°C.

The mean age was 54 ± 4 years, the mean ISS 18 ± 2, and the mean NISS 23 ± 3. One patient was diagnosed with a head AIS of more than 2. This patient suffered from diffuse axonal injury (DAI) without signs of bleeding, edema or compression on CT scan (AIS of 5). Diagnosis was made several days after injury based on clinical presentation. Since the high score assigned to DAI is related to an increased risk of mortality caused by direct brain injury rather than to severity of inflammation, this patient was not excluded from further analysis.

The mean Apache II score was 9 ± 2 and the mean hospital stay amounted to 16 ± 2 days. Six patients needed mechanical ventilation during their hospital stays for a mean duration of 6 ± 2 days. None of the patients developed ARDS. Pneumonia was diagnosed in two patients. One patient developed sepsis due to thoracic empyema and underwent thoracotomy for debridement of the pleural cavity.

Receptor expression on the neutrophil surface

L-Selectin and CD11b

L-Selectin expression was decreased at 9 h postinjury (p = 0.002) and remained decreased until 24 h (p = 0.012) (Fig. 1a). αM expression was significantly decreased at 9 h postinjury compared to control values (Fig. 1b). Although neutrophil activation is typically characterized by αM upregulation, expression of αM declined at 9 h postinjury (p = 0.020) to a minimum in this group of patients. This overall decrease in αM expression is probably due to an increased amount of young neutrophils, which express αM at low levels [29, 30].
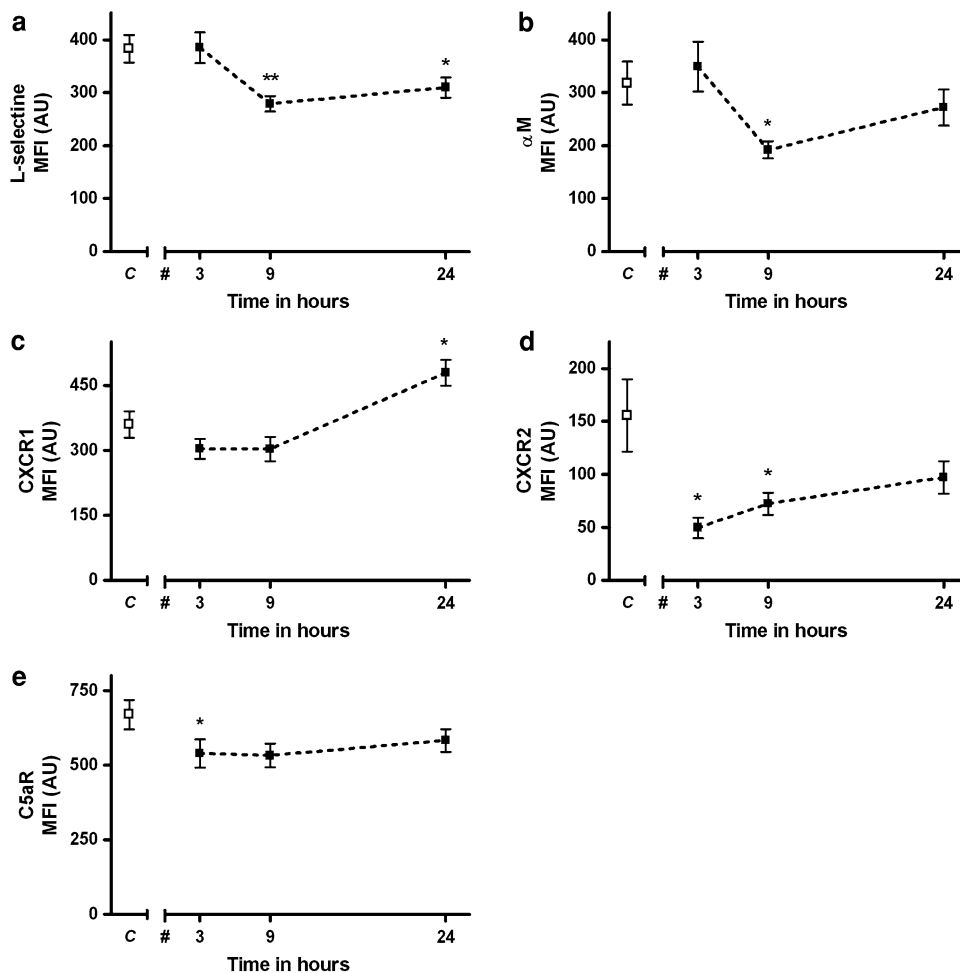
CXCR1, CXCR2 and C5aR

Neutrophil activation is associated with reduced surface expression of CXCR1, CXCR2 and C5aR [31–33]. After chest injury, circulating neutrophils showed a temporary decline in the expression of chemokine receptors CXCR1 and CXCR2 and complement receptor C5aR (Fig. 1c–e). This decline was statistically significant for CXCR2 and C5aR at 3 h postinjury, but not for CXCR1. CXCR2 expression remained low until 9 h postinjury. It has been demonstrated that, upon activation, CXCR2 internalizes more rapidly than CXCR1 [34], which may explain the

Table 1 Patient characteristics

Pt	Gender	Age	Mechanism of injury	Diagnosis	ISS	NISS	Thorax AIS	Apache II	Leukocyte count (×10^9)/l at admission	Complications
1	M	25	MVA	8 unilateral rib fractures Bilateral pneumothorax	16	25	4	3	12.0	None
2	M	62	MVA	3 unilateral rib fractures Clavicular fracture Orbital roof fracture	17	17	3	15	8.0	Pneumonia
3	M	51	Fall from height	2 unilateral rib fractures Unilateral pneumothorax	9	9	3	6	9.0	None
4	F	47	Fall from horse	10 unilateral rib fractures Flail thorax	17	17	4	3	19.3	None
5	M	60	MVA	5 Unilateral rib fractures Unilateral pneumothorax Unilateral lung contusion Pancreatic contusion	21	29	4	9	17.6	Infected epidural catheter
6	M	31	MVA	3 unilateral rib fractures Unilateral pneumothorax Unilateral lung contusion Humeral luxation Clavicular fracture Diffuse axonal injury	38	43	3	18	8.0	None
7	F	67	MVA	5 unilateral rib fractures Flail thorax Unilateral pneumothorax Bilateral lung contusion Fracture of proc. transversus	20	36	4	5	15.6	None
8	M	59	Fall from height	6 unilateral rib fractures Unilateral pneumothorax Unilateral lung contusion	16	25	4	18	15.9	Thorax empyema, sepsis
9	M	69	MVA	3 unilateral rib fractures	5	5	2	5	5.0	Infected epidural catheter
10	F	62	Bicycle accident	6 unilateral rib fractures Minor laceration of kidney Facial hematoma	14	14	3	7	8.5	Pleural effusion
11	F	53	Fall from height	4 unilateral rib fractrures 1 contralateral rib fracture Scapular fracture Fracture of cervical vertebral body	17	17	3	2	11.2	Pleural effusion
12	M	62	MVA	3 rib fractures Bilateral lung contusion Minor liver laceration	20	29	4	15	7.8	Pneumothorax
13	M	59	Attacked by cow	Multiple bilateral rib fractures Bilateral flail thorax Bilateral pneumothorax Bilateral lung contusion Sternal fracture Minor liver laceration	29	38	5	6	18.9	Pneumonia

Fig. 1 Expression of L-selectin (**a**), αM (CD11b) (**b**), CXCR1 (**c**), CXCR2 (**d**), and C5aR (**e**) on the neutrophil surface over time. *Open squares* indicate control values from healthy controls ($N = 8$), whereas *black squares* represent patients ($N = 13$) at 3, 9 and 24 h postinjury. *C*, control; *hash symbol*, time of injury. Data are presented as mean ± SE (*$p < 0.05$,**$p < 0.01$)

more pronounced decline of CXCR2 compared to CXCR1 in our results. The expression of CXCR2 and C5aR was gradually restored during the first 24 h after injury, indicating a transient activation of circulating neutrophils. CXCR1 expression increased above control values at 24 h after injury ($p = 0.039$).

FcγRII, active FcγRII and FcγRIII

Expression of FcγRII was markedly decreased until 24 h after injury ($p < 0.010$; Fig. 2). Expression of the active form was slightly lower in trauma patients compared to control values, although this decline did not reach statistical significance. Expression of fMLP-induced active FCγRII, however, was significantly decreased until 9 h after injury ($p = 0.006$). Expression of FcγRIII evidently dropped during the first 24 h after chest trauma ($p < 0.001$). FcγRIII is normally expressed at lower levels on young (banded) neutrophils compared with more mature forms [35]. Therefore, this decrease in overall FcγRIII suggests an influx of young neutrophils.

IL-6 levels

IL-6 levels were significantly enhanced at 3 h postinjury compared to control values [mean concentration of 44 ± 15 versus 0 pg/ml ($p < 0.001$)]. IL-6 levels further increased to a maximum mean concentration of 86 ± 31 pg/ml ($p < 0.001$ compared to control values) 24 h after blunt chest injury (Fig. 3).

Discussion

In this study we show that blunt chest injury leads to a systemic activation of circulating neutrophils, characterized by the shedding of L-selectin and the downregulation of CXCR2 and C5aR. It furthermore shows that blunt chest injury is associated with the mobilization of young (FcγRIII-low) neutrophils and with a reduced responsiveness of circulating neutrophils to an inflammatory stimulus.

Although seven patients had a chest AIS of ≥4, and two of these had a bilateral pulmonary contusion, none of these

Fig. 2 Intrinsic expression of FCγRII (**a**), active FCγRII (**b**), fMLP-induced expression of active FCγRII (**c**), and expression of FCRγIII (**d**) on the neutrophil surface during time. *Open squares* indicate control values from healthy controls ($N = 8$), whereas *black squares* represent patients ($N = 13$) at 3, 9 and 24 h postinjury. *C*, control; *hash symbol*, time of injury. Data are presented as mean ± SE ($*p < 0.05, **p < 0.01$)
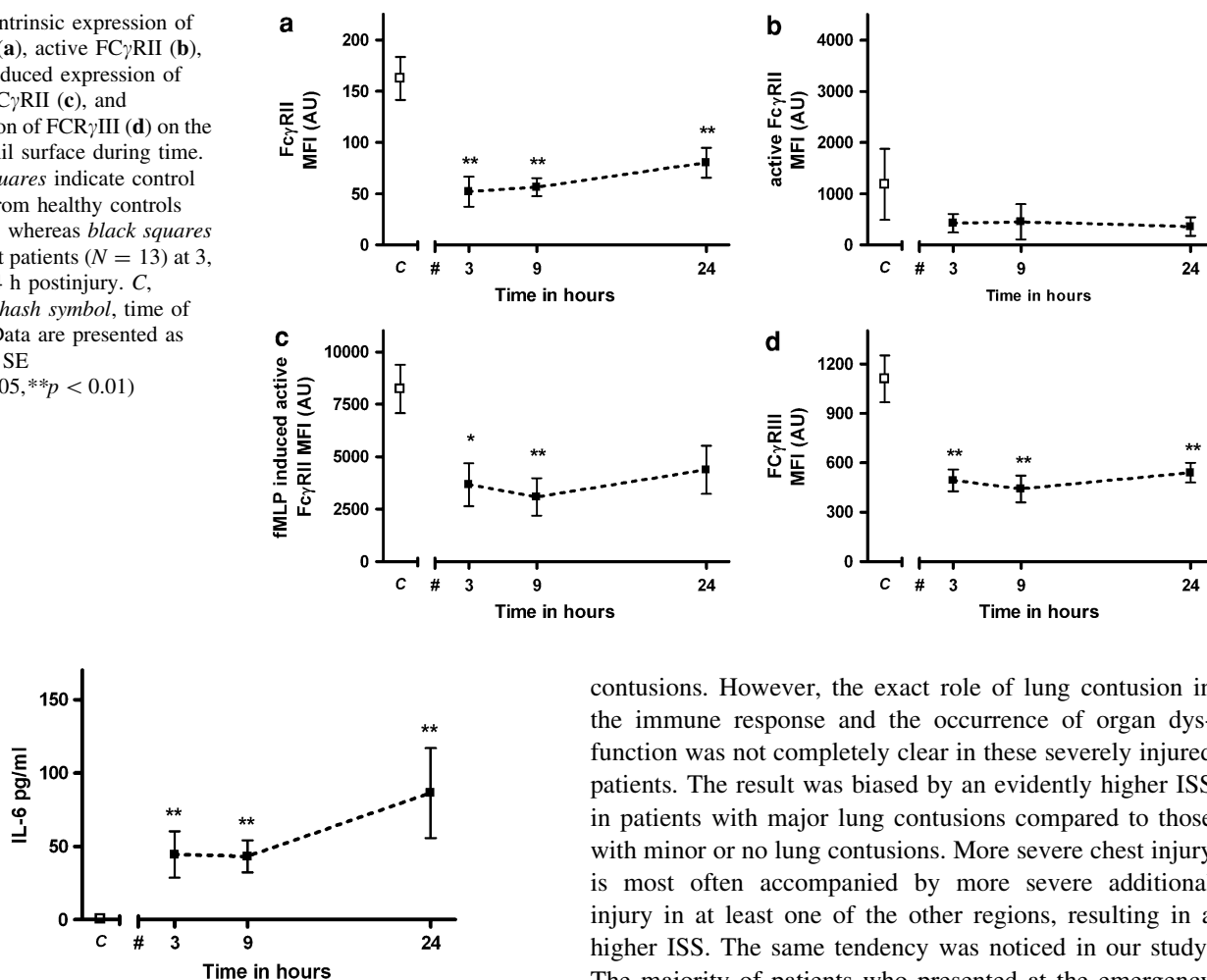
Fig. 3 Levels of circulating IL-6. *Open squares* indicate control values from healthy controls ($N = 8$), whereas *black squares* represent patients ($N = 13$) at 3, 9 and 24 h postinjury. *C*, control; *hash symbol*, time of injury. Data are presented as mean ± SE ($**p < 0.01$)

patients developed ARDS. Lung injury results in endovascular changes, tissue barrier failure, and locally increased cytokine levels, enabling systemic activated neutrophils to infiltrate the parenchyma. Despite vast local damage in the abovementioned cases, the innate immune response provoked was apparently not abundant enough to cause ARDS. Similar findings were found in an earlier study performed by Maier et al. [4]. This study showed that isolated lung contusion resulted in an increase in circulating IL-6, but it did not cause ARDS. These findings involved patients with minor as well as major lung contusions (based on CT lung injury score). The same study also described an enhanced inflammatory response, with significantly increased levels of circulating IL-6 and IL-8 plus significantly elevated multiple organ failure (MOF) scores in multi-trauma patients with major lung contusions compared to multi-trauma patients with minor or no lung

contusions. However, the exact role of lung contusion in the immune response and the occurrence of organ dysfunction was not completely clear in these severely injured patients. The result was biased by an evidently higher ISS in patients with major lung contusions compared to those with minor or no lung contusions. More severe chest injury is most often accompanied by more severe additional injury in at least one of the other regions, resulting in a higher ISS. The same tendency was noticed in our study. The majority of patients who presented at the emergency department with severe chest injury could not be included due to considerable injuries in regions other than the thorax. These challenging inclusion and exclusion criteria resulted in the inclusion of fewer patients, but also a more homogeneous group.

Earlier studies concerning multi-trauma patients demonstrated that fMLP-induced active-FcγRII expression was decreased in patients compared to controls, and that the expression was negatively correlated with ISS and adverse outcome [11, 20]. A clear difference in the median fMLP-induced expression of active FcγRII, measured during the first 24 h after injury, was seen between patients with ALI/ARDS (low expression) and patients with an uneventful course (high expression) [20]. The median values for the fMLP-induced expression of active FcγRII measured in this study are comparable to multi-trauma patients with an uneventful clinical course. These findings support the hypothesis that isolated chest injury induces only a restricted activation of the systemic immune response.

The increased number of circulating neutrophils after chest injury is most likely due to mobilization of young neutrophils, as displayed by a decrease in overall FcγRIII

expression. However, delayed neutrophil apoptosis has been described after trauma [36, 37]. Although this phenomenon remains to be established in vivo, we cannot exclude that the total number of neutrophils are biased by disturbed apoptosis, since apoptotic markers such as annexin V were not measured.

The fact that isolated chest injury rarely leads to ARDS contrasts with other conditions in which the lung is locally affected and this complication is frequently seen, such as during pneumonia or after aspiration [25, 38, 39]. Presumably, the systemic innate immune response evoked by isolated chest injury is less extensive than that during infection or after aspiration. In a rodent model, Hoth et al. [40] demonstrated that lung injury followed by exposure to *E. coli* lipopolysaccharide (LPS) leads to massive neutrophil infiltration and lung damage, whereas tissue infiltration and damage was far less after LPS or lung injury alone. In addition, serum IL-6 levels were significantly increased compared to LPS exposure or lung injury alone. They concluded that lung injury primes the systemic innate immune response, as was suggested by Maier et al. [4]

In this study we show that the systemic innate immune response caused by isolated chest injury is transient and short. Although IL-6 levels remained elevated until 24 h after injury, activation of circulating neutrophils was partially restored. However, this mild systemic response may be sufficient to enhance the innate immune response caused by a second hit such as concomitant tissue damage, fat emboli, or infection.

We therefore suggest that lung damage alone is not likely to result in an ARDS, but a synergism between inflammation caused by lung injury and an additional stimulus caused by a second hit results in a markedly increased risk of developing ARDS.

Conclusion

In this study we demonstrated that isolated blunt chest injury caused transient systemic activation of neutrophils together with a mobilization of young neutrophils into the peripheral circulation. In addition, only severe chest injury (AIS > 4) results in an increased number of circulating neutrophils. However, it seems that chest injury alone is not sufficient to cause ARDS in these cases; a second hit may be needed.

Conflict of interest None.

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