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EFFECTS OF THORACIC TRAUMA ON *IN VITRO* EFFEROCYTIC AND PHAGOCYTIC ACTIVITY OF ALVEOLAR MACROPHAGES UNDER NORMOXIC, HYPOXIC AND HYPERCAPNIC CONDITIONS

DISSERTATION

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"*Wisdom begins in wonder*"

Socrates

"*The true sign of intelligence is not knowledge, but imagination*"

Albert Einstein

"I don't pretend we have all the answers.

But the questions are certainly worth thinking about."

Arthur C. Clarke

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Abbreviations

AM	Alveolar macrophage/s
ANOVA	Analysis of variance
APC	Allophycocyanine
aPMN	Apoptotic polymorphonuclear neutrophils
ARDS	Acute respiratory distress syndrome
AT2-cells	Alveolar type two cells
BAL	Bronchoalveolar lavage
CARS	Compensatory anti-inflammatory response syndrome
CO_2	Carbon dioxide
COPD	Chronically obstructive pulmonary disease
DAMP	Damage-associated molecular pattern
DiD	Lipophilic tracer: 1, 1' – Dioctadecyl - 3, 3, 3', 3' – tetramethylin - dodicarbocyanine, 4 - chlorobenzensulfonate
ECMO	Extracorporeal membrane oxygenation
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia
ELISA	Enzyme-linked immunosorbent assay
et al.	Et alii
FACS	Fluorescence activated cell sorting
FITC	Fluorescein-isothiocyanate
FSC	Forward scatter
GRO/KC	Growth-regulated oncogene/keratinocyte-derived chemokine
h	Hour
HBSS	Hank's Balanced Salt Solution
ic	Intra cutaneous

IL	Interleukin
iv	Intra venous
LPS	Lipopolysaccharide
MCP-1	Macrophage chemoattractant protein 1
MIP-1	Macrophage inflammatory protein 1
MOF	Multiple organ failure
NaCl	Sodium chloride
O_2	Oxygen
PAH	Pulmonal artery hypertonia
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffering solution
PE	Phycoerythrin
PMN	Polymorphonuclear neutrophils
PS	Phosphatidylserine
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RBC	Red blood cells
PBMC	Peripheral blood mononuclear cell
ROS	Reactive oxygen species
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute (cell culture medium)
sham	Imitation control group without intervention
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential sepsis-related Organ Failure Assessment Score
qSOFA	Quick Sequential Sepsis-related Organ Failure Assessment Score
SSC	Side-scatter
TNF-α	Tumor necrosis factor α
TxT	Thoracic Trauma (Blunt chest trauma)

1. Introduction

1.1. Epidemiology

Thoracic trauma (TxT) is frequent and ranges widely from some organ abrasions to life-threatening injuries. Probably the oldest recorded and proven incident of death via blunt chest trauma (TxT) involved the Egyptian "boy-king" Tutankhamun. His reign was short-lived and his decease, according to Harer et al. was consectaneous to "massive crushing and tearing injury to his chest" [30]. The numerous possible complications after chest injury include tension pneumothorax, massive hemothorax, acute airway obstructions, flail chest, vascular injury and cardiac tamponade to name just a few [80]. Other sequelae are potential life-threatening contusions and ruptures. According to the German Trauma Registry [26] the most common injury mechanisms are blunt accounting for 95.2% of reported cases, of which 61.6% involve the thoracic area [45]. Approximately 33% of these injuries necessitate hospital admission. Overall, blunt thoracic injuries are directly responsible for 20-25% of all deaths, and are involved as a major contributor in further 50% of deaths [46].

The vast majority of TxT in the United States is induced by motor vehicle accidents, thus representing a frequent reason for admission to the emergency room. According to the National Safety Council report 2009, 1 out of 108 deaths were caused by a motor vehicle crash.

Multiply injured patients with concomitant chest trauma have an increased risk of developing pneumonia and acute respiratory distress syndrome (ARDS). TxT can also result in systemic inflammatory response syndrome (SIRS) and consecutive multiple organ failure (MOF).

1.2. Blunt chest trauma - a clinical approach

Blunt chest trauma is a commonly presented entity for a physician. Occupying most of the thoracic cavity, the lung is very susceptible to trauma. A patient, who suffers from thoracic trauma (TxT) usually presents - depending on the injury severity – with cyanosis, pain and

hematoma on the afflicted area, loss of breath, flail chest and bone fractures. Patients after TxT may show macroscopic hemorrhage and petechial bleeding of the lungs (Figure (Fig.) 1), caused by the pure physical trauma impact, initiating tissue tearing and micro vascular rupture, which can also be seen in conventional chest x-ray as massively contused lungs (Fig. 2). If trauma was severe and rupture to the lung tissue or the vascular system occurred, it can lead to a pneumo- or hemothorax, causing atelectasis, which then results in impaired gas exchange and impaired oxygen supply. The drastic ventilation-perfusion mismatch resulting from the inadequate gas exchange, significantly diminishes lung function and subsequently may lead to respiratory failure [39]. Respiratory failure then develops hypoxic and hypercapnic milieux in the undersupplied areas of the lung. Diagnostically, a sudden drop in arterial blood gas oxygen levels below 60 mmHg and increase in carbon dioxide levels above 50 mmHg may reflect these pathophysiological and functional changes [9]. Initial treatment strategies of ARDS should include lung protective ventilation, using low tidal volumes and low-pressure peaks at high breathing frequencies. Ultima ratio is the extracorporeal membrane oxygenation (ECMO) providing an artificial O_2/CO_2 exchange [66].

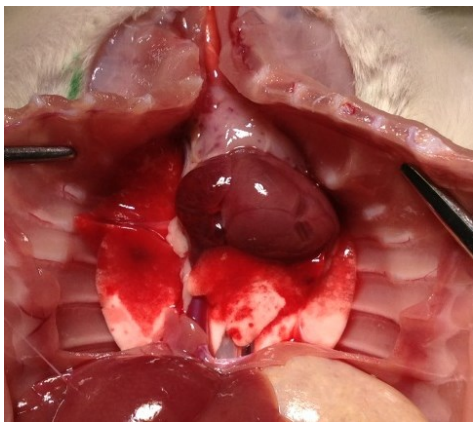


Figure 1: Thorax after sternotomia, showing contused lung parenchyma and petechial bleeding in the lungs of a rat.
Illustration by Michael Eichner

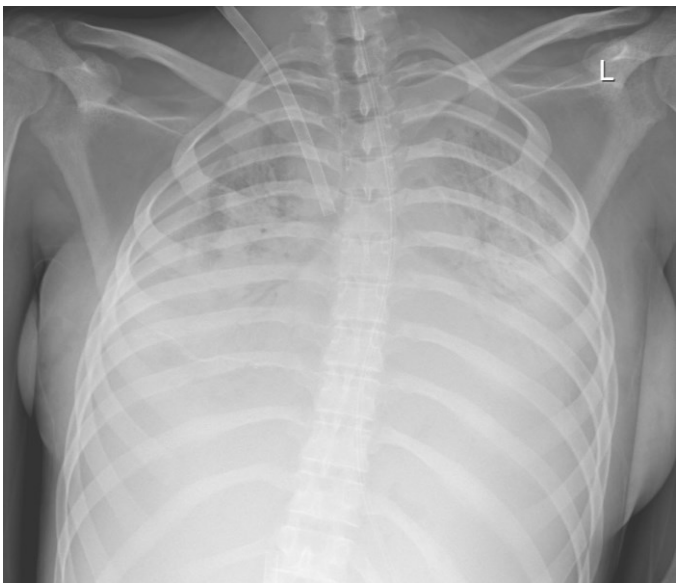


Figure 2: Conventional chest x-ray supine (age 19, female) showing subtotal bilateral opacity with atelectatic areas after thoracic contusion. Image morphologically reconcilable with ARDS. ECMO in situ.
Courtesy of Kantonsspital St. Gallen, Klinik für Radiologie und Nuklearmedizin, reprinted with permission.

1.3. The lungs - Morphology and physiology

Air streams through the trachea towards the carina, which splits into left and right primary bronchus. The primary bronchus divides further into 2 lobar bronchi on the left and 3 on the right. Anatomically the right primary bronchus is steeper compared to the left primary bronchus. The 5 lobar bronchi shade into 19 to 20 segmental bronchi, which further divide into more bronchioles, ending blind in alveoli. The lungs microarchitecture is constructed of roughly 300 million alveoli, which measure between 0.2-0.3 mm in diameter. Considering an area, coated with the inner lining of all alveoli combined, it is estimated to measure between 80 to 140 m^2 in the adult lungs. Adjacent to the alveolus the 5 µm thin capillaries are located. Alveoli are lined with flat alveolar type I and rectangular type II pneumocytes. The first facilitate blood-gas-exchange, while the function of the latter is mainly production and secretion of surfactant, a heterogeneous lipid-protein blend important to reduce surface tension and thus preventing alveolar collapse during expiration.

1.4. The lungs - Pathophysiology

1.4.1. Lung edema

Pulmonary edema denotes pathological accumulation of enclosed liquid, too much to be capably evacuated by lymphatic drainage, either inside the alveoli or the interstitium around the alveoli. The underlying cause can be cardiogenic or non-cardiogenic. A cardiogenic edema normally originates from too high hydrostatic pressure, triggered for example by left ventricular output failure or stenosis in pulmonary veins. Non-cardiogenic edema develops via an imbalance in the osmotic equilibrium. This can be due to a protein concentration mismatch or increased vascular permeability as seen in ARDS [32, 42].

1.4.2. Acute respiratory distress syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) represents a massive pulmonary inflammation triggered by primary pulmonary or systemic damaging factors. Besides lung embolism, aspiration, pneumonia, and medications (e.g. bleomycin), ARDS can rise from polytrauma. By definition, however, the underlying pathomechanism has originated from non-cardiogenic conditions to be termed as ARDS [86].

Depending on the severity, the clinical symptoms of patients with ARDS may vary and are categorized in three stages. During stage one arterial hypoxemia sets in, accompanied by compensatory hyperventilation and consecutive respiratory alkalosis. Stage two comprises progressive dyspnea, a rapid decrease in partial oxygen pressure and increased heart rate with beginning signs of bilateral diffuse infiltration on chest x-rays (Fig. 2), positive air bronchogram and pleural effusion. Stage three encompasses global respiratory insufficiency, which occurs in 31% of all patients administered with severe thoracic trauma. Patients in this stage are most likely to be cyanotic and unconscious, due to acute circulatory failure, arising in 26% of patients [87]. A common complication of ARDS is pneumonia, raised from nosocomial infections in non-invasively ventilated and intubated patients [15].

Pathomechanically, ARDS proceeds in three stages as well [42, 66]. The first stage represents the exudative stadium, in which protein-rich interstitial lung edema originates, due to diffuse damage of the lung parenchyma [66].

The second stage is the progression of the latter into an alveolar lung edema, inactivation of surfactant, the formation of hyaline membranes and atelectasis, as well as disturbances in perfusion, permeability and coagulation at the site of the alveoli. The post-traumatic apoptosis of alveolar type 2 pneumocytes, which constitute roughly around 15% of the lung's parenchymal cells, is triggered via the extrinsic death receptor pathway [73, 74] and causes loss of surfactant and enhancement of the phagocytic activity of AM [73].

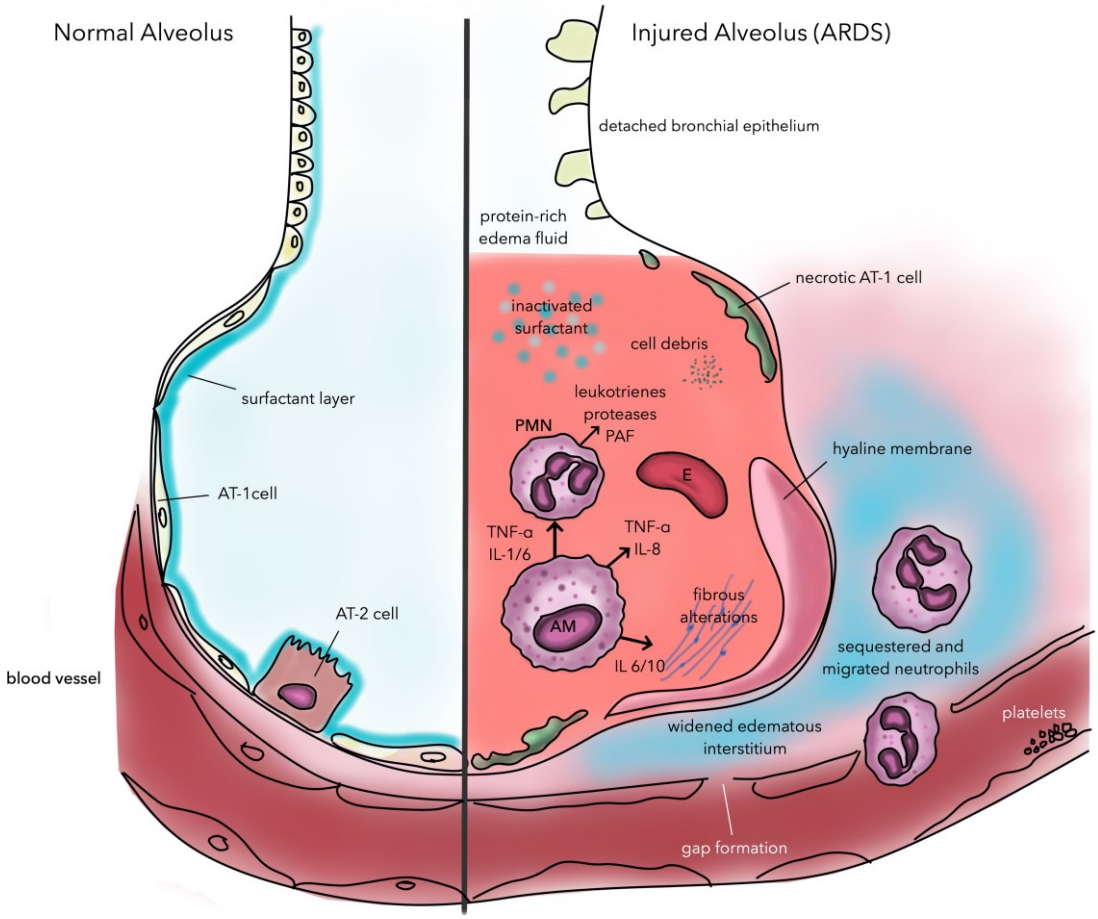


Figure 3: Alveolus, normal and compromised through the effects of ARDS. Conforms ARDS stage 3. AM: alveolar macrophage; AT: alveolar type; E: erythrocyte; PAF: platelet-activating factor; PMN: polymorphonuclear neutrophils

Illustration by Michael Eichner

The third stage represents the proliferative stadium with the development of fibrotic alterations, hence irreversible lung damage. In ARDS significantly increased levels of IL-8 in bronchoalveolar lavage (BAL) were found [33]. An additing of the intra-cellular adhesion molecule ICAM-1 seems key along with locally expressed β-2-integrins, to promote post-traumatic immigration of neutrophils and monocytes to the site of trauma in post-capillary venules [35, 60, 68, 71, 72, 74].

In the clinical setting of ARDS the severity of lung injury and the consecutive physiological dysfunction appeared highly dependent on the presence of neutrophils (PMN), which are mainly recruited by chemotactic factors and receptors for neutrophils (CXCL 1 and 2 and CXCR2). The number of recruited neutrophils detected in the alveolus of the patient is

directly proportional to an increased mortality [88]. The invaded PMN not only may damage tissue as a bystander effect to clear bacteria but also undergo apoptosis [71, 73].

1.5. Inflammation post TXT - SIRS, CARS and Sepsis

Trauma can challenge the human body by both, septic and sterile environments, the latter referring to blunt impacts. Open wound or septic trauma usually exposes the patient to pathogen-associated molecular patterns (PAMP), whereas blunt, or sterile trauma rises from damage-associated molecular patterns (DAMP), which originate mainly from the cytoplasm of damaged cells. PAMPs and DAMPs are both responsible for the genesis of systemic inflammatory response syndrome (SIRS).

SIRS describes a generalized hyper-inflammation triggered by self-sustaining and uncontrolled cytokine release. If in addition bacteria (e.g. positive blood cultures) are detected, the condition is defined as sepsis. Complement activation products (especially C5a), leukotriene B4, MIP-1α, TNF-α, IL-1β and IL-6 are predominantly involved in the early response of SIRS [35]. IL-6 is immediately released after trauma and can be used to evaluate the impact of injury [25]. IL-6 and IL-1β are pyrogenic cytokines and known to be responsible for up-regulation of the thermoregulatory preoptic-anterior hypothalamus, thus inducing fever [9]. TNF-α, MIP-1α and IL-1β activate circulating neutrophils and monocytes and enhance their sequestration into damaged tissue areas and especially the lung. The monocytes later mature into AM [39, 73].

ARDS is often associated with SIRS. Patients suffering from SIRS must fulfill at least two of the following criteria:

- Body temperature below 34°C, or above 38°C
- White blood cell count below 4000 cells/mm^3, or above 12000 cells/mm^3
- Tachypnea with more than 20 breaths per minute, or pCO_2 less than 4.2 kPa (32 mmHg)
- Heart rate above 90 beats per minute

In February 2016 the American Medical Association has renewed the sepsis definition as

life-threatening organ dysfunction caused by a dysregulated response to infection and modulated the criteria for a quicker and more clinical approach. The old SIRS-criteria basically focused on the inflammatory response parameters in the human body, as well as the basic vital parameters. According to an international task force, an inflammatory reaction helps the body to overcome a septic challenge and is not always harmful, thus should not be used to monitor the severity of sepsis and the patient's outcome. Subsequent to infection, organ dysfunction can be identified as an acute change using the Sequential sepsis-related Organ Failure Assessment Score (SOFA), which monitors pulmonary function via partial oxygen pressure, basic mental alterations using the Glasgow Coma Score, circulatory function by measuring middle arterial pressure or the use of vasopressors, liver function via bilirubin levels, blood coagulation by monitoring thrombocyte count and renal failure via creatinine blood levels [75].

A new quick SOFA score (qSOFA) was also implemented as a quick roundup solely focusing on the patient's altered mental status, systolic blood pressure below 100 mmHg and an elevated respiratory rate above 22 per minute. In future the qSOFA should help to identify a septic challenge much quicker [75].

Positive about the new scoring system is the monitoring of general organ dysfunction, which can also be used to document the clinical course more easily. However, the new scoring system has yet to prove to be practicable and beneficial in everyday clinical use.

Generalized edema may develop during SIRS and sepsis, leading to hypovolemia, thus activation of the hemodynamic system and the coagulation cascade, which can exacerbate into disseminated intra-vascular coagulation (DIC). Worsening prognostic factors include thrombocytopenia, acute encephalopathy, metabolic acidosis and acute renal dysfunction. Shock is the main feared deuteropathy, which can trigger and drive multi-systemic organ dysfunction.

Synchronically to SIRS, co-exists the compensatory or counter-regulatory anti-inflammatory response syndrome (CARS). It describes the systemic deactivation of the immune system and release of various anti-inflammatory mediators such as IL-10 [85], in order to recreate homeostasis. CARS was previously thought to succeed SIRS, but new research has enlightened an almost parallel progressive pattern. (Fig. 4)

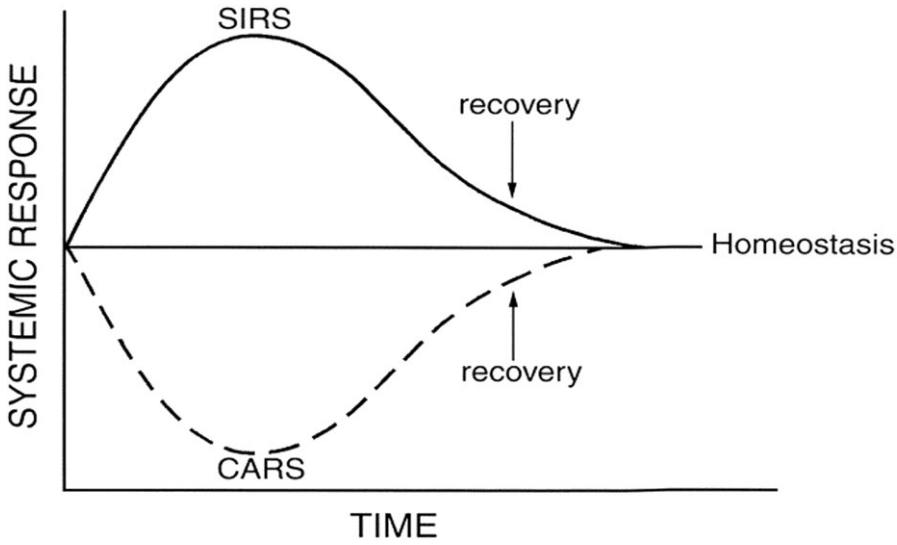


Figure 4: SIRS and CARS and their chronological order.

From the Journal of Bone and Joint Surgery, 2005 Feb; 87(2): 434-449

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1.6. *Escherichia coli*

For the lung is one of the few organs largely interfacing directly with the surrounding environment, it is highly prone to infection. In an intensive care study, 4.4% of patients developed pneumonia due to *E. coli* infection, making it the second most common cause of bacteriogenic pneumonia [47]. Those particular patients were in general older and female. In this context, a relation between continuous application of proton pump inhibitors and other acid reducers could cause ascending infections with *E. coli* [47]. In addition, the activity of inflammatory cells, necessary to combat gram-negative bacteria is reduced e.g. after TxT. This results in decreased bacterial clearance and consecutively to increased mortality [19].

1.7. Hypoxia

Hypoxia is known to increase inflammatory levels in mountain sickness, further developing vascular leakage and causing pulmonary and cerebral edema [28, 29]. Healthy subjects,

who experienced three days and nights at an altitude of around 3400 m showed increased IL-6 and CRP concentrations in blood samples [31].

During hypoxia, the IL-8 response is up-regulated by AM, caused by oxygen-sensing prolyl hydroxylases [33]. Under normoxic conditions, they are responsible for hydroxylation of the alpha subunit of the hypoxia-inducible transcription factor (HIF). HIF is built of two subunits, usually HIF-1α, which is ubiquitously represented, or HIF-2α, which is bound to certain tissues and HIF-1β. A hydroxylation in the α-subunit causes the subunit to bind to Von-Hippel-Lindau protein, which in turn leads to ubiquitylation and proteasomal degradation and thus no transcriptive induction of pro-inflammatory mediators [78]. However, during hypoxia HIF works as a nucleosomal transcription factor, further inducing TNF-α and NF-κB pathways. These are involved in TLR regulation and promote an increase in phagocytosis, leukocyte recruitment and overall trigger adaptive immunity [5, 34].

The HIF pathway also contributes to the angiogenesis in various neoplasias via vascular endothelial growth factor (VEGF) induction, is necessary for keratocyte immigration to wounds, [6] but also in the feared TLR4 up-regulation, as seen in ischemia of donor kidneys [41]. Elevated inflammatory levels may also be seen among obese individuals, because, the fatty tissue is physiologically undersupplied with oxygen and thus triggers the HIF pathway, as recently discussed on 15th congress of the European shock society in Vienna [70].

1.8. Hypercapnia

Hypercapnia is derived from the greek “ηζπερ” and “καπνοσ”, meaning “above” “smoke”. It is the condition of abnormally elevated blood levels of carbon dioxide (CO_2) and plays a highly important role in the body's metabolism. Carbon dioxide blood gas levels above 45 mmHg define hypercapnia. Excess supply of CO_2 as achieved by hypoventilation leads to respiratory acidosis. It is also seen in patients with chronic obstructive pulmonary disease (COPD), which affects more than 20 million people in the U.S. and is also present in ARDS. In contrast drop of CO_2 causes respiratory alkalosis and can be caused by hyperventilation. Some studies have shown that hypercapnia markedly enhances the inflammatory response 24 h after exposure to hypercapnia in endotoxin stimulated rats [57]. Hypercapnia causes

an up-regulation of IL-1 β and IL-6 and a down-regulation of IL-10. Hypercapnia may also protect against ventilation-induced acute lung injury, which can be attributed to the acidosis-mediated protection from stretch injury by a NF-κB related pathway [16, 57]. In clinical setting, hypercapnia is considered a risk factor for mortality in hospitalized patients, suffering from community-acquired pneumonia [84].

1.9. The alveolar macrophage – a gatekeeper

Alveolar macrophages (AM) represent the lungs first line of host defense. Myrvik et al. were the first to describe the possibility to harvest AM via bronchoalveolar lavage (BAL) in 1961 [54]. Since then BAL has been widely used for pre-clinical and clinical research. The average amount of AM harvested in BAL fluid is around 10^5 cells per ml BAL fluid in nonsmoking subjects. Smokers have shown to host around four times as many [56].

AM originate from blood monocytes, stemming from precursor cells in hematopoietic organs [27, 71]. The term macrophage originates from the greek “πηαγειν”, which roughly can be translated into “eating”. AM are mostly resident in the lungs' alveoli and play a substantial role in the immune defense against inhaled particles and pathogens such as bacteria and aerogenous transmitted viruses. Pathogens can also be carried into the lungs after sharp thoracic trauma and via hematologic distribution during septic challenge. PAMPs and DAMPs exposure ultimately triggers a pro-inflammatory reaction elicited through AM’s release of multiple mediators, which next to other functions recruit further defense cells and stimulate resident cells, creating a pro-inflammatory micro-milieu (Fig. 4). Cytokines like IL-1β, IL-6 and TNF-α and chemokines like KC, MIP-2, MIP-1α and MCP-1, complement-activating factors, alarmins and arachidonic acid metabolites contribute to the development of the pro-inflammatory milieu.

An important function of the AM is to remove not only foreign particles and substances, but also endogenous apoptotic cells. The term efferocytosis, which describes the physical removal of apoptotic cells only, is however rarely used in this manner. During efferocytosis, apoptotic cells like aged PMN release specific signaling molecules, to indicate their status and to be removed. Signals like nucleotides, lysophosphatidylcholine and

fractalkine (CX3CL1) help the AM to find the apoptotic cell [23]. Signals on the apoptotic cell surface, including phosphatidylserine (PS) and calreticulin, indicate its need to be removed (the so called “eat me” signals). Counterparts on the AM surface are specific receptors, such as PS- and vitronectin receptors, mer and stabilin-2, which can bind via bridging molecules, to commence removing the apoptotic cells [81]. Efferocytosis is mainly regulated by a balance of Rac-1 and RhoA, two important GTPases necessary for signal transduction in diverse cells. Rac-1 acts as a general promoter for efferocytosis, whereas RhoA can inhibit the efferocytic activity. [81]

Since AM lie adjacent to the alveolar capillary bed/sustenance system, transmission of released chemoattractants and mediators to distant organs and host defense cells operates instantaneously. Post-traumatic AM activation leads to MCP-1 and MIP-2 release as well as pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines [56]. Increased expression of FAS receptor on the AM surface and release of FAS ligand ensues. This triggers an alteration in cytokine release, in which IL-6 release is enhanced and IL-10 levels decrease. MCP-1 levels remain unaltered [72]. Released MCP-1, MIP-1α, MIP-2 and complement activating factors (e.g. C5a) cause migration of peripheral monocytes and neutrophils. Infiltrated and recruited neutrophils mount an oxidative burst response and may undergo subsequent apoptosis. The earlier described FAS ligand release affects nearby AT-2 cells and triggers additional apoptosis. Residual cell waste of infiltrated PMN and AT-2 cells is then phagocytized by AM. Studies have shown a measurable up-regulation in toll-like receptor 4 (TLR4) - expression 48 h post-pulmonary contusion. The TLR4 pathway plays a pivotal role in the host defense against gram-negative bacteria like *Pseudomonas aeruginosa* and *E. coli* [79].

AM not only find function in defense against invading pathogens and chest trauma, they also play a pivotal role in ischemia-reperfusion injury (e.g. occurring after cardiopulmonary surgery). This provokes a massive up regulation of the nuclear transcription factor kappa-light-chain-enhancer NF-κB, which is vital for cytokine production and cell survival. It is also involved in JNK/AP-1 activation via FAS signaling [58].

Furthermore, ischemia reperfusion injury contributes to an increase in phospholipase A_2 activity in the cytosol of the AM. This subsequently increases Thromboxane release, thus causing pulmonary artery hypertonia (PAH). Close to AM, AT-2 cells in the alveolar wall are

activated by TNF-α and in response release chemoattractants especially KC and MIP-2, supporting neutrophil migration [36, 76]. Increase in vascular permeability (induced by leukotrienes), neutrophil migration to the lungs and PAH all contribute to the genesis of pulmonary edema, which dramatically aggravates the patient's outcome [4].

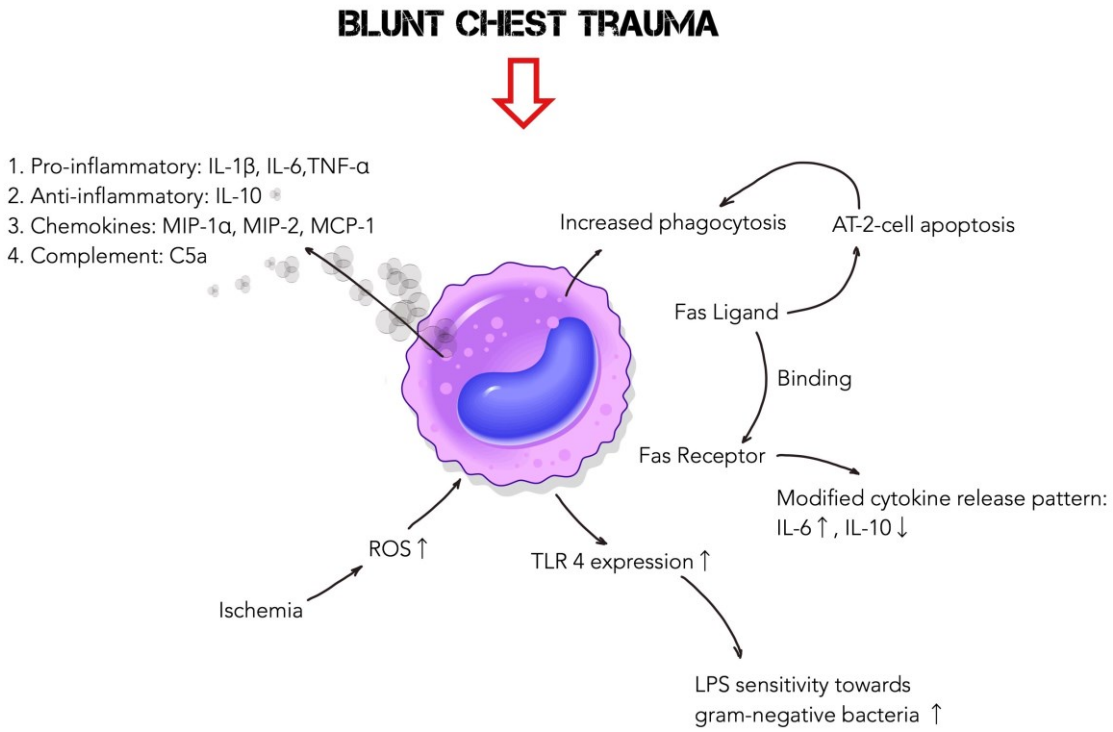


Figure 5: Alveolar macrophage and its reaction to blunt chest trauma.

Illustration by Michael Eichner

Another important aspect of trauma can be hemorrhagic diathesis in lungs during shock development. An insufficient perfusion means malnutrition of the lung tissue and oxygen deprivation. In consequence, cellular hypoxia develops and the concentration of reactive oxygen species (ROS) increases. The oxidative stress triggers defensive algorithms of AM, as seen in an enhanced lipopolysaccharide-induced inflammatory response after ischemia-reperfusion injury [56]. This increased lipopolysaccharide (LPS) sensitivity may be explained by the ROS-induced TLR4 expression [62]. If bacterial LPS now stimulate AM, TLR4 signaling pathway activation leads to TNF-α release [77] and IL-10 increase, as well as a decrease in IL-1 β release [49, 53, 55].

In the context of trauma, the AM plays also an important role in burn injury. During a thermal injury, toxic metabolites, mostly oxygen derived, may arise, which activate AM 3-4 days after trauma [56, 67]. Roughly after one week, an increase in IL-6, MIP-1 β and RANTES release can be measured, without enhancement in TNF-α, KC, MCP-1 and MIP-1α [62]. After burn injuries, the AM phagocytic activity seems generally diminished [52], leading to insufficient clearance of bacterial agents. This may finally lead to subsequent pneumonia and sepsis.

1.10. Study design and hypothesis

This dissertation relies on a prospective, randomized, controlled, observational experimental study and is based on recent findings regarding the induction of AT-2 cell apoptosis and migration of PMN to the alveoli following TxT [74]. Apoptotic AT-2 cells and latex beads were co-incubated with alveolar macrophages (AM) after TxT to show that TxT enhances the clearance activity of AM [73].

The next step for understanding the altered post-traumatic function of AM is to focus on the efferocytic and phagocytic behavior *in vivo* after TxT. Efferocytosis describes the ingestion of apoptotic cells only and should be distinguished from phagocytosis, which represents all other remainders of cells and particles.

Apoptotic polymorph nuclear neutrophils (aPMN) and *Escherichia coli* (*E. coli*) strains can be used to differentially examine the change on efferocytic and phagocytic behavior of AM *in vivo* after separate instillation into rat's lungs.

Furthermore, trauma to the lung inflicts atelectasis, or less ventilated areas, thus creating sites of hypoxia and hypercapnia. These changed environmental conditions may in addition affect the ingestion activity, as well as cytokine release of AM.

Therefore the following hypotheses are proposed:

1. TxT leads to an alteration of the lung immune system in terms of up-regulation of the efferocytic and phagocytic activity of AM.

2. The up-regulation of efferocytic and phagocytic activity is affected by partial hypoxic and hypercapnic conditions.

3. The assumed post-traumatic anti-inflammatory response of AM is modified by hypoxic and hypercapnic conditions.

2. Material and methods

2.1. Laboratory animals

The animals subjected to this well-established trauma model were wild type, male Sprague-Dawley rats (CD-Rats, *Rattus norvegicus*, Charles River, Sulzfeld, Germany) with a weight of 251 to 353 g. The experiments were approved by the Federal Animal Care and Use Committee (permit number 1051). The animals rest in the animal care facility for 14 days post arrival, to limit the stress factor induced by relocation. The animals were given ad libitum access to food (Altromin 1314 Forti total pathogen free, Altromin International, Lage-Lippe, Germany) and water. The supervising veterinarians ensured the strict hygiene standards.

2.2. Flow cytometry

The basic idea behind fluorescence-activated cell sorting (FACS) is to analyze thousands of particles in real time according to their individual properties. The optical signal emission of individual cells is used to identify each particle while passing through a triple laser array. A FACSCanto™ II (Fig. 6A) by the company Beckton Dickinson was used in this study.

A carrier fluid is used to guide the cells to be examined singularly with a laminar flow into a micro cuvette by creating a sheath around the probe liquid. (Fig 6B) The cells then pass the triple laser array, consisting of a blue (488 nm, air-cooled, 20 mW solid state), a red (633 nm, 17 mW HeNe) and a violet (405 nm, 30 mW solid state) laser component [3]. The lasers excite either a specially designed fluorescent dye, which had been applied beforehand, or the cell itself, due to its special properties. The emitted light signal is then captured by a complex trigon or octagon detector array.

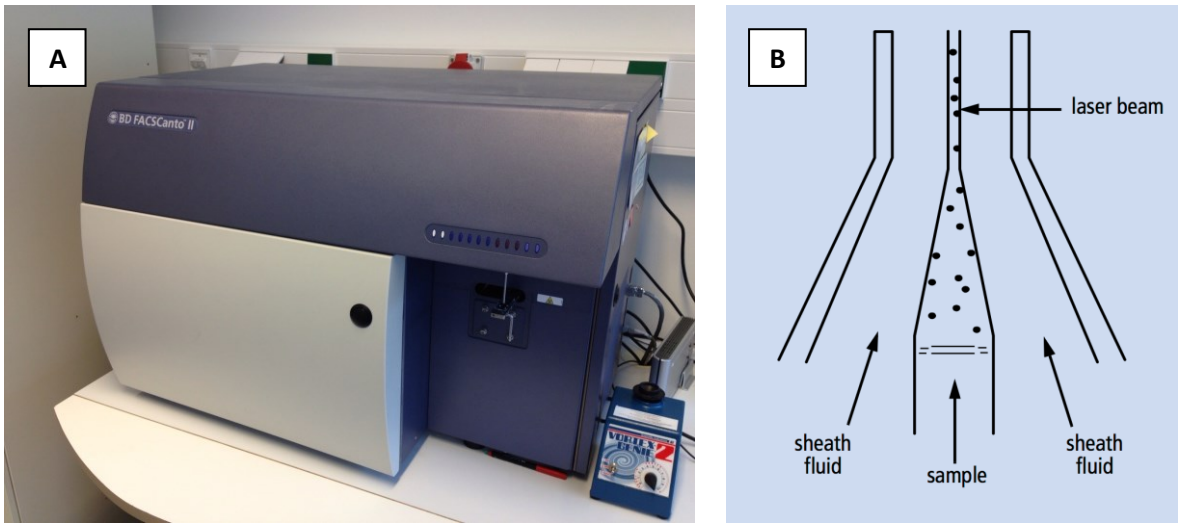


Figure 6A: FACS analyzing machinery at our research facility.

Figure 6B: Measuring micro cuvette of a flow cytometer. The probe liquid is guided into the FACS by a surrounding carrier (sheath) fluid.

Courtesy and © Becton, Dickinson and Company, reprinted with permission.

The resulting output is a scatter plot that is generated during the flow experiment by the detection and measurement of light signals along the longitudinal axis, called forward scatter (FSC) and light signals measured at a 90° angle to the incident light, called side scatter (SSC). The FSC detects the cell's size via light diffraction, whereas the SSC measures the cell's inner complexity and granularity (e.g. ingested cells) by light reflection.

Due to their autofluorescence, AM can be detected by a 488 nm blue argon-ion laser.

Apoptotic PMN (aPMN) were labeled with 1, 1’ - Dioctadecyl - 3, 3, 3’, 3’ - tetramethylin - dodicarbocyanine, 4 - chlorobenzensulfonate (DiD), a lipophilic tracer which shows a fluorescence excitation at 644 nm, an emission maximum at 665 nm [18] and is detectable by the red helium-neon-laser (633 nm), normally used to trace allophycocyanine (APC), thus also referred to as APC channel.

The *E. coli* BioParticles from pHrodo are highly sensitive, fluorogenic particles and are perfect for the detection of phagocytic ingestion in FACS. Their approximate fluorescence excitation is at 560 nm and the emission maximum is at 585 nm and are measured in the phycoerythrin (PE) channel [7].

2.3. Immunology Multiplex assay

To analyze the chemokine and cytokine concentrations in BAL and cell culture supernatants, we used the immunology Multiplex assay. The assay used magnetic microbeads, which were tagged with a precisely mixed color code of only two fluorescent dyes and were distinguishable to 100 different color creations. Additionally, they were labeled with a specific capture antibody. When the analyte was introduced, the target antigen was captured via the bead's antibody. After multiple washing steps, the biotinylated detection antibodies were added, which bind on the target antigen, previously captured by the antibody-coated beads. The bead-antigen-antibody blend was then incubated with Streptavidin-PE conjugate, a reporter molecule that could be detected and quantified by flow-cytometry based analysis. In similar ways like the FACS instruments, the bead-antibody-chemokine particle was flushed through a laser array, with two components. The first laser excited the color in the beads. The second one detected the different colored beads. A computing device quantified and analyzed the results, based on the radiated reporter-bead signals [50, 51].

2.4. Overview of preparation and analytic machinery

Cell Analyzer (FACS)	BD FACSCanto II, BD Biosciences, Bedford, MA, USA (Fig. 6)
Centrifuge	Multifuge 3 S-R centrifuge Heraeus, Thermo Scientific, USA
Incubator	Herasafe KS class II, Type A2 biological safety cabinet, Thermo Scientific, USA
Bio-Plex Suspension array system	Biorad, Munich, Germany
Milliplex map rat cytokine/chemokine magnetic bead panel	Merck Millipore, Darmstadt, Germany

2.5. Used chemicals, solutions and buffers

Ampuwa solution	Aqua ad iniectabilia, 1L Plastipur, Fresenius, Bad-Homburg, Germany
Aprotinine	Sigma-Aldrich, Life Science, St. Louis, MO, USA
Crystal Violet	Pharmacy University of Ulm, Ulm, Germany
Dextran	Leuconostoc spp., Sigma Life Science, St. Louis, MO, USA
DiD	1, 1' - Dioctadecyl - 3, 3, 3', 3' - tetramethylin - dodicarbocyanine, 4 -chlorobenzensulfonate, Invitrogen, Darmstadt, Germany
E. coli	pHrodo Red *E. coli* BioParticles conjugate, Molecular Probes, Life Technologies, Darmstadt, Germany
Ficoll-Paque Plus	Endotoxin tested (<0.12EU/ml), density 1.077 g/ml, lymphocyte isolation, GE Healthcare Bio-Sciences AB, Uppsala, Sweden
HBSS ++	Hank's balanced salt solution, with calcium^{2+}, with magnesium^{2+}, Gibco, Life Technologies, Darmstadt, Germany
HEPES	1 M, Gibco, Life Technologies, Darmstadt, Germany
Isotonic saline solution	0.9% NaCl, Fresenius, Bad-Homburg, Germany
Leupeptine	Sigma-Aldrich, Life Science, St. Louis, MO, USA
PBS	2,67 mM KCl; 1,47 mM KH_2PO_4; 137,93 mM NaCl; 8,06 mM $Na_2HPO_4 \bullet 7H_2O$ in Aqua dest. Fresenius, Bad-Homburg, Germany
Pepstatine	Sigma-Aldrich, Life Science, St. Louis, MO, USA
RPMI 1640	Gibco, Invitrogen, Life Technologies, Darmstadt Germany
Sevorane 100%	Sevoflurane 250 ml, Abbott, Wiesbaden, Germany
Temgesic	1 vial: 0.3 mg Buprenorphine, Fresenius, Bad-Homburg, Germany
Trypan Blue Solution	0.04%, Sigma-Aldrich, Life Science, St. Louis, MO, USA
Trypsin Inhibitor	Sigma-Aldrich, Life Science, St. Louis, MO, USA
Trypsin-EDTA	Sigma-Aldrich, Life Science, St. Louis, MO, USA

2.6. Additional materials

Cannula 22 G	B. Braun, Melsungen, Germany
Cannula 26 G	B. Braun, Melsungen, Germany
Cell lifter	Corning, Sigma-Aldrich, Life Science, St. Louis, MO, USA
EDTA tubule	7.5 ml, Sarstedt, Nürnbrecht, Germany
Eppendorf 0.5 ml	Eppendorf AG, Hamburg, Germany
Eppendorf 1.5 ml	Eppendorf AG, Hamburg, Germany
Eppendorf 2 ml	Eppendorf AG, Hamburg, Germany
FACS-Tubes	BD Biosciences, Bedford, MA, USA
Falcon tube 15 ml	Falcon, BD Biosciences, Bedford, MA, USA
Falcon tube 50 ml	Falcon, BD Biosciences, Bedford, MA, USA
Filtertips 10 µl	BD Biosciences, Bedford, MA, USA
Filtertips 100 µl	BD Biosciences, Bedford, MA, USA
Filtertips 1000 µl	BD Biosciences, Bedford, MA, USA
Intra venous catheter	24 Gauge (G), B. Braun, Melsungen, Germany
Neubauer cell counting chamber	Sarstedt, Nürnbrecht, Germany
Petri dish 150 mm	Corning, Sigma-Aldrich, Life Science, St. Louis, MO, USA
Petri dish 35 mm	Corning, Sigma-Aldrich, Life Science, St. Louis, MO, USA
Safety glasses	n.s.
Safety Multyfly cannula	21 Gauge (G), Sarstedt, Nürnbrecht, Germany
Sterile urine cup	Sarstedt, Nürnbrecht, Germany
Stripette 5 ml	Corning, Sigma-Aldrich, Life Science, St. Louis, MO, USA
Stripette 10 ml	Corning, Sigma-Aldrich, Life Science, St. Louis, MO, USA
Stripette 25 ml	Corning, Sigma-Aldrich, Life Science, St. Louis, MO, USA
Syringe 1 ml	B. Braun, Melsungen, Germany
Syringe 20 ml	B. Braun, Melsungen, Germany
UV-Lamp	380-315 nm, 3.26-3.94 eV, Kendro Laboratory Products, Hanau, Germany

2.5. Methods

2.5.1. Blunt chest trauma

For sedation, every animal was placed into an anesthetizing container, which was flooded with 96% oxygen and 4% Sevoflurane (Sevorane®, Abbott, Wiesbaden, Germany). The state of anesthesia was verified carefully. The animals were then transferred into an inhalation mask and fixed with medical tape (Leukosilk®) in supine position on a perspex plate. The Sevoflurane mixture was further supplied with a continuous flow of 1 l/min.

To ensure that animals do not suffer any pain and to reduce the post-traumatic stress levels, the analgesic Buprenorphine (1 ml ≙ 0.03 mg per kg bodyweight) was subcutaneously injected into the animal's neck 30 minutes before blunt chest trauma or sham treatment. In the first 24 h after TxT or sham treatment, Buprenorphine had to be injected every 8 h.

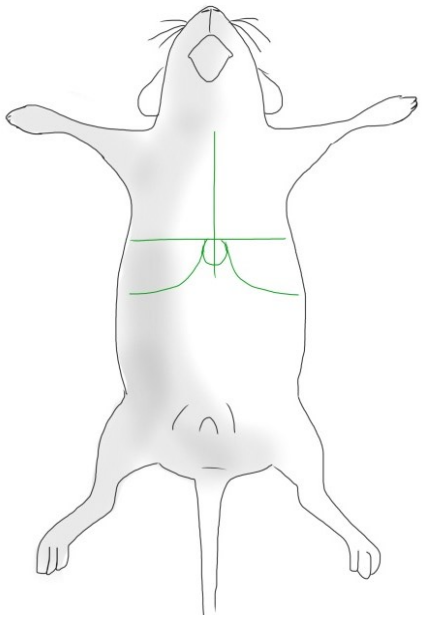


Figure 8: Mapping of the laboratory animal, using the ribcage and xyphoid bone as anatomical landmarks for a reproducible positioning of the blast cylinder.
Illustration by Michael Eichner

We shaved the chest and abdomen, to apply markings to constitute a constant alignment for a blast cylinder, generating the thorax trauma. The caudal outline of the ribcage and the xyphoid bone of the animal were used as general guidelines, marking a cross with the mid-sternal line (Fig. 8).

For the induction of the chest trauma, we used an in-house designed blast cylinder (scientific workshop University of Ulm, Ulm, Germany), which could generate blast waves of consistent force. The cylinder consists of two parts, divided by a sheet of Mylar film (DuPont GmbH, Bad Homburg, Germany), creating two chambers. The lower opened part was aligned two centimeters above the animal's thorax (Fig. 9). The upper, closed part of the cylinder was connected to a pressure regulator (Zinser, Ersbach, Germany) of a gas bottle (pressure: 13 bars).

Via a peripheral trigger the electric high-speed valve (Hee-D-24, Festo, Esslingen, Germany) was opened. By operating the trigger, the upper part of the blast apparatus is instantly filled with air until the pressure exceeds the Mylar film's bursting pressure. This leads to the sudden rupture of the film, releasing a reproducible pressure wave onto the rat's chest.

The valve was triggered during full inspiration. The time of apnea was noted, as well as the time of sedation. The similar sedation time was used for the sham animals as well, to adjust the influence of Sevoflurane effects [89].

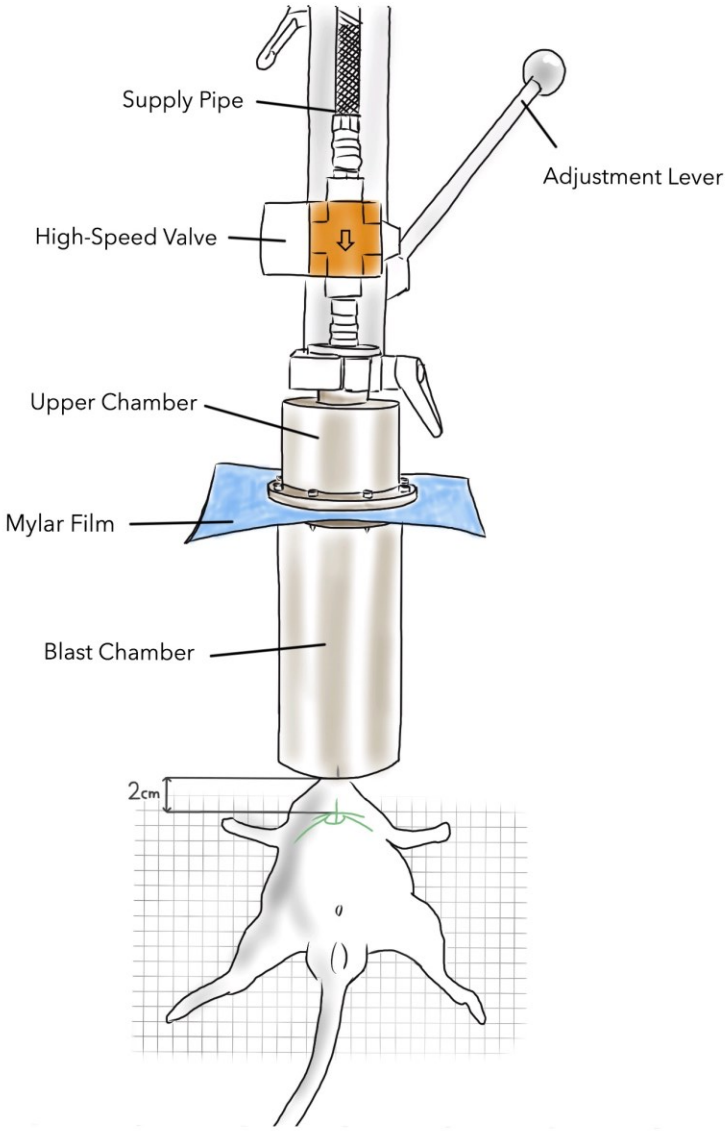


Figure 9: Blast apparatus set-up. The Mylar film is pictured in blue. The High-speed valve is colored in orange.

Illustration by Michael Eichner

2.5.2. *In vivo* efferocytosis and phagocytosis

2.5.2.1. Isolation of polymorphonuclear neutrophils

For the isolation of PMN, Sham and TxT procedure, we needed two animals per experiment cycle.

The animals were anesthetized as mentioned above and fixated in supine position. After disinfecting, the abdomen was opened with a 3 cm cut on the mid-sternal line. The abdominal aorta was exposed and punctured, using a 24 Gauge (G) intra-venous (iv) catheter to bleed the rats.

For the PMN isolation, we mixed the drawn EDTA blood in equal ratio with 0.9% NaCl solution. This sodium chloride and blood mixture was then carefully transferred to a Falcon containing 15 ml of Ficoll (Ficoll Paque Plus®, GE Healthcare), forming two layers, Ficoll on the bottom and the blood on top. After spinning in the centrifuge at 340 x g for 30 minutes at room temperature a distinctive distribution pattern occurs in the Falcon, with the Plasma on top followed by PBMC, Ficoll, granulocytes and red blood cells at the bottom. (Fig. 10)

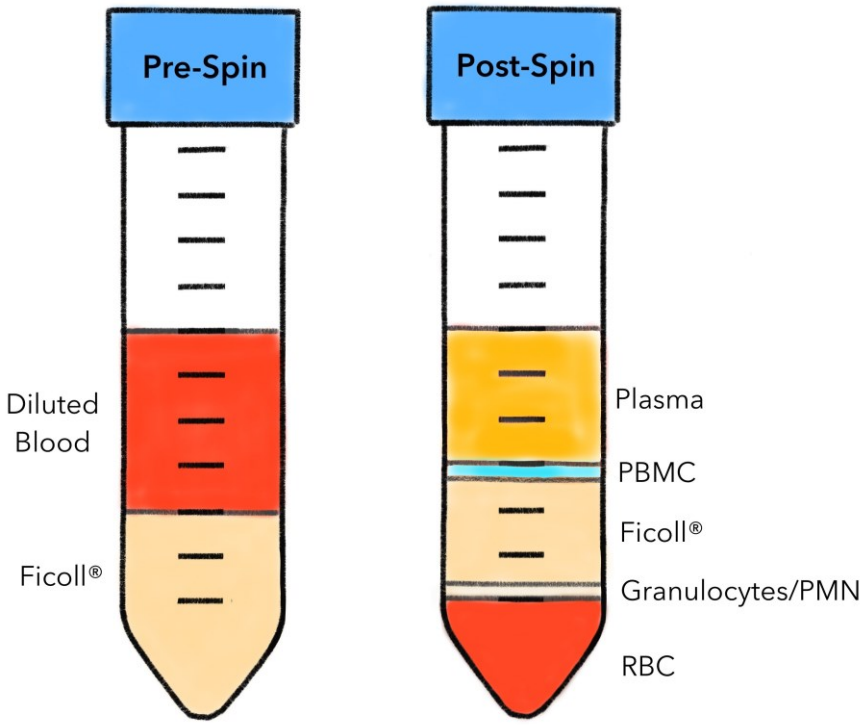


Figure 10: Distributive pattern after centrifuge spinning of blood components.

Illustration by Michael Eichner

The PBMC layer, mostly consisting of monocytes and lymphocytes as well as the plasma and Ficoll supernatants were discarded, remaining the red blood cells on the bottom and about 1 ml of liquid above, containing the granulocytes or PMN cells. The residuum was filled up with 0.9% NaCl solution, to a total volume of 30 ml. Subsequent adding of 7.5 ml of 5% of Dextran (Dextran® from Leuconostoc spp, Sigma) made the red blood cells clot and sediment after 45 minutes at room temperature. The supernatant (hosting mostly neutrophils and few scattered erythrocytes) was transferred into a new 50 ml Falcon, filled up with 0.9% NaCl solution to a total volume of 40 ml. After centrifuging at 530 x g for five minutes at room temperature the supernatant was discarded, since the pellet now contained all neutrophils and a few remaining erythrocytes.

To lyse the remaining erythrocytes, they were taken up in 10 ml of distilled water, followed by an immediate addition of 5 ml of 2.7% NaCl solution to stop the lysing process. After filling up to a total volume of 40 ml with 0.9% NaCl solution the probe was centrifuged at 530 x g for five minutes at room temperature. After discarding the supernatant and dissolving the pellet in 2 ml of HBSS ++, the cells were counted using trypan blue.

2.5.2.2. Intracellular staining of PMN

For FACS analysis, PMN needed to be stained beforehand. We continued with 12×10^6 PMN and centrifuged at 530 x g for five minutes at room temperature. We diluted 5 µL of 1, 1' - Dioctadecyl - 3, 3, 3', 3' - tetramethylin - dodicarbocyanine, 4 -chlorobenzensulfonate (DiD, Molecular Probes, Liefesciences) in 2 ml HBSS++ (Ca^{2+}, Mg^{2+}), creating a dilution of 1:400. The PMN were dissolved in 2 ml of fluorescent cell tracker DiD solution and incubated for 30 minutes at 37°C in a water bath. Afterwards the solution was filled up with HBSS++ to a total volume of 14 ml and was subsequently centrifuged at 530 x g for five minutes at room temperature. The supernatant was discarded.

2.5.2.3. Induction of apoptosis in polymorphonuclear neutrophils

After intracellular staining, the PMN pellet was dissolved with 1.5 ml of HBSS++ and transferred to a petri dish with 35 mm of diameter. In said petri dish, the cells were radiated with UV light (315 to 400 nm) for 25 seconds. The solution was transferred into a 15 ml Falcon, for incubation in the water bath (37°C) for another 60 minutes. We then divide the apoptotic PMN (aPMN) cells into two equal parts. Those were then centrifuged at 530 x g for five minutes at room temperature. The supernatant was again discarded. One pellet was dissolved with 1200 µl of HBSS solution. The other pellet was solubilized with 1200 µl of HBSS solution +25 mM HEPES.

2.5.2.4. Preparing *E. coli*

To analyse the phagocytosis of AM concerning bacteria, we used labeled and stabilized *E. coli*. (pHrodo Red *E. coli* Bio Particles conjugate, molecular probes, Eugene, OR, USA)

We diluted 120 µl of the conjugate in 1080 µl RPMI + 10% FCS. A second flask was used to prepare the same solution with HEPES buffer.

2.5.2.5. Intratracheal instillation of apoptotic neutrophils or *E. coli*

Male Sprague-Dawley rats underwent sham treatment or blunt chest trauma. Before instillation of aPMN or *E. coli* to the lung, PMN were harvested and as described above, labeled with DiD. 2, 24 and 48 h post-trauma or during sham procedure, we instilled the fluorescent-labeled apoptotic PMN or *E. coli* intratracheally. For this purpose, animals were fixed on a perspex plate and anesthetized with Sevoflurane as described in 2.5.1. The rats were set at an angle of 60° with the head tilted back. To perform instillation, the mouth was opened and the tongue protruded. 300 µl of the respective cell suspension was applied twice. With obstruction of the nasopharyngeal air supply, the applied suspensions

were aspirated. Two hours after instillation rats were sacrificed by exsanguination under deep anesthesia.

2.5.2.6. Bronchoalveolar lavage

To extract the bronchoalveolar lavage, we performed a sternotomia, exposing heart and lungs including the trachea. For the cannulation of the trachea and, therefore, performing the bronchial lavage, we used butterfly needles normally used for blood drawing. After inserting the cannula of the butterfly needle into the trachea we ligated and fixated both with thin thread. (Fig. 12)

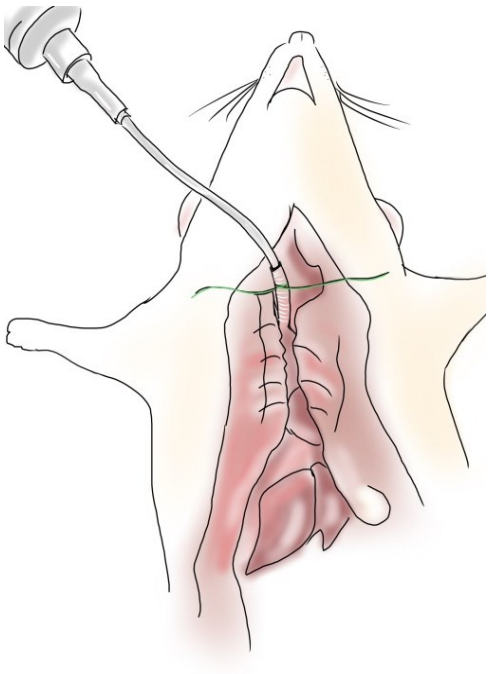


Figure 12: Cannulation of the trachea in rats after sacrifice.
Illustration by Michael Eichner

After cutting the left atrium, the heart's right ventricle was punctured and 10 ml of ice-cold PBS solution were injected to flush the pulmonary circulatory system. Thereafter the first bronchoalveolar lavage was performed with 5 ml of ice-cold PBS solution. This first sample is treated with a protease inhibitor and centrifuged at 450 x g at 4°C for 10 min. The supernatant is subsequently used for Multiplex and Enzyme-linked Immunosorbent Assay (ELISA) analysis. The pellet with the consisting AM is pooled with AM harvested by the next eight lavages, with 8 ml of PBS. After the third lavage, we additionally began massaging the chest, allowing more macrophages to detach from the alveoli. The now harvested lavage is then stocked on ice until further processing.

The BAL was centrifuged at 450 x g for 10 minutes at 4°C. The supernatants were discarded and the pellets were taken up in 2 ml HBSS++. Another centrifugation cycle with 450 x g for 10 minutes at 4°C was performed. To lyse the erythrocytes, we added 10 ml sterile distilled water to the pellet and added 5 ml of 2.7 % NaCl solution. We filled up to a

total volume of 40 ml with 0.9% NaCl solution. A centrifugation step with 450 x g for 10 min at 4°C followed. After disposal of the supernatant, we dissolved the pellet with 2 ml HBSS++ solution, followed by the cell count with crystal violet (blended 1:1 with DPBS) at a 1:10 ratio.

2.5.3. *In vitro* efferocytosis and phagocytosis study design

The isolation, staining and apoptosis of PMN were conducted as described in 2.5.2.1. We assorted three cohorts for the animal procedures, subdivided under the aspect of time in three cohorts and additional three on the level of cellular incubation and finally another three on AM engulfment level. The first three cohorts were basically divided into PMN donor rats (I) and rats, which went through thoracic trauma (TxT) procedure (II) or sham procedure (III). The PMN donor rats were only destined to provide material for the Sham and TxT animals to engulf and are therefore not further mentioned and counted into assessment. The TxT and Sham animals underwent exact same procedures, like analgesia, anesthesia and shaving, except the actual trauma. 2, 24 and 48 h following sham procedure or TxT, animals were sacrificed and AM were harvested. The isolation and preparation of AM was conducted as described in chapter 2.5.2.6. Each group was further divided into three different cell incubation conditions: normoxia (21% O_2), hypoxia (5% O_2) and hypercapnia (20% CO_2). Finally, we divided on the phagocytic level, in which one group of AM had to starve (this group was defined as control cohort), one was provided apoptotic PMN (from the donor rats) and the last, which were provided *E. coli* (Fig. 11).

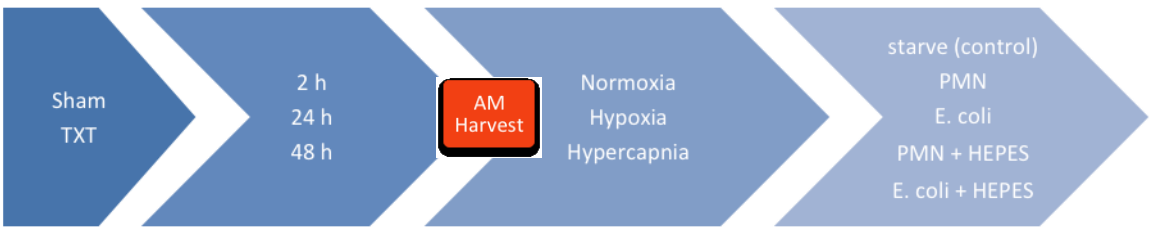


Figure 11: Groups in this study

Each approach was repeated five times (n=5). Every repetition contained two animals per group, which were pooled after cell counts. Additionally, we divided all TxT and sham groups into two, in which one received 25 mM HEPES buffer.

2.5.3.1. Phagocytosis assay

AM were pooled from two animals, which undergone the same treatment (AM of the BAL harvested from 2, 24, or 48 h after sham treatment or blunt chest trauma). The cell suspensions were filled up with HBSS++ to gain a total volume of 15 ml.

Table 1: Allocation of media, ingestive probes and treatment of incubation conditions on cell culture dishes.

Dish number	Culturing condition	Treatment of AM	Buffer
1	21 % O_2	No ingestion, HBSS++	No
2	21 % O_2	Add 10^6 labeled apoptotic PMN, HBSS++	No
3	21 % O_2	Add E.coli in RPMI +10% FCS	No
4	21 % O_2	Add 10^6 labeled apoptotic PMN	25 mM HEPES
5	21 % O_2	Add E.coli in RPMI +10% FCS	25 mM HEPES
6	2.5 % O_2	No ingestion, HBSS++	No
7	2.5 % O_2	Add 10^6 labeled apoptotic PMN, HBSS++	No
8	2.5 % O_2	Add E.coli in RPMI +10% FCS	No
9	2.5 % O_2	Add 10^6 labeled apoptotic PMN	25 mM HEPES
10	2.5 % O_2	Add E.coli in RPMI +10% FCS	25 mM HEPES
11	20 % CO_2	No ingestion, HBSS++	No
12	20 % CO_2	Add 10^6 labeled apoptotic PMN, HBSS++	No
13	20 % CO_2	Add E.coli in RPMI +10% FCS	No
14	20 % CO_2	Add 10^6 labeled apoptotic PMN	25 mM HEPES
15	20 % CO_2	Add E.coli in RPMI +10% FCS	25 mM HEPES

To culture the cells under different conditions the suspensions had to be portioned in 15 petri dishes. 1 ml of the AM solution was transferred into petri dish 1, 2, 6, 7, 11, 12 and 0.5 ml HBSS++ was added respectively.

The remaining 9 ml of the AM solution were divided into three 15 ml Falcons, considered as tube A, B and C, and centrifuged at 450 x g for 5 min at room temperature.

The supernatant of tube A was discarded and we diluted the remainder with 4.5 ml of RPMI + 10% FCS (culture media for E.coli). The suspension is then divided into three equal parts and separated into dishes 3, 8, and 13.

Tube B was handled equally, with the exception of using 4.5 ml of HBSS++ + 25mM HEPES. The suspension was divided into dishes 4, 9, and 14.

Tube C the cells were re-suspended in 4.5 ml RPMI + 10% FCS with 25mM HEPES buffer. Then the suspension was divided into the petri dishes, 5, 10, and 15.

We used the buffer HEPES on dishes 4, 5, 9, 10, 14, and 15 to rule out the problem of massive acidotic shifts, especially on those treated with CO_2 later.

The AM were incubated for 2 h at 37°C in normoxic (21% O_2, 5% CO_2), hypercapnic (20% CO_2) or hypoxic (2.5% O_2) conditions (Table 1).

After the pre-incubation time of two hours, the supernatants were removed and he solutions of aPMN or *E. coli* were added as shown in table 1.

The follow-up treatment consisted of another 2 h in the incubator.

To verify the impact of the hypercapnic condition we measured the pH values in the culture supernatant. This supernatant was centrifuged at 1000 x g for 5 min and stored for later use in Milliplex analysis at -80°C.

The adherend AM were washed by adding HBSS to cleanse from not-ingested material and residual cell culture medium.

To resolve the cells from the bottom, we added 2 ml Trypsin-EDTA, incubated for 30 seconds at 37°C and 5% CO_2, then 1 min on ice. Adding RPMI 10% attenuated the trypsinization process. With a spatula, the cells were gently removed from the petri dish bottom and then gently resolved using a pipette. The suspension is transferred into a 15 ml Falcon tube and centrifuged at 450 x g for 5 min. After supernatant disposal, the pellet was resolved with 150 µl DPBS, then transferred into a FACS tube for measurement.

2.5.4. Statistical analysis

All results were processed using the Microsoft® based Systat Software Sigma Plot 12.0 and provided in the figures as means ± standard error of the mean (SEM). Statistical significance was evaluated with two-way analysis of variance (ANOVA) technique, using Student Newman Keuls method. The two-way ANOVA examines the influence of two independent variables on one dependent variable. It assesses the effects of the variables on each other and also the interaction between these variables. Significance was assumed when $p < 0.05$.

3. Results

3.1. Alveolar macrophage cell count

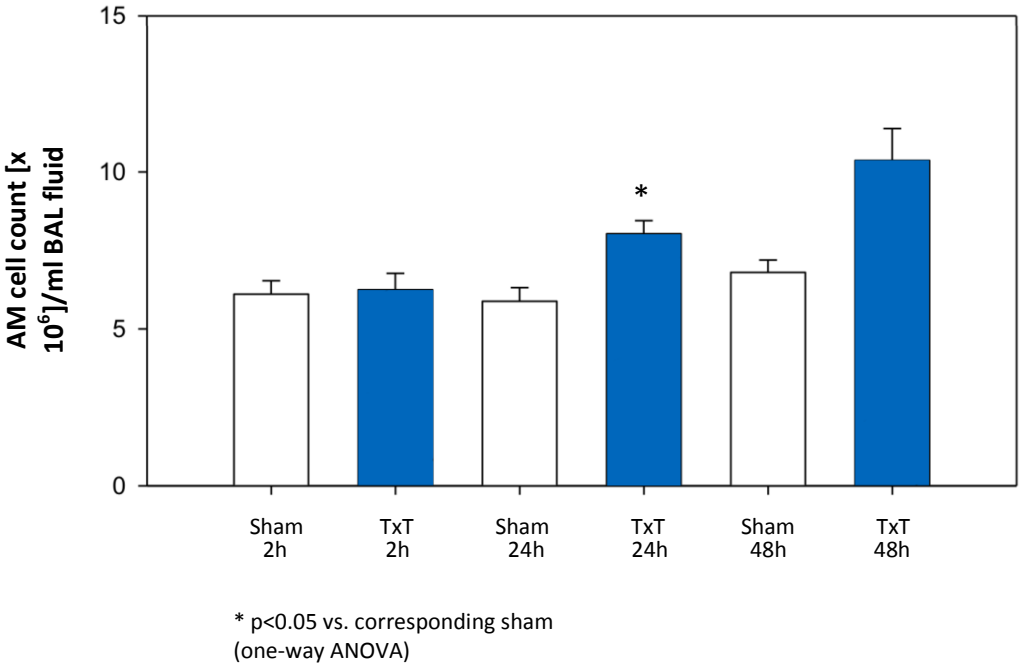


Figure 13: Number of AM isolated from BAL. Values are expressed as means ± standard error of the mean (SEM) of n=20 animals per group.

After the harvesting of AM via BAL, cells were counted using a Neubauer chamber. The number of recruited cells remained constant in sham groups, the recruited cells in post-traumatic groups, however, show a tendency to increase over time. With increasing time period after TxT, the higher was the gained AM cell amount. The 24 hours (h) TxT cell count was significantly higher over the corresponding 24 h sham group, using one-way ANOVA.

3.2. *In vivo* efferocytosis

To examine the alteration in efferocytic activity of AM after blunt chest trauma, aPMN were instilled intratracheally 2, 24 and 48 hours (h) after sham procedure or after TxT.

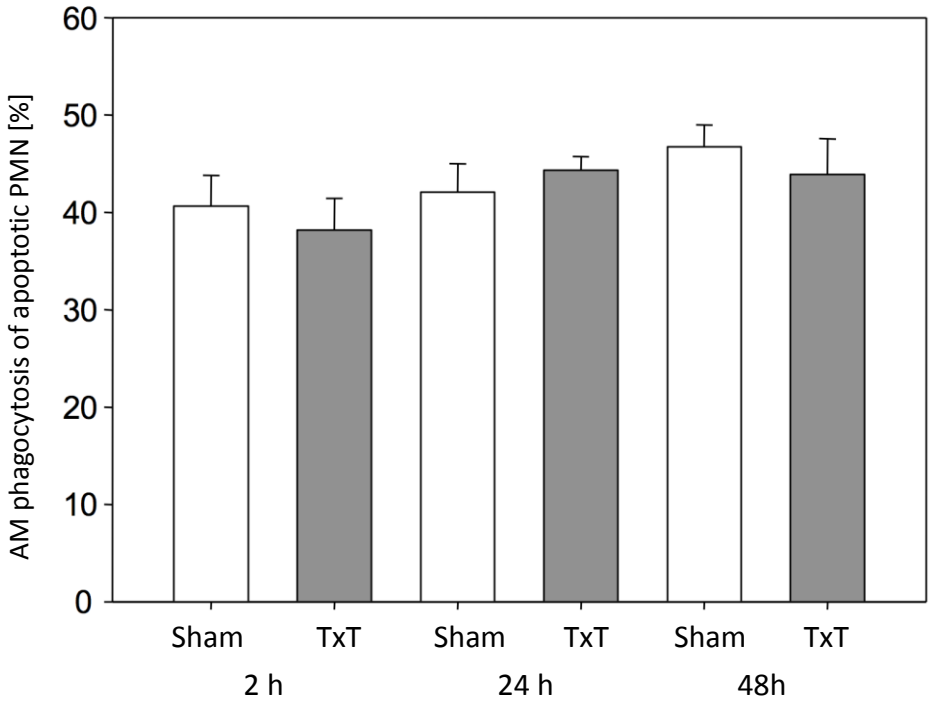


Figure 14: *In vivo* efferocytosis of alveolar macrophages (AM) after blunt chest trauma (TxT): percentage of AM ingesting fluorescent-labeled apoptotic neutrophils (aPMN), intratracheally instilled 2, 24 or 48 h after sham treatment or chest trauma. Ingestion of aPMN was analyzed by flow cytometry. Values are expressed as means ± standard error of the mean (SEM) of n = 10 rats per group.

Figure 14 shows no significant changes in AM efferocytosis post-trauma towards sham procedure *in vivo*. The values shown are given in percentage of AM ingesting aPMN (%).

3.3. *In vivo* phagocytosis

To analyze alterations of the phagocytic activity of AM, *E. coli* were instilled intratracheally 2, 24 and 48 hours (h) after sham procedure or blunt chest trauma.

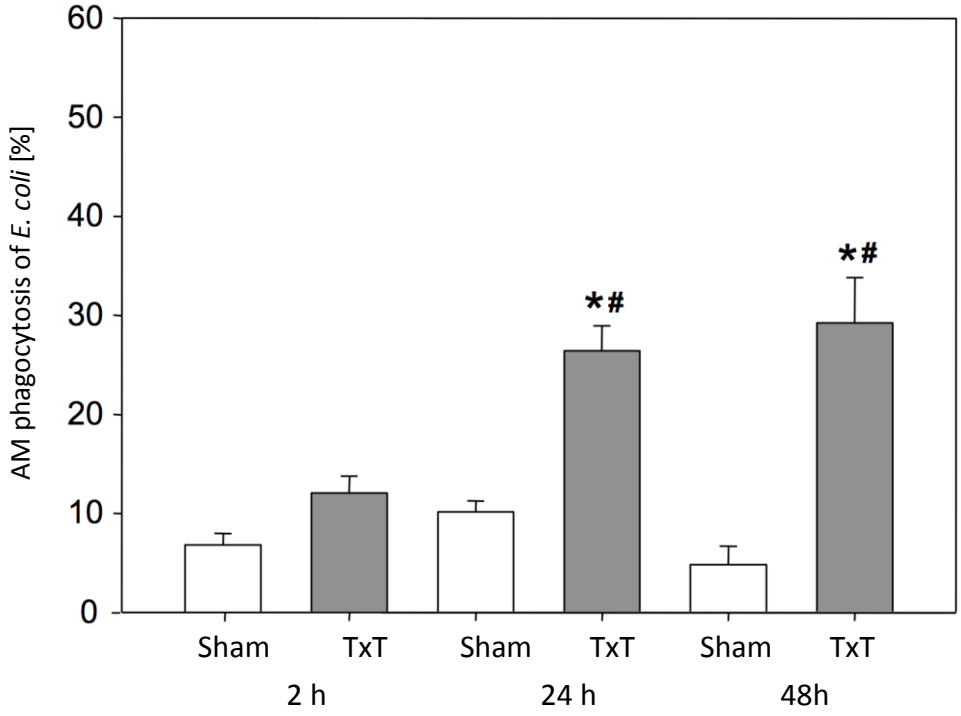


Figure 15: *In vivo* phagocytosis of alveolar macrophages (AM) after blunt chest trauma (TxT): percentage of AM ingesting fluorescent-labeled *E. coli*, intratracheally instilled 2, 24 or 48 h after sham treatment or chest trauma. Ingestion of *E. coli* was analyzed by flow cytometry. Values are expressed as means ± standard error of the mean (SEM) of n = 5 rats per group.

Figure 15 shows an increase of *E. coli* phagocytosis by AM after TXT. The post-trauma peaks are increased over the corresponding sham groups. The percentage of AM, that ingested *E. coli* was significantly increased 24 h after trauma and remained elevated until 48 h after blunt chest trauma. The values are presented in percentage of AM ingesting *E. coli* (%).

3.4. *In vitro* efferocytosis under normoxic, hypoxic and hypercapnic conditions

We examined the relative efferocytic activity of AM under normoxic, hypoxic and hypercapnic conditions. AM were harvested from rats, 2, 24 and 48 hours (h) after they underwent sham or trauma (TxT) treatment. A color code was applied for lucidity. Normoxic conditions sham in blue, normoxic conditions TxT in dark blue, hypoxic conditions sham in orange, hypoxic conditions TxT in dark orange/brown, hypercapnic conditions sham in green and hypercapnic conditions TxT in dark green.

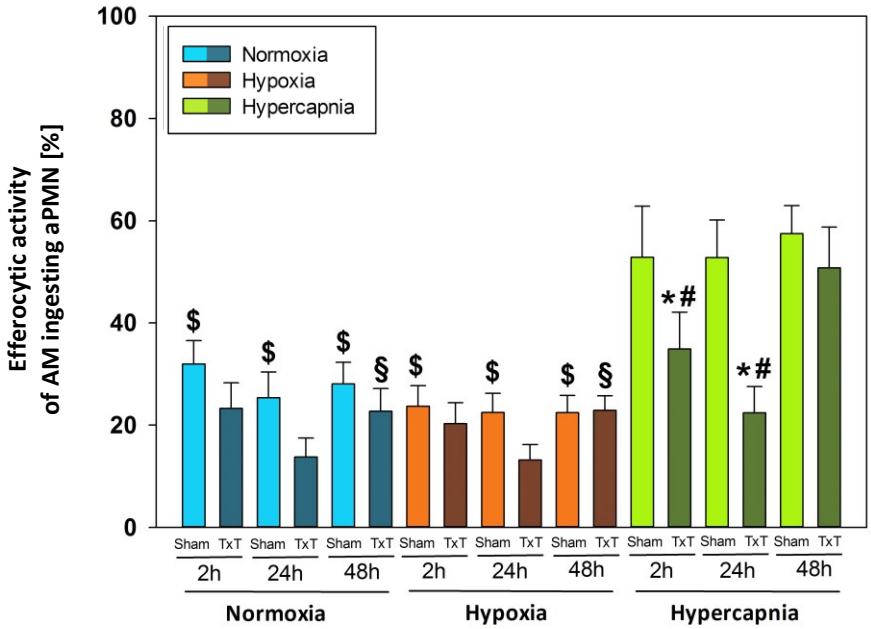


Figure 16: Phagocytosis of aPMN: *In vitro* efferocytosis of alveolar macrophages (AM) isolated after blunt chest trauma (TxT) or sham treatment, cultured under normoxic, hypoxic and hypercapnic conditions for 2 h. AM were harvested from rats, 2, 24 or 48 hours (h) after they underwent sham or trauma (TxT) procedure and then incubated with fluorescent-labeled apoptotic neutrophils (aPMN) under normal oxygen (21% O_2, 5% CO_2), low oxygen (2.5% O_2) or elevated carbon dioxide (20% CO_2) levels. The percentage of AM ingesting aPMN was detected by flow cytometry. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

AM incubated under normoxic and hypoxic conditions tend to engulf less aPMN post TxT compared to AM isolated after sham procedure. In hypercapnia, a significant drop-off in TxT compared to Sham at 2 and 24 h was detected. A general increase of the percentage of efferocytic active AM in hypercapnia compared towards normoxia could be observed. This result was significant in 48 h TxT hypercapnia versus 48h normoxia. The efferocytosis increase in Sham hypercapnia towards the Sham groups in normoxia and hypoxia was also significant. Looking at the TxT site in hypercapnia the 48 h animals showed a significant increase in PMN ingestion over 2 and 24 h animals. A slight, but not significant decrease was also seen in 2 and 24 hours TxT versus Sham in normoxia and hypercapnia. The values are presented in percentage of AM ingesting aPMN (%).

3.5. *In vitro* phagocytosis under normoxic, hypoxic and hypercapnic conditions

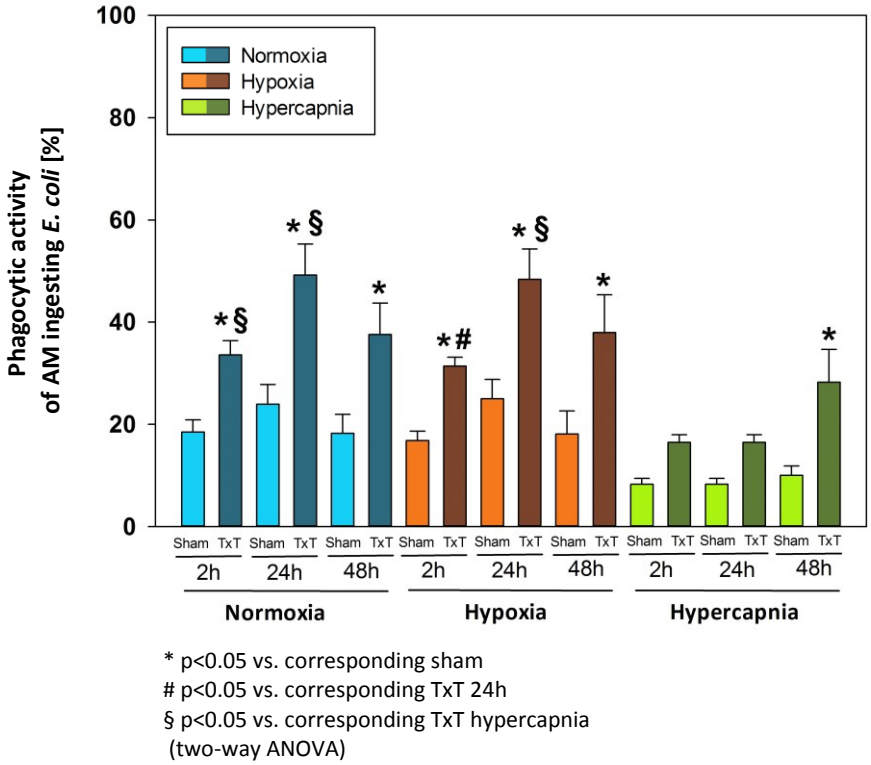


Figure 17: Phagocytosis of *E.coli:* *In vitro* phagocytosis of alveolar macrophages (AM) isolated after blunt chest trauma (TxT) or sham treatment, cultured under normoxic, hypoxic and hypercapnic conditions for 2 h. AM were harvested from rats, 2, 24 or 48 hours (h) after they undergone sham or trauma (TxT) treatment and then incubated with fluorescent-labeled *E. coli* under normal oxygen (21% O_2, 5% CO_2), low oxygen (2.5% O_2) or elevated carbon dioxide (20% CO_2) levels. The percentage of AM ingesting *E. coli* was detected by flow cytometry. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

The *E. coli* phagocytosis by AM showed a significant increase of devouring activity in AM after TxT. In hypoxic conditions, a significant increase was shown, concerning 2 h TxT versus 24 h TxT. A decrease in phagocytic activity from normoxia/hypoxia towards hypercapnic state could be detected, which was significant in 2 h and 24 h normoxia versus corresponding hypercapnia. The values are presented in percentage of AM ingesting *E. coli* (%).

3.6. Cytokine concentrations in AM culture supernatants

After incubating the AM under normoxic, hypoxic and hypercapnic conditions with aPMN or *E. coli*, cell culture supernatants were analyzed with immunology multiplex assay (see chapter 2.3.3.).

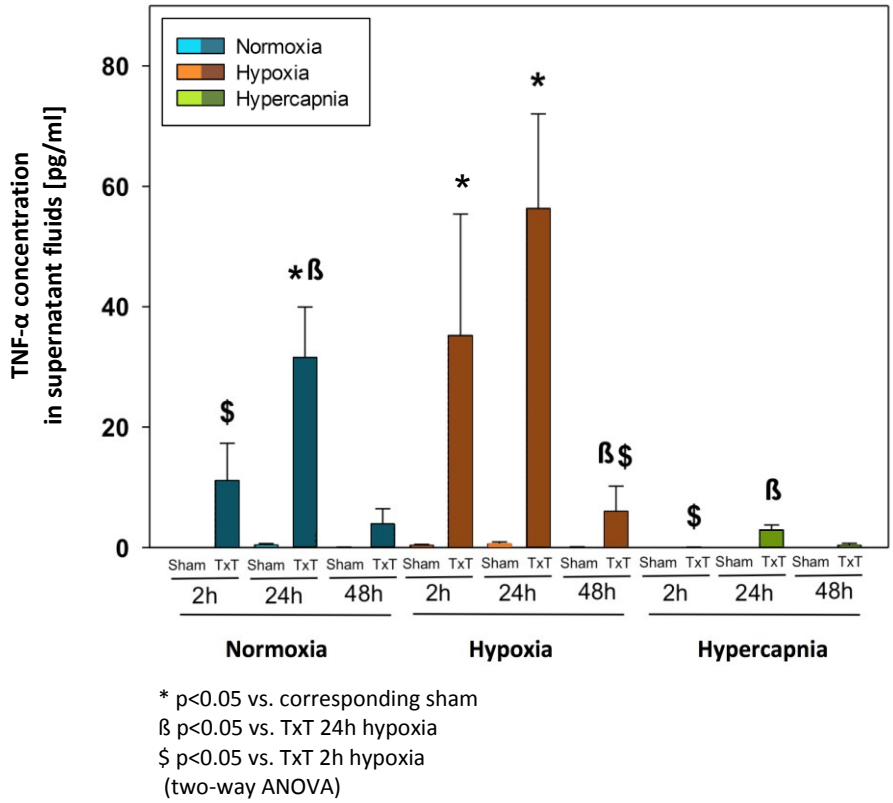


Figure 18 A: TNF-α without ingestion: Tumor necrosis factor alpha (TNF-α) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions without addition of fluorescent-labeled apoptotic neutrophils (aPMN) or *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured without aPMN or *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assays. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Tumor necrosis factor alpha (TNF-α) cytokine concentration of AM culture supernatants isolated 24 hours (h) post-trauma and incubated under normoxic conditions (short: 24h TxT normoxia group) and the TNF-α concentrations of AM culture supernatants isolated 2h and 24 h post-trauma incubated under hypoxic conditions (short: 2 and 24h TxT hypoxia

groups) are significantly increased in comparison to their corresponding sham TNF-α concentrations. The 24 h posttraumatic peaks showed the highest increase of cytokine release. 24 h normoxia and hypercapnia post-trauma, as well as 48 h post-trauma hypoxia, were significantly decreased over 24 h hypoxia. 2h normoxia and hypercapnia post-trauma and 48 h hypoxia post-trauma were significantly decreased over 2h hypoxia TxT.

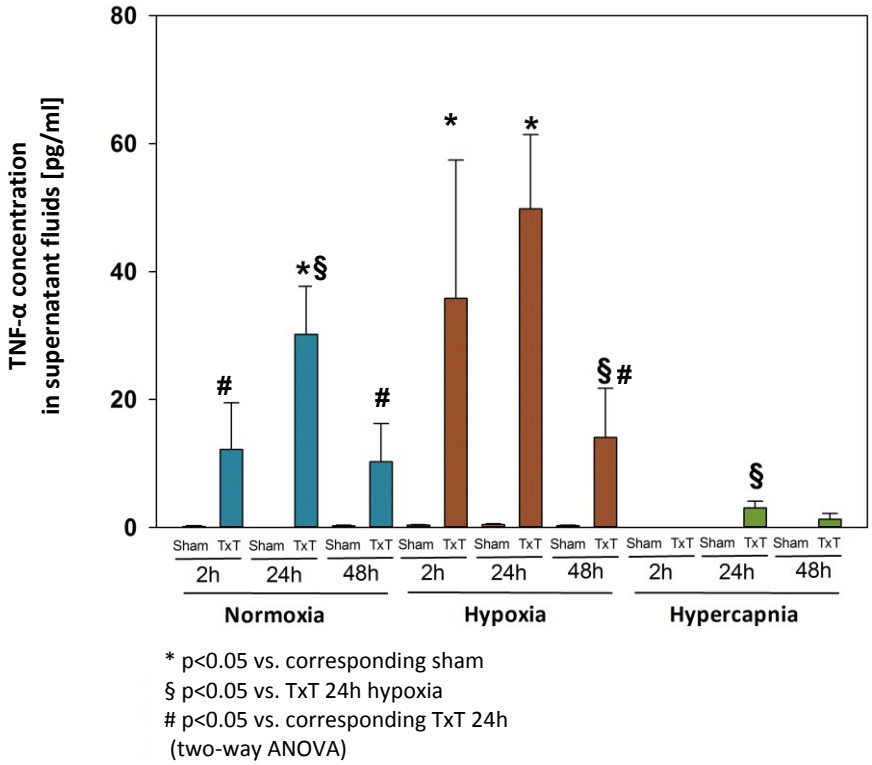


Figure 18 B: TNF-α during aPMN engulfment: Tumor necrosis factor alpha (TNF-α) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled apoptotic neutrophils (aPMN). AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with aPMN under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

The TNF-α release of AM 24 h TxT, cultivated under normoxic conditions is significantly increased over it's corresponding 2 and 48 h groups. 24 h TxT hypoxia was significantly increased compared to 48 h TxT hypoxia. The TNF-α response during aPMN ingestion displayed the propensity of a downfall in TNF-α release in the hypercapnia cohort

compared to an increased release during hypoxia and normoxia. In all cases the tendency of TNF-α increase in TxT compared to sham procedure was visible. Statistics showed a significant increase of TNF-α release in 24 h normoxia and hypoxia TxT compared to corresponding sham groups. Over all AM isolated 24 h after TxT and cultured under hypoxic conditions showed the highest increase in TNF-α release and was significant when comparing to 24 h normoxia, 24 h hypercapnia and 48 h hypoxia post-trauma. Comparing to figure 18 A (without ingestion) we saw a corresponding pattern. The culture supernatants of AM in 24 h post-trauma groups showed the highest peak in cytokine release.

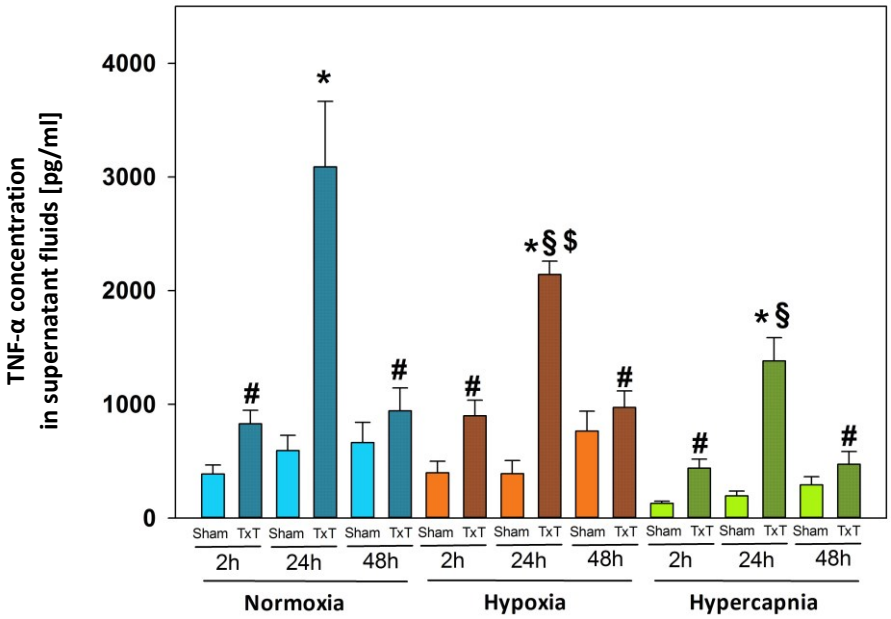


Figure 18 C: TNF-α during *E. coli* engulfment: Tumor necrosis factor alpha (TNF-α) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as mean ± standard error of the mean (SEM) of n = 5 animals per group.

TNF-α concentrations in AM culture supernatants incubated with *E. coli* showed higher cytokine concentration levels, when compared to incubation with aPMN. In aPMN feeding, values ranged from almost 0 up to 57 pg/ml. In contrast *E. coli* exposure resulted in mean concentrations of almost 3200 pg/ml. TNF-α concentrations after 24 h post-trauma were significantly increased over their corresponding 2 and 48 h TxT groups during normoxic, hypoxic and hypercapnic conditions. In the 24 h cohorts with normoxic, hypoxic and hypercapnic conditions the results in TxT were significantly increased over sham. 24 h TxT in normoxia showed the highest release of TNF-α and was significantly increased over 24 h TxT during hypoxic and hypercapnic conditions. 24 h TxT hypoxia was significantly increased over 24 h TxT hypercapnia.

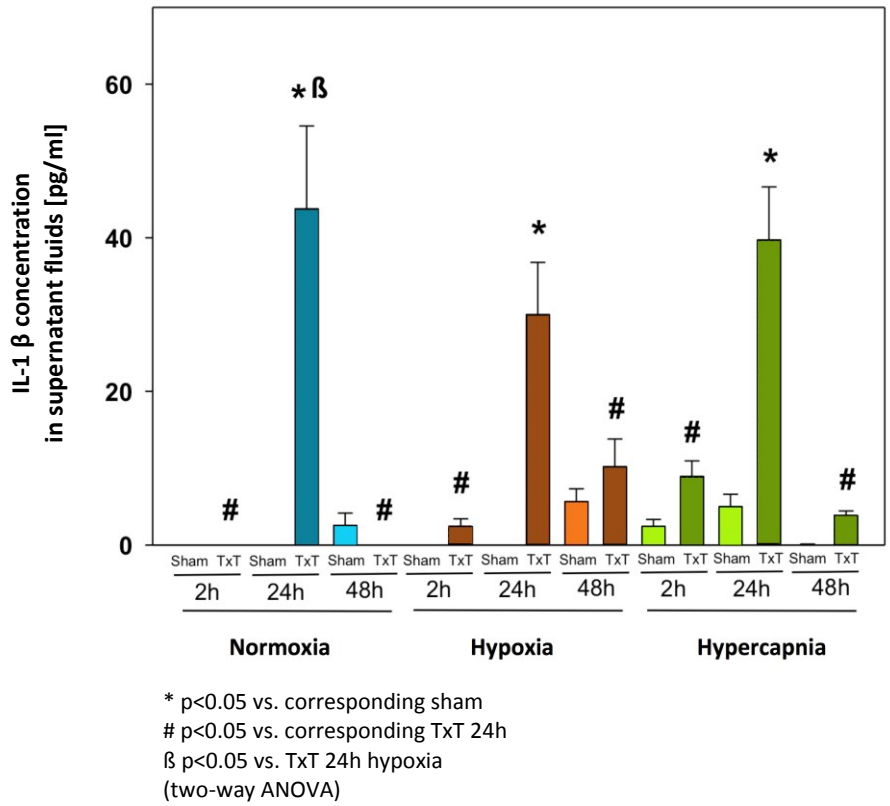


Figure 19 A: IL-1 β without ingestion: Interleukin 1 beta (IL-1 β) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h without addition of fluorescent-labeled apoptotic neutrophils (aPMN) or *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured without aPMN or *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Fig. 19 A shows IL-1 β concentrations of AM culture supernatants, incubated without ingestive material. 24 h post-trauma AM supernatants showed the highest cytokine response significant over 2 and 48 h in their corresponding groups. 24 h TxT cohorts were significant over their corresponding sham groups. 24 h TxT normoxia was significantly higher over 24 h TxT hypoxia.

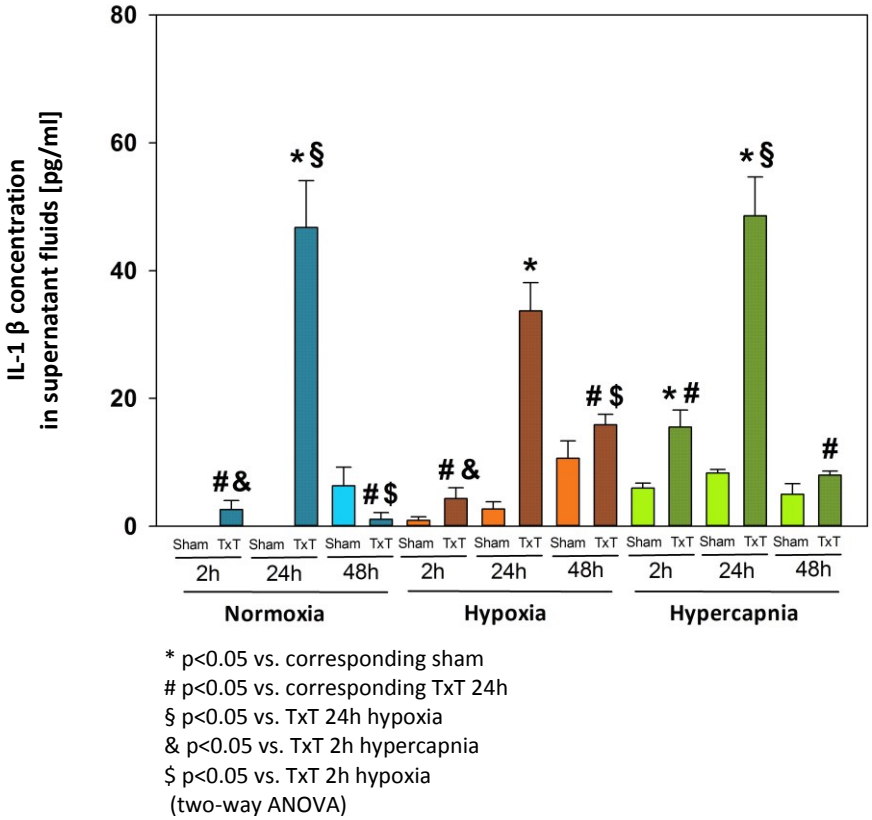


Figure 19 B: IL-1 β during aPMN engulfment: Interleukin 1 beta (IL-1 β) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled apoptotic neutrophils (aPMN). AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with aPMN under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

The strongest increase in IL-1 β release was seen in TxT 24 h model in each of the three main groups. Hypercapnia and normoxia being the lead. TxT in 24 h normoxia, 24 h hypoxia, 24 h hypercapnia, 2 h hypercapnia were significantly increased over corresponding sham procedure and over corresponding TxT in 2 and 48 h in each individual group. 24 h normoxia and hypercapnia TxT showed significantly increased IL-1 β levels compared to 24 h TxT hypoxia. 48 h TxT in normoxia showed a notable and significant decrease in IL-1 β release compared to 2 h TxT in hypoxia, whereas compared to 48 h TxT hypoxia. In 2 h TxT, we could calculate a significant increase in hypercapnia towards hypoxia and normoxia. In general IL-1 β release with PMN ingestion resembled

closely to the cytokine response without any ingestive material present. 24 h groups showed the highest peak in cytokine release.

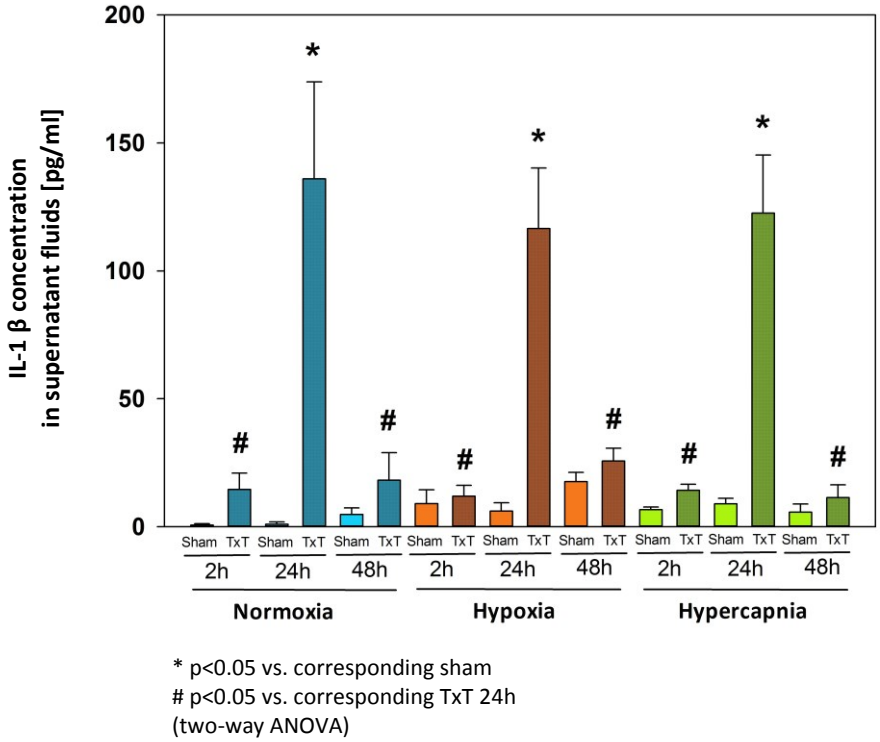
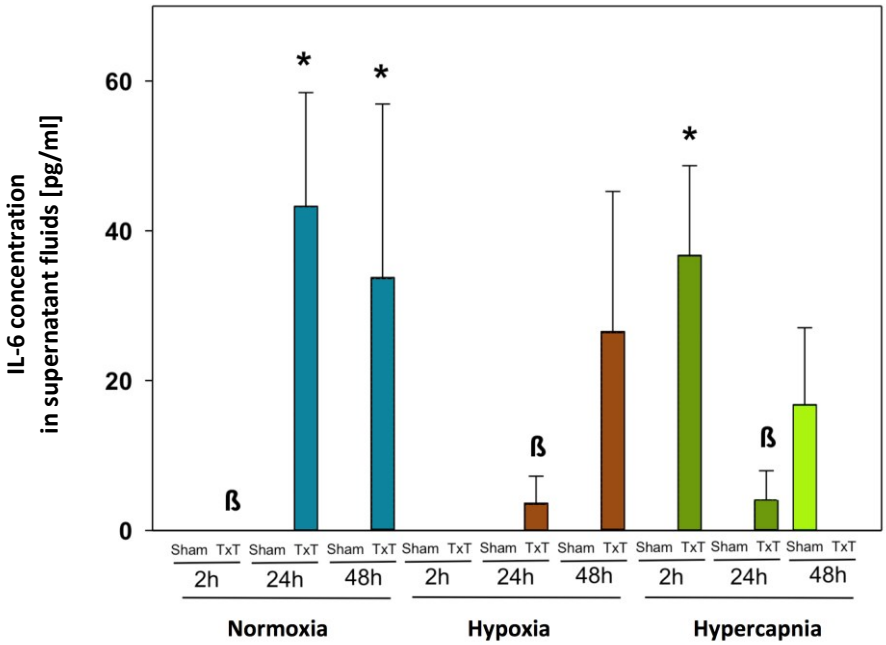


Figure 19 C: IL-1 β during *E. coli* engulfment: Interleukin 1 beta (IL-1 β) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

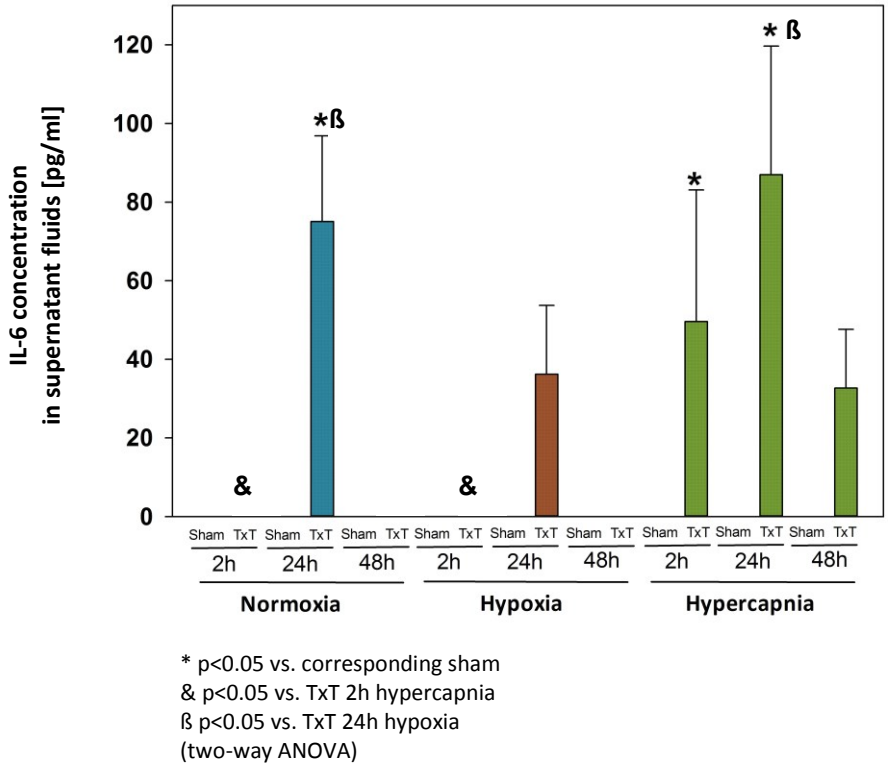
The strongest IL-1 β release during *E. coli* phagocytosis was seen in 24 h TXT throughout normoxic to hypercapnic conditions. Two-way ANOVA showed a significant increase of IL-1 β release in 24 h TXT towards the corresponding sham procedure and towards the TXT in 2 and 48 h animals in each of the major groups. 24 h groups showed the highest peak in cytokine release.



* p<0.05 vs. corresponding sham
ß p<0.05 vs. TxT 24h normoxia
(two-way ANOVA)

Figure 20 A: IL-6 without ingestion: Interleukin 6 (IL-6) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h without addition of fluorescent-labeled apoptotic neutrophils (aPMN) or *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured without aPMN or *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 20 A shows the IL-6 response without efferocytosis or phagocytosis. The post-trauma normoxia groups at 24 and 48 h as well as the 2 h hypercapnia TxT were significantly increased over their respective sham groups. 2 h normoxia, 24 h hypoxia and 24 h hypercapnia TxT showed significantly decreased IL-6 response compared to 24 h TxT normoxia.



* p<0.05 vs. corresponding sham
& p<0.05 vs. TxT 2h hypercapnia
ß p<0.05 vs. TxT 24h hypoxia
(two-way ANOVA)

Figure 20 B: IL-6 during aPMN engulfment: Interleukin 6 (IL-6) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled apoptotic neutrophils (aPMN). AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with aPMN under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 20 B shows the IL-6 concentration in AM culture supernatants during aPMN efferocytosis. TxT 2 h, 24 h and 48 h hypercapnia groups displayed the strongest increase in IL-6 release. The analysis showed a significant increase in 24 h TxT normoxia and 2 h and 24 h TxT hypercapnia over corresponding sham procedure. 2 h normoxia and hypoxia post-trauma showed significantly decreased results, compared to the corresponding 2h hypercapnia TxT. 24 h normoxia and hypercapnia TxT were both significantly increased over 24 h hypoxia post-trauma.

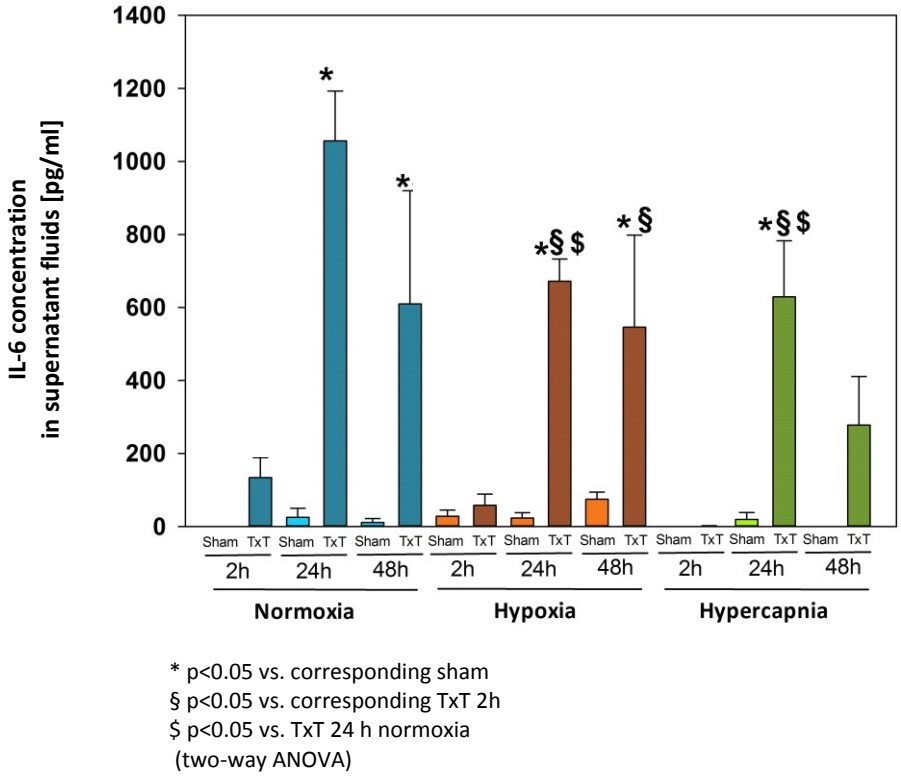


Figure 20 C: IL-6 during *E. coli* engulfment: Interleukin 6 (IL-6) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 20 C shows the IL-6 response during *E. coli* phagocytosis. Compared to figure 20 B hypercapnia did not represent the peak levels in IL-6 release. 24 h TxT of normoxia, hypoxia and hypercapnia responded with a significantly increased IL-6 secretion, compared to corresponding sham procedure. This also counted for 48 h TxT in normoxia and hypoxia. 24 and 48 h TxT in the hypoxia group were significantly higher than 2 h TxT hypoxia. Also seen in 24 h TxT in hypercapnia compared to corresponding 2 h TxT. TxT 24 h normoxia exhibited the highest IL-6 response and was significantly increased compared to 24 h TxT hypoxia and hypercapnia.

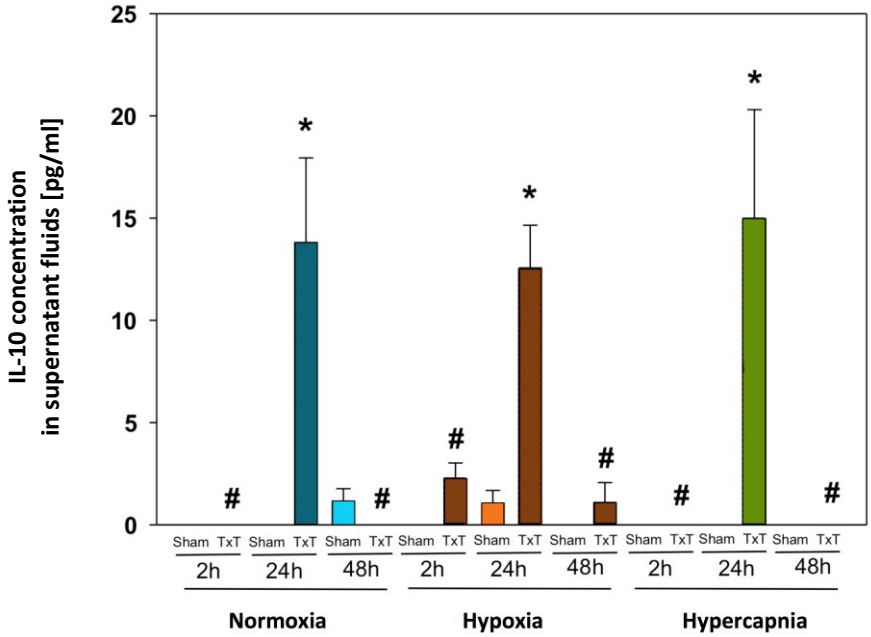


Figure 21 A: IL-10 without ingestion: Interleukin 10 (IL-10) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h without addition of fluorescent-labeled apoptotic neutrophils (aPMN) or *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured without aPMN or *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 21 A illustrates the IL-10 release pattern in absence of efferocytosis or phagocytosis. The 24 h post- traumatic groups showed the highest peak in cytokine release and were significant over their respective 2 and 48 h relatives. 24 h post-trauma were significantly elevated over their respective sham groups.

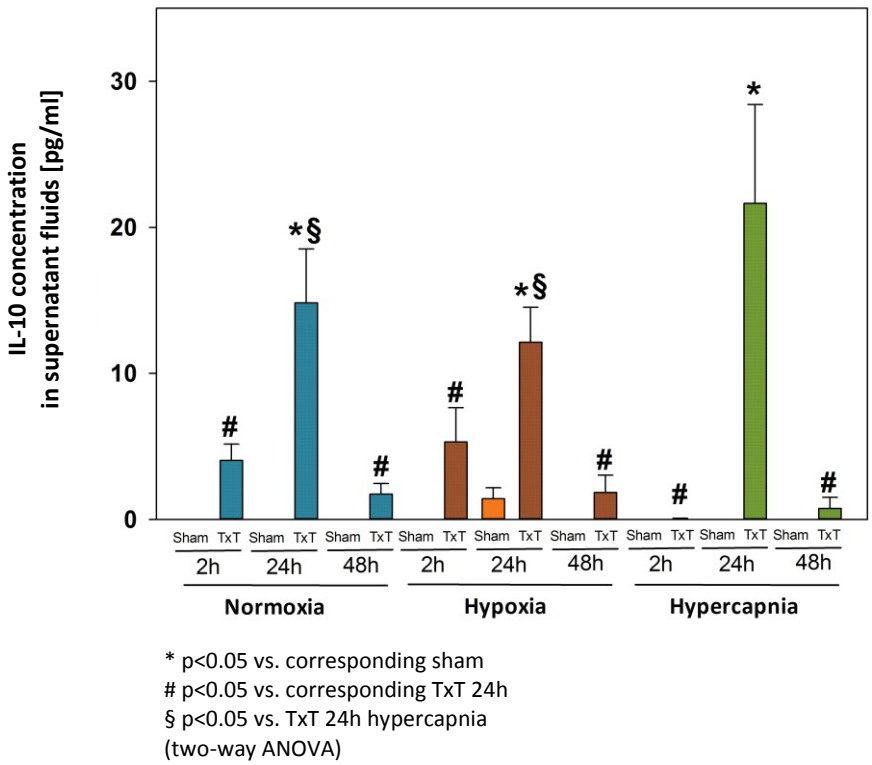


Figure 21 B: IL-10 during aPMN engulfment: Interleukin 10 (IL-10) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled apoptotic neutrophils (aPMN). AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with aPMN under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 21 B displays the IL-10 concentration in AM culture supernatants during aPMN efferocytosis. The 24 h post-trauma groups during normoxia, hypoxia and hypercapnia showed a significantly increased IL-10 release pattern, compared to each of their corresponding sham groups. In all groups, the 2 h and 48 h TxT responses were significantly less than the corresponding 24 h TxT. Amongst all 24 h TxT, hypercapnia showed the highest IL-10 release level and was significantly different compared to 24 h TxT in normoxia and hypoxia.

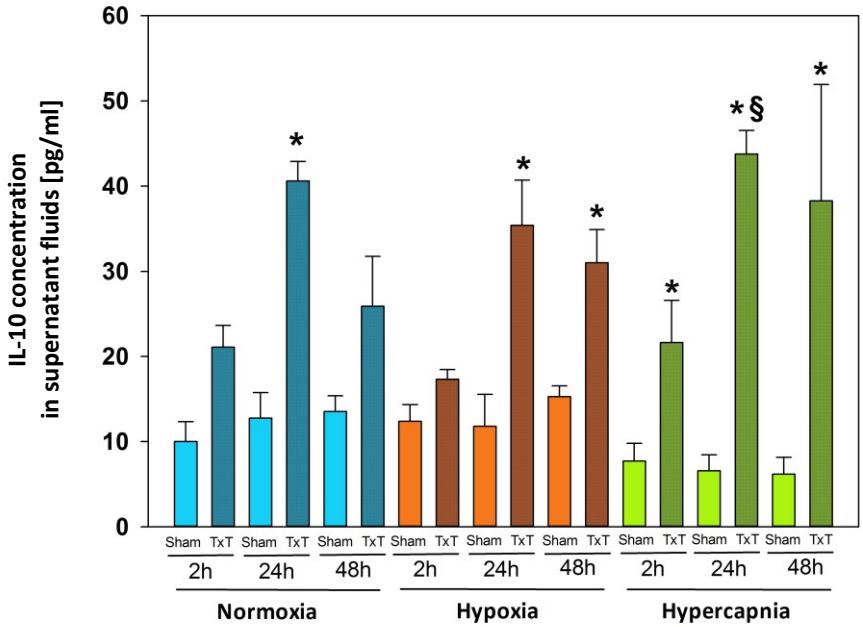
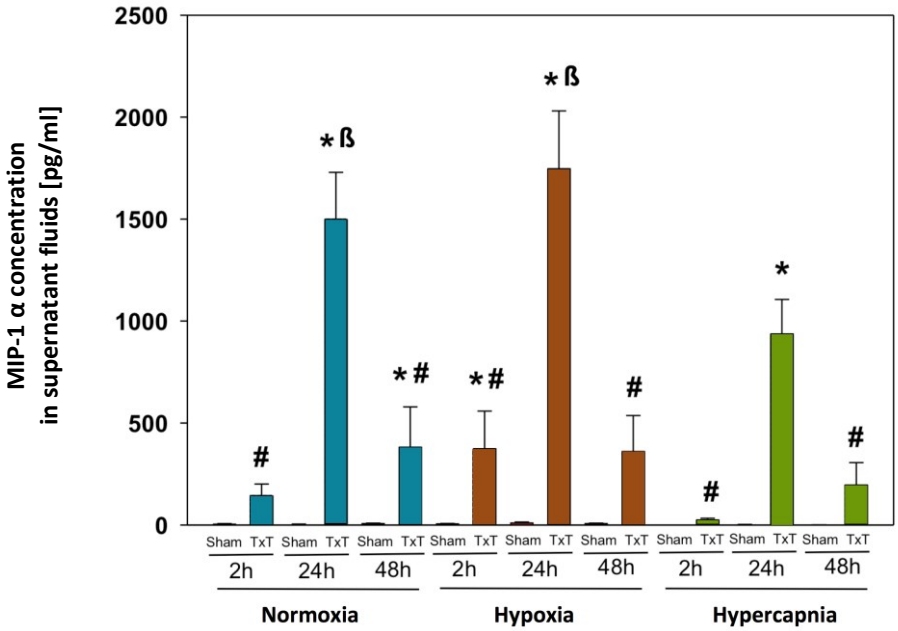


Figure 21 C: IL-10 during *E. coli* engulfment: Interleukin 10 (IL-10) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Compared to figure 21 B, *E. coli* exposure did not reveal a similarly pronounced difference between Sham and TxT. Nevertheless, a significant increase was seen in TxT towards sham in 24 h normoxia, 24 and 48 h hypoxia and 2, 24 and 48 h hypercapnia. TxT in 24 h hypercapnia was also significantly elevated over 2 h TxT in the same group.



* p<0.05 vs. corresponding sham
# p<0.05 vs. corresponding TxT 24h
ß p<0.05 vs. TxT 24h hypercapnia
(two-way ANOVA)

Figure 22 A: MIP-1 α without ingestion: Macrophage inflammatory protein 1 alpha (MIP-1 α) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h without addition of fluorescent-labeled apoptotic neutrophils (aPMN) or *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured without aPMN or *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 22 A illustrates the chemokine MIP-1 α release pattern in absence of PMN or *E. coli*. The post-traumatic groups at 24 and 48 h normoxia, 2 and 24 h hypoxia, as well as 24 h hypercapnia, were significantly elevated over their respective sham groups. 24 h post-traumatic groups showed the highest cytokine values and were significant over their respective 2 and 48 h relatives. 24 h TxT hypercapnia was significantly diminished over the respective group in normoxia and hypoxia.

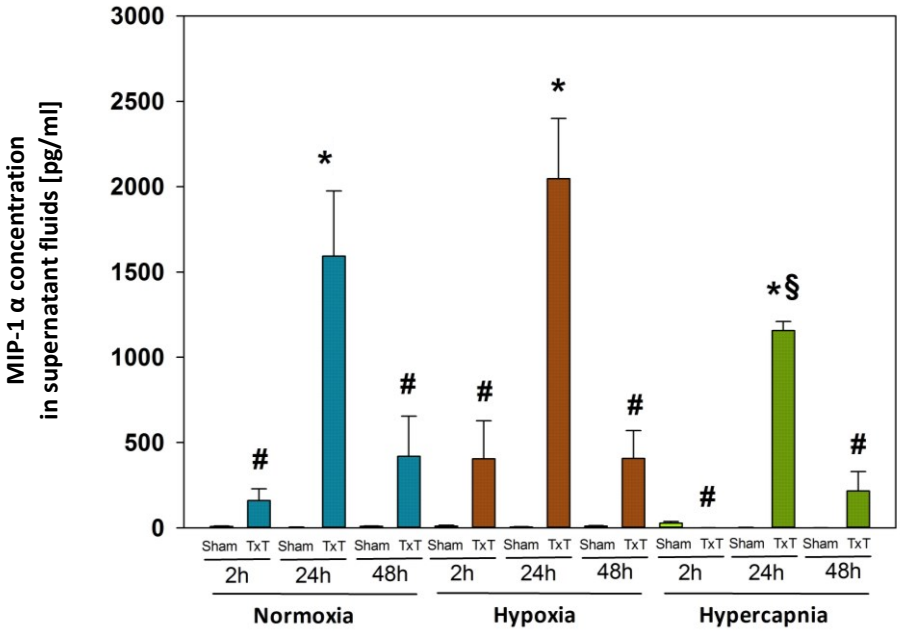


Figure 22 B: MIP-1 α during aPMN engulfment: Macrophage inflammatory protein 1 alpha (MIP-1 α) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled apoptotic neutrophils (aPMN). AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with aPMN under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

The MIP-1 α concentration of AM culture supernatants was significantly increased post-trauma compared to sham procedure in 24 h in normoxia, hypoxia and hypercapnia groups. 24 h TxT was significantly elevated compared to 2 h and 48 h TxT in normoxia, hypoxia and hypercapnia. Furthermore, 24 h TxT showed the strongest increase in MIP-1 α concentration and was significantly elevated over corresponding 24 h TxT hypercapnia. Comparing to figure 22 A the MIP-1 α release pattern was not altered when adding aPMN.

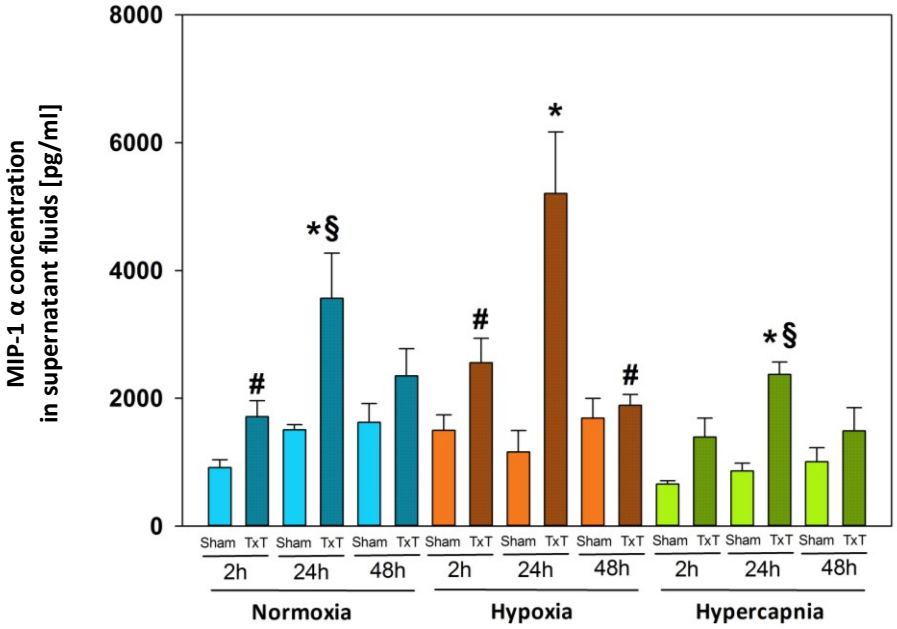


Figure 22 C: MIP-1 α during *E. coli* engulfment: Macrophage inflammatory protein 1 alpha (MIP-1 α) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 22 C shows the MIP-1 α response during *E. coli* phagocytosis. Compared to figure 22 B, there was a significant increase in cytokine release in TXT versus sham at 24 h in all three major groups. Similar to 22 B, the 24 h TXT showed the most reaction. It was significantly elevated over the corresponding 24 h TXT in normoxia and hypercapnia. During hypoxia, MIP-1-α release at 24 h TXT was significantly higher than corresponding TXT at 2 and 48 h. The same counts for 24 h TXT normoxia versus 2 h TXT in normoxia incubation condition.

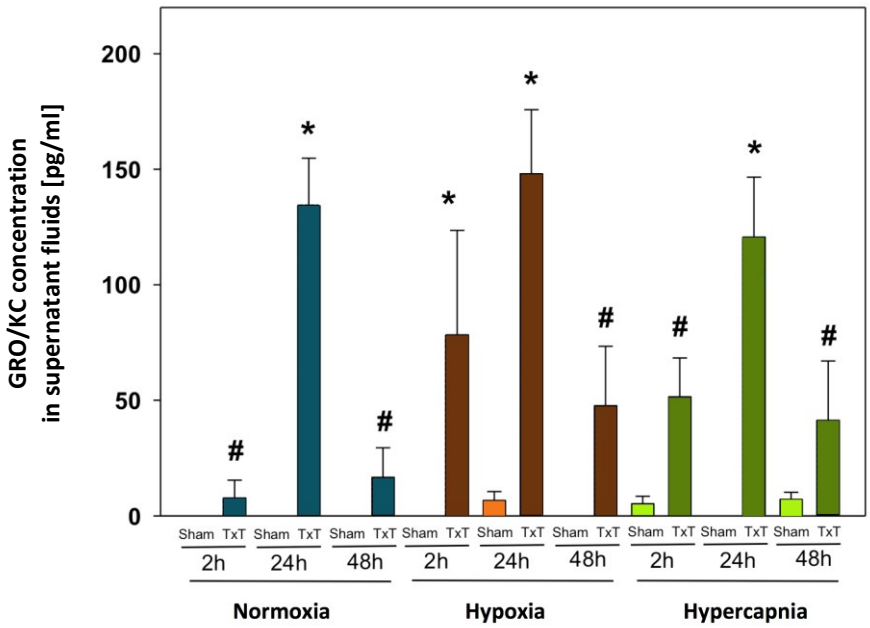


Figure 23 A: GRO/KC without ingestion: Keratinocyte chemoattractant (KC)/human growth-regulated oncogene (GRO) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h without addition of fluorescent-labeled apoptotic neutrophils (aPMN) or *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured without aPMN or *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 23 A illustrates the GRO/KC response in absence of aPMN or *E. coli*. 24 h normoxia, hypoxia and hypercapnia, as well as 2 h hypoxia TxT were significantly increased over their corresponding sham groups. 24 h post-trauma GRO/KC levels were also significantly elevated over their respective 2 and 48 h groups, except for 24 h hypoxia versus 2 h hypoxia.

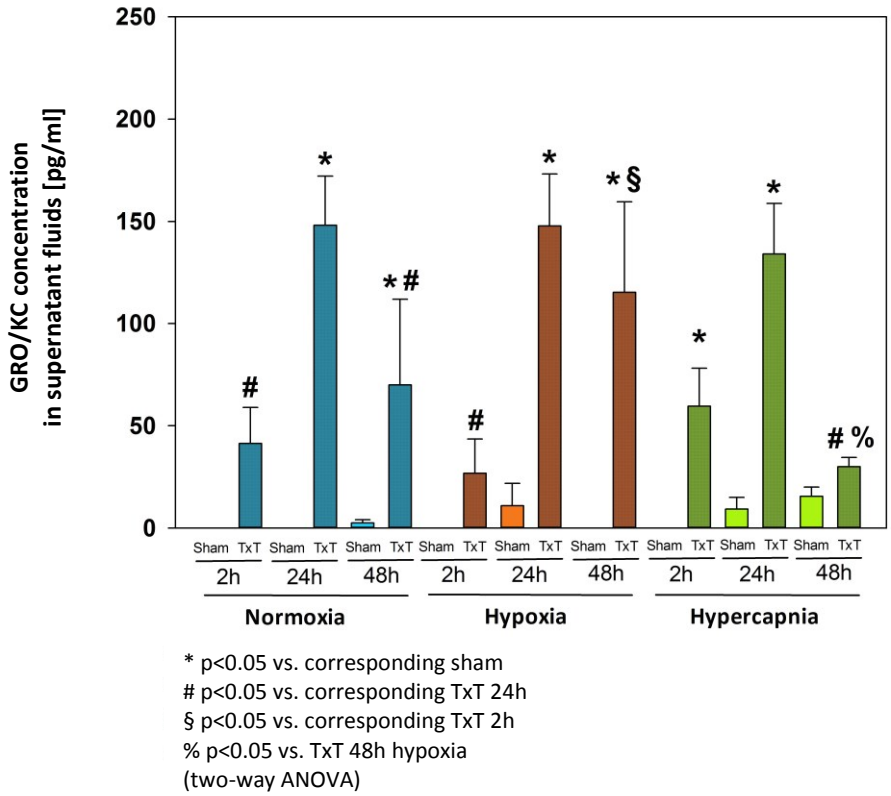


Figure 23 B: GRO/KC during aPMN engulfment: Keratinocyte chemoattractant (KC)/human growth-regulated oncogene (GRO) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled apoptotic neutrophils (aPMN). AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with aPMN under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 23 B displays the GRO/KC release in aPMN engulfment. GRO/KC was significantly higher in TxT towards sham at 24 and 48 h in normoxia and hypoxia and 2, 24 h hypercapnia. The 24 h TxT showed elevated levels in all three groups and was significant towards 2 and 48 h TxT in normoxia, 2 h TxT in hypoxia and at 48 h TxT in hypercapnia. The analysis showed also a relevant increase at 48 h TxT in hypoxia towards 2 h TxT in the same group. 48 h TxT hypoxia showed significantly elevated levels of GRO/KC towards 48 h TxT in hypercapnia.

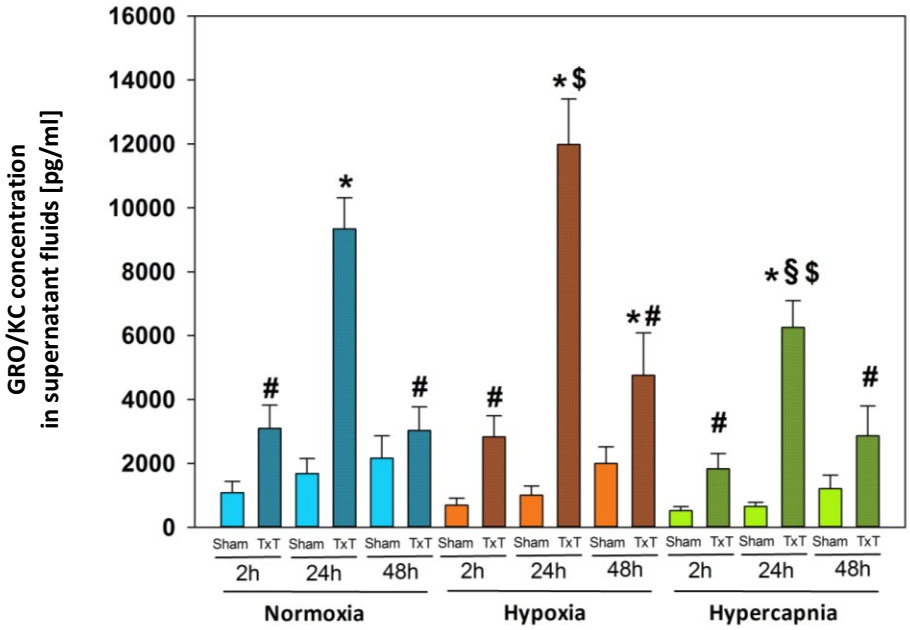


Figure 23 C: GRO/KC during *E. coli* engulfment: Keratinocyte chemoattractant (KC)/human growth-regulated oncogene (GRO) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 23 C represents the GRO/KC release pattern during *E. coli* treatment. TxT resulted in an increase versus sham at 24 h in normoxia, hypoxia and hypercapnia and 48 h hypoxia. In all three main groups 24 h TxT values were significantly elevated over similar condition at 2 h and 48 h. 24 h TxT in hypoxia was notably elevated and showed a relevant increase of GRO/KC release than seen at 24 h TxT in hypercapnic state. 24 h TxT and hypoxia were significantly higher versus 24 h TxT in normoxia, which showed a significantly higher response in GRO/KC release towards 24 h TxT in hypercapnia.

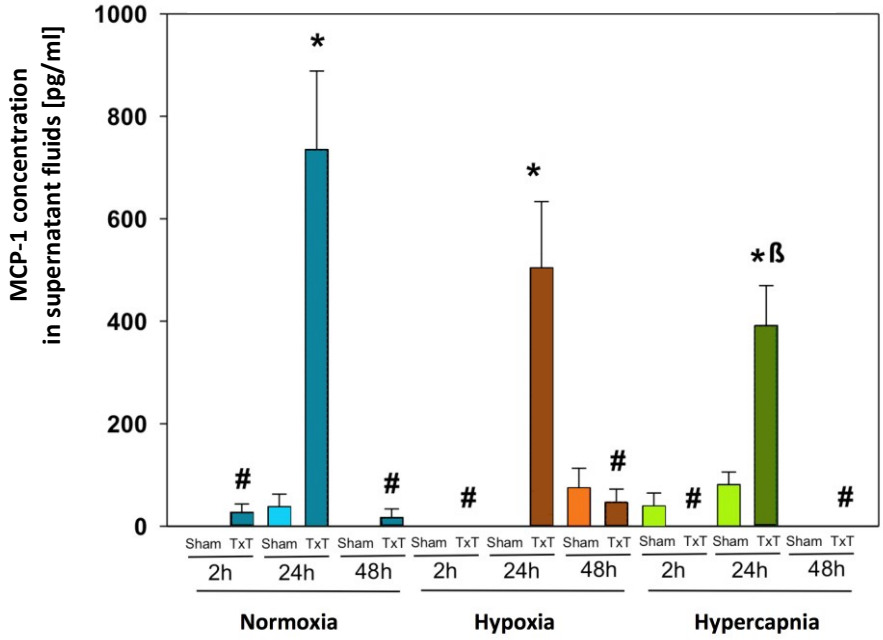


Figure 24 A: **MCP-1 without ingestion:** Monocyte chemoattractant protein 1 (MCP-1) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h without addition of fluorescent-labeled apoptotic neutrophils (aPMN) or *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured without aPMN or *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

The 24 h post-traumatic MCP-1 release from AM was significantly increased during normoxia, hypoxia and hypercapnia and was significantly increased over corresponding sham and was significantly greater than their respective 2 and 48 h groups. 24 h hypercapnia was significantly decreased compared to the 24 h normoxia post-traumatic group.

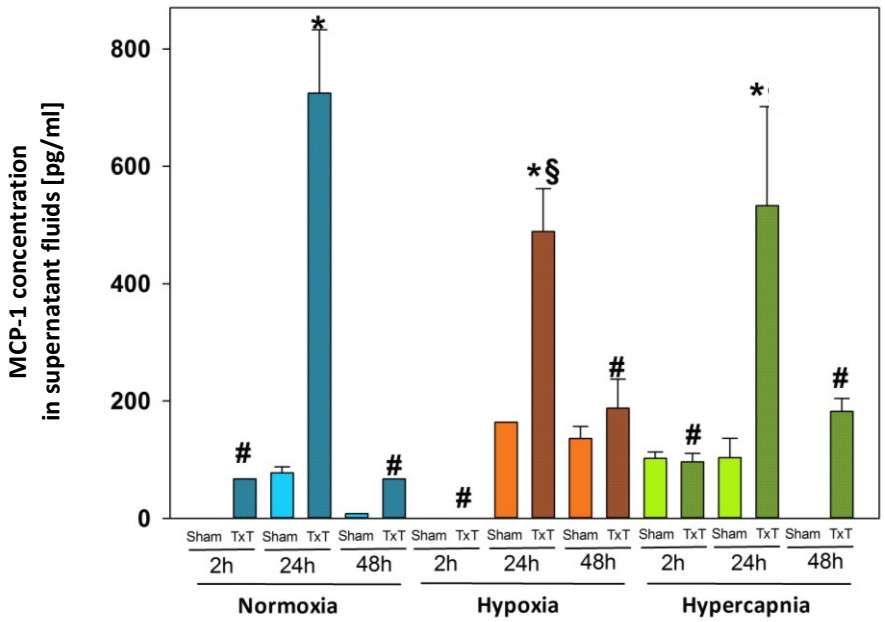
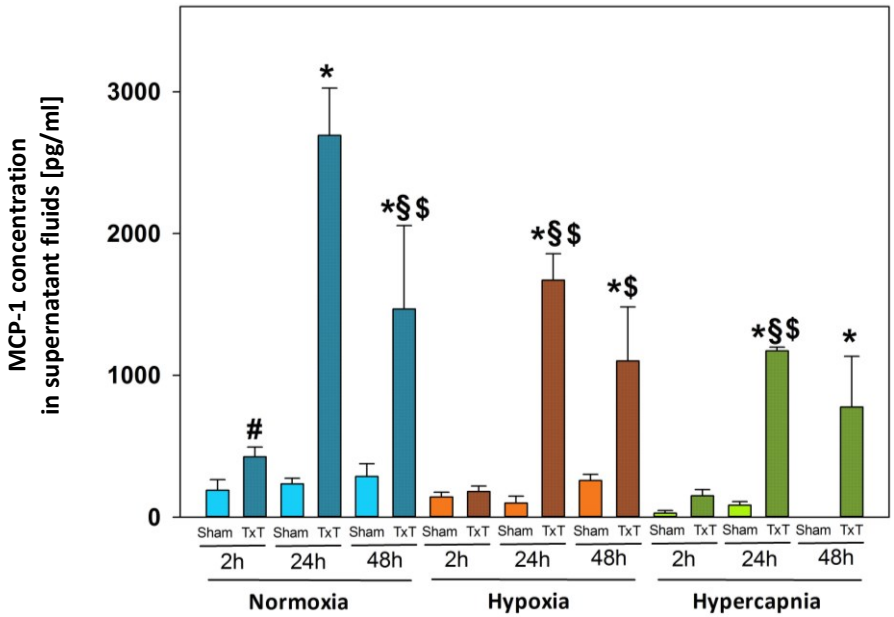


Figure 24 B: MCP-1 during aPMN engulfment: Monocyte chemoattractant protein 1 (MCP-1) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled apoptotic neutrophils (aPMN). AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with aPMN under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 24 B displays the reaction of MCP-1 in presence of PMN engulfment. Cytokine release at 24 h TxT in normoxia, hypoxia and hypercapnia was sigificantly increased over their corresponding sham and corresponding 2 and 48 h TxT groups.



* p<0.05 vs. corresponding sham
$ p<0.05 vs. corresponding TxT 2h
§ p<0.05 vs. TxT 24h normoxia
(two-way ANOVA)

Figure 24 C: MCP-1 during *E. coli* engulfment: Monocyte chemoattractant protein 1 (MCP-1) concentrations in supernatant fluids of alveolar macrophages (AM) isolated after blunt thoracic trauma (TxT) or sham treatment, cultured under normoxic, hypoxic or hypercapnic conditions for 2 h with addition of fluorescent-labeled *E. coli*. AM were isolated 2, 24 or 48 h after TxT or sham treatment and cultured with *E. coli* under normoxic, hypoxic or hypercapnic conditions. Cytokine concentrations were analyzed by multiplex assay. Values are expressed as means ± standard error of the mean (SEM) of n = 5 animals per group.

Figure 24 C displays the MCP-1 conditions during *E. coli* exposure. Compared to the conditions in figure 24 B the threshold levels are set much higher with peaks around 3000 pg/ml.

An additional set of experiments was conducted, in which every respective cohort was given a buffering solution. The buffer only had little to no effect on the pH values of the culture supernatants. There was also no significant change in the outcome of phagocytic activity or cytokine/chemokine response, compared to the resembling groups without the buffering solution.

3.7. Data summary

In vivo and *in vitro* efferocytosis and phagocytosis activity

In vivo efferocytosis activity of alveolar macrophages (AM) with intratracheal instillation of fluorescent-labeled apoptotic neutrophils (aPMN) 2, 24 or 48 h after blunt chest trauma (TxT) or sham treatment in rats was altered neither by blunt chest trauma (TxT) nor sham treatment. The phagocytosis activity of AM with fluorescent-labeled *E. coli* on the other hand, was significantly up regulated after TxT when compared to sham treatment.

In vitro efferocytosis activity of alveolar macrophages (AM) isolated 2, 24 or 48 h after blunt chest trauma (TxT) or sham treatment and cultured under normoxic, hypoxic and hypercapnic conditions with fluorescent-labeled apoptotic neutrophils (aPMN) was down regulated after trauma, when compared to sham treatment. The phagocytosis activity of AM with fluorescent-labeled *E. coli* on the other hand, was significantly up regulated after TxT when compared to sham treatment.

Hypercapnic culturing conditions have shown to increase efferocytosis activity of AM isolated after TxT or sham treatment and cultured with aPMN significantly. Phagocytosis of *E. coli* on the other hand, was significantly down regulated under influence of elevated CO_2. Hypoxic conditions did not alter the efferocytic or phagocytic behavior of AM.

Cytokine profiles of AM after TxT and sham treatment during normoxic, hypoxic and hypercapnic conditions

Cytokine/chemokine levels in AM supernatants were significantly up regulated after trauma. Cytokine/chemokine release was at maximum in supernatants of AM isolated 24 h after TxT.

Table 2: Effects of hypoxia and hypercapnia on cytokine/chemokine release in supernatant fluids from alveolar macrophages (AM) isolated 24 h after blunt chest trauma and co-incubated with aPMN, E. coli, or none.

24 h post TxT			
Expression	**Co-incubation**	**Hypoxia**	**Hypercapnia**
↑	none	TNF-α	
	aPMN	TNF-α	IL 10
	E. coli	MIP-1 α	
↓	none	IL 1 β IL 6	TNF-α IL 6 MIP-1 α MCP 1
	aPMN	IL 6 MCP 1	TNF-α
	E. coli	TNF-α IL 6 MCP 1	TNF-α IL 6 GRO/KC MCP 1

4. Discussion

Blunt chest trauma (TxT) is one of the most common forms of injury e. g. resulting from motor vehicle accidents. Therefore, it is important to fully understand the inflammatory processes involved after TxT to provide better treatment and to improve the outcome for the trauma patient. An important protagonist in pulmonary immune defense is the alveolar macrophage (AM). The ability of AM to take up apoptotic material and bacteria, such as apoptotic neutrophils (aPMN) and *E. coli,* is a crucial part in the resolution of inflammatory challenges e.g. after acute lung injury [90]. We therefore investigated both, the efferocytic (ingestion of apoptotic PMN) and phagocytic (ingestion of *E. coli*) capability of AM in male Sprague Dawley rats *in vivo* by intratracheal instillation of either apoptotic neutrophils (aPMN) or *E. coli* 2, 24 or 48 h after trauma or sham treatment. Furthermore, TxT could affect intrapulmonary ventilation and create hypoxic and hypercapnic areas. Thus, we isolated AM 2, 24 or 48 h after trauma or sham procedure to investigate afterwards *in vitro* any alterations in efferocytosis and phagocytosis under normoxic, hypoxic or hypercapnic conditions alongside their inflammatory response.

Following hypotheses were proposed:

1. TxT leads to an alteration of the lungs' immune system in terms of up-regulation of the efferocytic and phagocytic activity of AM.

2. Said up-regulation is attributable to the partial hypoxic and hypercapnic conditions in the contused lung.

3. Post-traumatic changes in the AM cytokine release pattern towards a pro-inflammatory response may be modified by hypoxic and hypercapnic conditions towards an anti-inflammatory cytokine release.

4.1. Efferocytosis and phagocytosis *in vivo* and *in vitro*

We anticipated blunt thoracic trauma (TxT) to alter alveolar macrophages (AM) of rats in terms of an up regulation of their efferocytic and phagocytic activity *in vivo* and *in vitro*.

However, by intratracheal instillation of fluorescent-labeled apoptotic neutrophils (aPMN) post trauma or sham treatment, the efferocytic activity of AM *in vivo* was not significantly increased after trauma and remained unchanged.

In vitro experiments showed, that the post-traumatic efferocytic activity of aPMN in AM isolated 2, 24 or 48 h after trauma procedure or sham treatment, was equally unchanged for both, normoxic and hypoxic culturing conditions. Hypercapnic conditions on the other hand, significantly lowered the efferocytic activity of AM isolated 2 and 24 h after trauma compared to AM, which underwent sham procedure.

Seitz et al. suggested, that *in vitro* AM efferocytosis of apoptotic alveolar type 2 cells (AT2-cells) was significantly increased after blunt chest trauma in rats, but an increase in AM efferocytosis of aPMN was only present, when AM were additionally stimulated with phorbol-12-myristate-3-acetate, a strong activator of protein kinase C [73].

Erwig et al. demonstrated in a different setting that rat bone marrow derived macrophages, which had the opportunity to ingest apoptotic PMN before the uptake of a "second hit" of apoptotic cells, would present a lower efferocytic activity, than starved macrophages. Their results indicated a reduction of 50% caused by a reduced residual capacity and by interrupting an integrin-dependent recognition pathway resolving in an impaired uptake of neutrophils [21]. Our results resembled the findings of Erwig et al. This would imply, that PMN, which had migrated to the site of the alveolus [35] subsequently to blunt chest trauma, could have given AM the preceding opportunity to ingest and "fill up on" aPMN prior to the commencement of our *in vitro* studies. AM eventually would have become saturated by the excessive intake, hence presenting reduced capacity to remove the supplied fluorescent-labeled aPMN. This assumption could explain why trauma did not significantly alter the efferocytic activity of AM in our data. But since the AM had prior contact to aPMN and our data did not reveal any significant decrease in efferocytic activity, the ingestion of aPMN could in fact be increased by the trauma stimulus

compared to sham treatment.

The trauma patient develops areas of hypoxia and hypercapnia consecutively to contusion in his lungs [39]. These circumstances raise the question, whether or not the AM are affected by the surrounding changes. There is growing evidence that hypercapnic acidosis may itself minimize or reduce lung injury as a result of suppressing inflammatory processes and may even be used as a therapeutic strategy [40]. Therapeutic hypercapnia is also used for ventilation-perfusion coupling in the lungs and plays an important role in promoting cerebral vasodilation [43]. Hypoxia on the other hand, has been known to increase inflammatory levels (e. g. in patients with mountain sickness), further causing pulmonary and cerebral edema, due to increased vascular leakage [28, 29].
In this context the hypothesis was proposed, that alterations of efferocytosis and phagocytosis after trauma were attributable to the partial hypoxic and hypercapnic conditions in the contused lungs. To verify this hypothesis we examined the behavior of AM in various settings.

Normoxic and hypoxic *in vitro* conditions during AM efferocytosis, mimicked the findings from our *in vivo* studies. However, hypercapnic conditions significantly increased the efferocytic activity levels in AM during sham procedure compared to hypoxia and normoxia. This could be explained by the Henderson-Hasselbalch paradigm of hypercapnia-induced shift to acidic pH levels. Park et al. found, that macrophages in low pH medium showed enhanced efferocytic activities using phosphatidylserine (PS)-coated beads, which are commonly used to simulate apoptotic cells. According to them, a minimal acidic microenvironment may lead to an increased transcription in macrophage surface proteins like stabilin-1 and to a subsequent enhancement of efferocytic activity. After blockage of stabilin-1, the up-regulated efferocytosis was suspended in macrophages [59]. We could also show, that the efferocytic activity of AM isolated 2 and 24 h after trauma was significantly lowered compared to sham group.
Hypercapnic conditions therefore alter the efferocytic activity of AM differently and are not reconcilable with our *in vivo* results. We assumed, that trauma combined with hypercapnia affected the efferocytic synapsis of AM in a different way and thus might have directly modulated transcription factors necessary for PS- and vitronectin receptors, Mer and Stabilin-1 and 2 expression [81]. In other words, trauma could be a trigger to decrease

aPMN clearance during hypercapnia by affecting the transcription of receptors, or bridging molecules of the efferocytic synapsis.

More than 4% of all community-acquired pneumonias in the elderly patient are due to E. coli strains, making it the second most common microorganism to cause bacteriogenic pneumonia, [47] it is of major concern to understand the processes involved in post-traumatic, bacteriogenic pneumonia. Therefore, E. coli can be considered as an eligible model organism for present investigations.

Here, the phagocytic activity, assessed by E. coli intake, was significantly up regulated post-trauma. The *in vitro* study showed similar results during normoxic and hypoxic conditions. This was also seen in AM isolated 48 h after trauma during hypercapnia. Only AM isolated 2 and 24 h after trauma failed to show any significant alteration in E. coli ingestion.

Islam et al. could demonstrate *in vitro,* that the phagocytic activity of porcine AM was significantly increased following a 24 h incubation period with low concentrations of LPS [37]. Their results support our findings of a significantly up-regulated phagocytic activity *in vivo* and *in vitro*. These results could point to a massively increased inflammatory response, triggered by the stimulation with LPS found on E. coli surfaces. Further, this increased reaction towards LPS after trauma could also be caused by a physiological phenomenon, which is called the Euler-Liljestrand reflex. The Euler-Liljestrand reflex represents the lungs' physiologic shutdown of blood supply to compromised or less ventilated lung tissue. Compromised areas may arise from contusion itself, or from hemorrhagic infiltration, which would cause atelectasis, thus further impeding ventilation. The Euler-Liljestrand induced consecutive ischemia can now enhance the TLR4 expression of AM (which is especially sensitive to LPS from gram-negative bacteria, such as *E. coli*), due to an increased genesis of ROS [56]. Previous research has also confirmed, that the TLR4 receptor was found to be up regulated in mouse AM 24 h post-trauma [80]. These mechanisms could clearly cause a significant gain in phagocytic activity of AM.

In terms of incubation conditions during the phagocytosis experiments with *E. coli,* we could show, that normoxic and hypoxic conditions reflected the findings of our *in vivo* studies. AM isolated 2 and 24 h after blunt chest trauma and incubated under hypercapnic conditions on the other hand did not show any significant increase compared to their respective sham groups. Only 48 h post trauma AM did significantly increase *E. coli*

ingestion, compared to the non trauma group.

Hypercapnia decreased the phagocytic activity of AM compared to normoxia and hypoxia conditions, which led to a reduced *E. coli* ingestion *in vitro*. According to Wang et al., elevated CO_2 levels decreased phagocytosis of opsonized polystyrene beads, as well as heat-killed bacteria in mouse AM after being stimulated with LPS [84].

This might be attributable to the CO_2 dependent expression of anti-apoptotic factors, such as Bcl-2 and Bcl-xl, which next to increasing bacterial clearance also negatively regulate autophagy in macrophages by binding to Beclin 1. Targeting the Bcl-2/Bcl-xl-Beclin 1 interaction could ameliorate the CO_2 induced immunosuppression, thus improving the outcome of patients suffering from advanced lung disease [10].

4.2. Alterations in cytokine release post trauma and influences of hypoxia and hypercapnia

Trauma to the lungs can cause atelectasis and displace major airways. This may lead to inadequate ventilation, causing hypoxic and hypercapnic areas in the surrounding tissue. We hypothesized that the post-traumatic change in efferocytosis and phagocytosis could be altered by the influence of hypoxia and hypercapnia. Changing conditions might also alter the cytokine and chemokine release patterns in AM. We hypothesized trauma to evoke a massive change in mediator release, which could be shifted by elevated CO_2 levels towards an anti-inflammatory pattern.

Our results showed that blunt chest trauma significantly altered the cytokine/chemokine response in AM supernatants of rats. Compared to respective sham groups, the supernatants of AM isolated and cultured 2, 24 or 48 h after trauma showed a significantly increased mediator release. These changes were also described in previous studies showing that IL-1 β, IL-6, IL-10, TNF-α, MCP-1, MIP-1 α and GRO/KC expressions from AM were significantly increased post trauma [25, 65, 72].

Present results indicated, that the significantly highest release of IL-1 β, IL-10, TNF-α, MCP-1, MIP-1 α and GRO/KC was reached in AM isolated 24 h after trauma. IL-6 were insignificantly altered, although Visser et al. found, that in patients the highest IL-6

response could be measured at 24 h post-trauma [82]. This could be imputed by the increased standard deviation in the IL-6 data, explainable by the inconsistency of IL-6 generation and release, e.g. changed by stress [69]. In the clinical setting of polytrauma, IL-6 was significantly increased and could be used as clinical marker accordingly [25].

In terms of efferocytosis versus phagocytosis, supernatant fluids of AM cultured with *E. coli,* revealed much higher stimulated mediator releases. Comparing the highest values of GRO/KC concentrations, we detected a 120 fold higher concentration of GRO/KC in supernatants co-incubated with *E. coli*, compared with aPMN co-incubation. LPS, as part of the *E. coli* surface, stimulated the GRO/KC chemokine release, like interferon α and TNF-α, reflecting the high chemokine response [2]. Similar effects were found for the pro-inflammatory cytokines TNF-α and IL-6. Driscoll et al found that rat AM expressed in particular increased levels of MIP-1 α in response to LPS stimulus [20]. This deviance might be attributable to the excessive LPS stimulation additional to the blunt chest trauma.

4.2.1. Hypoxia

The incubation of AM was conducted with 5 % oxygen concentration. Incubation of control AM (no ingestion) in these low O_2 conditions revealed a significantly stimulating effect on the TNF-α release of AM after blunt chest trauma. TNF-α was also up regulated in AM incubated with aPMN. This increase in TNF-α mediator release could be explained by the hypoxia-induced nucleosomal transcription factor HIF, which also induces the NF-κB pathway, hence increasing the amount of TNF-α released [5, 34]. In contrast, the incubation with *E. coli* showed significantly diminished TNF-α levels during hypoxia. Matuschak and Hirani et al. have described similar findings for TNF-α production in rat AM stimulated with *E. coli* lipopolysaccharide under hypoxic conditions [33, 48].

IL-1 β release was suppressed during efferocytosis in our studies. Low oxygen levels did not influence the IL-1 β release during the incubation of AM with *E. coli*. Matuschak et al. on the other hand showed a decrease in IL-1 β release of rat AM during *E. coli* phagocytosis. According to them, the DNA binding profile of NF-κB was reduced during hypoxia, thus decreasing IL-1 β release [38, 48].

In present data, hypoxia suppressed the IL-6 release of AM during efferocytosis and phagoytosis. Chao et al. showed that hypoxia increased the expression of HIF-1 α and NF-κB in the lungs and led to an up regulation of neurokinin-1 receptors in alveolar macrophages, causing IL-1, IL-6, and TNF-α to increase [13]. Some researchers also found increased IL-6 and CRP concentrations in humans when accommodating to high altitudes with associated low oxygen levels [31].

During hypoxia, LPS, or *E. coli* increased the MIP-1 α and GRO/KC release, compared to normal oxygen levels. This could be explained by the pro-inflammatory effects of the HIF-pathway. Abolhassani et al. published similar results for MIP-1 α release [1].

During efferocytosis and phagocytosis, reduced oxygen levels significantly diminished MCP-1 release, compared to normoxia. Opposed to that, Chao et al. found hypoxic AMs to release MCP-1 as a key mediator. According to their research, alveolar hypoxia caused a rapid increase of MCP-1 plasma concentrations [12, 14]. An explanation for the controversial findings may be the fact, that Chao et al. performed the hypoxic experiments *in vivo* without a trauma impact, whereas we incubated AM *in vitro* after trauma. Trauma and *in vitro* incubation must have different effects on MCP-1 release during hypoxia.

4.2.2. Hypercapnia

We could show that AM under hypercapnia significantly reduced the release of TNF-α, IL-6, MCP-1 and MIP 1-α. During efferocytosis AM showed significantly increased IL-1 β (at 2 h), IL-6 (at 2 and 48 h) and IL-10 levels. TNF-α and MIP 1-α release was suppressed during efferocytosis in hypercapnia.

Elevated CO_2 levels seem to suppress TNF-α, IL-6, GRO/KC, MCP-1 and MIP 1-α during *E. coli* ingestion.

We could show, that elevated CO_2 levels had an anti-inflammatory effect on AM mediator release. TNF-α concentrations were significantly suppressed under elevated CO_2 levels, whereas the reaction to low oxygen exposure showed a stimulation in TNF-α release. Gates, Kimura and Lang et al. have demonstrated such a behavior for TNF-α during hypercapnia [24, 38, 44]. According to Lang, the decrease in TNF-α release was

attributable to the influence on cellular acid-base status mediating the effects of CO_2 on cytokine secretion in AM [44].

We could show, that the IL-1 β (at 2 h), IL-6 (at 2 and 48 h) response in AM was significantly increased post trauma during PMN engulfment when incubated with elevated CO_2 levels. The control and *E. coli* incubation groups on the other hand were markedly inhibited by hypercapnia. Gates et al. confirmed that IL-6 would decrease under the influence of CO_2 [24]. Further research indicated, that elevated CO_2 levels selectively inhibited and down-regulated IL-6 and NF-κB pathways, which was consecutively leading to TNF-α down-regulation in LPS-stimulated AMs [84]. Opposed to that, Abolhassani and Norozian et al. reported converse results [1, 57]. Norozian et al. showed, that a pro-inflammatory reaction in the lungs (assumed on the sole expression of pro- and anti-inflammatory cytokines) would be amplified by the influence of elevated CO_2 levels. A 24 h exposure to therapeutic hypercapnia was, according to Norozian et al., associated with a pro-inflammatory immune response in the lung tissue and led to a significantly higher IL-1β and IL-6 expression [57]. We were not able to confirm their suggestion. In present study hypercapnia acted anti-inflammatory. Of note, Norozian et al. used specimens, which were previously stimulated with endotoxin [57].
Other researchers have shown a decrease in IL-10 cytokine concentrations in endotoxin-stimulated specimens treated with elevated CO_2 levels [38, 57]. Down-regulation of IL-10 was absent in our studies during phagocytosis of *E. coli*. But we could demonstrate an increase of IL-10 during aPMN ingestion. It has to be emphasized, that some researchers applied hypercapnia *in vivo*, by which other cells and cytokines can react and interfere on the general outcome. Whereas present study performed an *in vitro* exposure of CO_2, which only interfered with AM.

4.3. Limitations of the study

Sevoflurane was used as a sedative agent, which has been known to perform as suppressor for TNF-α, IL-6 and MCP-1 expression [89].

To evaluate the influence of altered pH levels on the experiment, in terms of hypercapnia induced acidosis, the post-incubation pH levels were determined and a control group implemented, in which HEPES was used as buffering solution. However, HEPES was unable to reduce the hypercapnia-triggered acidosis completely. We maintained the HEPES control groups nevertheless and were not able to capture a major deviance between buffered and non-buffered results. To further exclude a probable additional impact of acidosis to the cell cultures, further research should refer to bicarbonate based buffer systems, which could on the other hand annihilate the cellular influences of CO_2 itself.

4.4. Conclusion

In conclusion, we could show that blunt chest trauma stimulates the phagocytic activity of AM in rats *in vivo* and *in vitro,* as indicated by the up regulated *E. coli* ingestion. It is assumed that the LPS stimulus enormously enhances phagocytic activity and cytokine/chemokine response. Another contributing mechanism may be the Euler-Liljestrand reflex, which could enhance phagocytosis via TLR4 up-regulation. In contrast, the efferocytic activity of AM when ingesting aPMN remained unchanged *in vivo.* This was similar to *in vitro* findings during normoxic and hypoxic conditions.

Compared to respective sham groups, blunt chest trauma has shown to significantly raise the cytokine and chemokine mediator release, predominantly in AM isolated 24 h after trauma.

Incubation at low oxygen levels did neither alter the efferocytic nor phagocytic capabilities of AM, but instead influenced the mediator release significantly. Hypoxia significantly increased the TNF-α release in AM incubated with and without aPMN, whereas stimulation with LPS seemed to inhibit the TNF-α release. On the other hand, the pro-inflammatory cytokines IL-1 β and IL-6, as well as MCP-1 were mostly suppressed during aPMN and *E. coli* ingestion by the influence of hypoxia.

Incubation in hypercapnic conditions promoted the ingestion of aPMN in AM, then again inhibited the ingestion of *E. coli,* which could increase the probability of bacteremic lung

infections. Hypercapnia markedly suppresses the release of pro-infammatory mediators such as TNF-α. During phagocytosis IL-6, as well as MIP-1-α, GRO/KC and MCP-1 were significantly decreased by hypercapnia. On the other hand the levels of IL-10 were increased under the influence of elevated CO_2. This implies that hypercapnia could have anti-inflammatory influences on AM after trauma *in vitro*.

5. Summary

Blunt trauma to the thoracic area is frequent and directly responsible for 20-25% of all trauma deaths. Even mild trauma to the chest can cause severe local and systemic inflammatory alterations. Alveolar macrophages (AM) are the lungs first line of host defense and play an important role in overcoming the effects of blunt chest trauma (TxT). This dissertation is focused on the response of AM to TxT under various conditions.
Following hypotheses were proposed:

1. TxT leads to an alteration of the lungs' immune system in terms of up-regulation of the efferocytic and phagocytic activity of AM.

2. Said up-regulation is attributable to the partial hypoxic and hypercapnic conditions in the contused lung.

3. Post-traumatic changes in the AM cytokine release pattern towards a pro-inflammatory response may be modified by hypoxic and hypercapnic conditions towards an anti-inflammatory cytokine release.

Firstly, AM were examined after intra-tracheal instillation of polymorph nuclear neutrophils (PMN) and *Escherichia coli (E. coli)* post-trauma. AM were also examined in consideration of their capacity after previous ingestion of apoptotic PMN (aPMN). Secondly, efferocytosis, phagocytosis and mediator release of post-traumatic AM were analyzed *in vitro* under normoxic, hypoxic and hypercapnic conditions.
Male CD rats were subjected to blunt chest trauma by a one 'hit' single blast wave. For intra-tracheal instillation, PMN were harvested from untreated donor rats. After labeling the PMN with DiD, the apoptosis was induced with UV radiation. *E. coli* or aPMN were then instilled into the lungs 2, 24 or 48 hours (h) after trauma or sham treatment. Two hours after instillation, rats were sacrificed.
After trauma the percentage of AM ingesting aPMN remained unchanged. In contrast, the post-traumatic amount of AM ingesting *E. coli* was significantly increased 24 h after trauma and remained elevated *in vivo*. To verify, if these effects are attributable to the influences of decreased oxygen or elevated carbon dioxide levels post-trauma, AM were isolated 2, 24 or 48 h after induced trauma or sham treatment via bronchoalveolar lavage (BAL). The

harvested AM were cultured per se, with aPMN or *E. coli* and then incubated under normoxic (21% O_2, 5% CO_2), hypoxic (5% O_2) and hypercapnic (20% CO_2) conditions for 2 h. After incubation, AM were analyzed using flow cytometry. To examine the mediator response, culture supernatants were analyzed in immunology multiplex assay.

TxT did not alter the AM efferocytosis of aPMN *in vivo,* when compared to sham procedure. This result was reproduced *in vitro* during normoxia and hypoxia. Hypercapnic conditions partially increased aPMN efferocytosis.

Phagocytosis of *E. coli* in turn, was significantly increased by blunt chest trauma in AM *in vivo* and *in vitro*. Hypercapnia down regulated phagocytosis in AM significantly by CO_2 dependent induction of anti-apoptotic factor expression. Hypoxia did not alter phagocytosis in AM.

Blunt chest trauma significantly up regulated the mediator release in AM compared to respective sham groups. Maxima in mediator release were reached 24 h after trauma. Hypoxia and hypercapnia had various effects on AM mediator release. In our studies, incubation at low oxygen levels did not alter the efferocytic or phagocytic capabilities of AM, but influenced its mediator release significantly. AM co-incubated with and without aPMN have shown a significantly increased TNF-α release. AM stimulated with LPS in turn showed a lowered release in TNF-α. Other pro-inflammatory mediators, such as IL-1 β, IL-6 and MCP-1 were mostly suppressed by the influence of hypoxia during aPMN and *E. coli* ingestion. Hypercapnia altered the efferocytic and phagocytic capabilities of the lung's primary defense cells. Elevated CO_2 levels promoted the efferocytosis of aPMN, but suppressed the phagocytosis of *E. coli.* Hypercapnia markedly suppressed the release of TNF-α and MIP-1-α, as well as IL-6, GRO/KC and MCP-1 during *E. coli* ingestion and incubation without phagotropic nutrition. Levels of IL-10 were increased under the influence of elevated CO_2. In conclusion, hypercapnia revealed anti-inflammatory influences on AM after trauma *in vitro*. There is growing evidence that hypercapnia may itself minimize or reduce lung injury as a result of suppressing inflammatory events and may be used as a therapeutic strategy, but the impaired bacteremic clearance by AM might increase the risk for lung infections.

6. Reference list

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