Cover Letter

January 31, 2022

Amrita Ahluwalia, PhD
Editor-in-Chief
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Dear Dr. Amrita Ahluwalia,

We are pleased to submit an original article entitled "Immunopathology of Terminal Complement Activation and Complement C5 Blockade Creating a Prosurvival and Organ-protective Phenotype in Trauma" for consideration by the *British Journal of Pharmacology*.

Traumatic hemorrhage (TH) is the leading cause of potentially preventable deaths that occur early after injury with the majority of deaths happening in the prehospital phase of care. Current treatments still largely rely on organ/tissue supportive approaches and fluid resuscitation, and no effective pharmacological therapeutics are available for critical TH patients yet. Thus, development of pharmacological treatments to create a prosurvival and/or organ-protective phenotype is a serious unmet need in prehospital care of severe TH patients. In this manuscript, using a translational medicine approach (bedside to bench), we have i) identified complement C5 as therapeutic target in a cohort of military casualties with two major mechanisms of injury (69% blast injury and 26% gunshot wounds, 83% of these patients with traumatic brain injury), ii) selected a clinical-stage drug candidate (nomacopan, a bifunctional anti-inflammatory protein binding highly specifically to both C5 and leukotriene B4) with desirable properties (thermostability, easily transported/stored/reconstituted, amenable to manufacture in single use dual chamber autopen, and multiple routes of administration) that make it suitable for battlefield/prehospital use, and iii) first demonstrated that early blockade of C5 in a clinically relevant preclinical animal model of blast injury and hemorrhagic shock significantly improves survival (nomacopan *vs.* saline: 80% *vs.* 30%, $p<0.05$) and attenuated multiple-organ damage by reducing systemic and local inflammation, and improving hemodynamics and metabolism. Nomacopan administration represents a promising adjunct to damage control that that may significantly reduce the morbidity and mortality in severe TH patients while awaiting transport to critical care facilities.

Taken together, our manuscript falls within the *British Journal of Pharmacology*'s scope in the areas of "translational pharmacology research" / "drug discovery and validation" because it addressed complement C5 activation as the risk factor for severe traumatic hemorrhage that causes a tremendous loss in productive life and a poor life quality worldwide, and provided countermeasures to minimize risk from terminal complement activation. We believe that the translational approaches and findings presented in our paper will appeal to the international scientists who subscribed to *British Journal of Pharmacology*. Furthermore, our findings will allow your international audience to understand the translational research approach of trauma immunomodulation in identifying therapeutic targets, selecting clinical drug candidates, and designing target-based drug discovery/development.

As required in the submission guidelines of the *British Journal of Pharmacology*, I hereby warrant the following to be true:

The manuscript has been read and approved for submission to the *British Journal of Pharmacology* by all authors.

1. The text and images in the manuscript submitted are the original work of the authors.
2. This work has not been published elsewhere nor is it currently under consideration for publication elsewhere.

Thank you for your consideration of this manuscript.

Sincerely,

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Title: Immunopathology of Terminal Complement Activation and Complement C5 Blockade Creating a Prosurvival and Organ-protective Phenotype in Trauma

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Running Head: Complement C5 inhibition in traumatic hemorrhage

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ABSTRACT

Background and Purpose: Traumatic hemorrhage (TH) is the leading cause of potentially preventable deaths that occur during the prehospital phase of care. No effective pharmacological therapeutics are available for critical TH patients yet. Here, we identify terminal complement activation (TCA) as a therapeutic target in combat casualties and evaluate the efficacy of TCA inhibitor (nomacopan) on organ damage and survival in vivo.

Experimental Approach: Complement activation products and cytokines were analyzed in plasma from 54 combat casualties, and the correlations between activated complement pathway(s) and the clinical outcomes in trauma patients were assessed. Nomacopan was administrated to rats subjected to lethal TH (blast injury and hemorrhagic shock). Effects of nomacopan on TH were determined using survival rate, organ damage, physiologic parameters, and laboratory profiles.

Key Results: Early TCA was found to be associated with systemic inflammatory responses and clinical outcomes in this trauma cohort. Lethal TH in the untreated rats induced early TCA that correlated with severity of tissue damage and mortality. The addition of nomacopan to a damage control resuscitation (DCR) protocol significantly inhibited TCA, decreased local and systemic inflammatory responses, improved hemodynamics and metabolism, attenuated tissue and organ damage, and increased survival.

Conclusion and Implications: Our findings reveal that early TCA represents a rational therapeutic target for trauma patients; and nomacopan as a prosurvival and organ-protective drug, could emerge as a promising adjunct to DCR that may significantly reduce the morbidity and mortality in severe TH patients while awaiting transport to critical care facilities.

Keywords: traumatic hemorrhage, mortality, organ failure, prehospital care

Abbreviations: CH50, complement hemolytic 50% activity; DCR, damage control resuscitation; ETBV, estimated total blood volume; HMGB1, high mobility group box 1; ISS, injury severity score; LTB4, leukotriene B4; MAP, mean arterial pressure; MAPK, mitogen-activated protein kinase; MODS, multi-organ dysfunction syndrome; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; SIRS, systemic inflammatory response syndrome; TBI, traumatic brain injury; TCA, terminal complement activation; TH, traumatic hemorrhage; TLR, toll-like receptor.

What is already know

- Traumatic hemorrhage is the leading cause of potentially preventable deaths that occur during the prehospital phase of care.
- Terminal complement activation has potential to trigger inflammation and MODS.

What does this study add

- Treatment with nomacopan attenuates organ damage and increases survival.
- Nomacopan functions as a prosurvival and organ-protective drug.

What is the clinical significance

- Nomacopan could emerge as a promising adjunct to DCR that may significantly reduce the morbidity and mortality in severe TH patients while awaiting transport to critical care facilities.

INTRODUCTION

Traumatic hemorrhage (TH) is a major cause of potentially preventable death on the battlefield as well as in the civilian world. Blast injury was the predominant wounding mechanism during recent conflicts, accounting for 70-80% of military casualties in Iraq and Afghanistan. The pathophysiology of blast-induced injury is distinctive and appears more complex than that of most other forms of trauma (1).

TH involves tissue injury, ischemia, and subsequent reperfusion. Ischemia/reperfusion injury and damaged tissue activate a multifaceted network of plasma cascades (complement, coagulation, kinin, and fibrinolytic systems) that play a major role in the systemic inflammatory response syndrome (SIRS) and the compensatory anti-inflammatory response syndrome. SIRS and anti-inflammatory response syndrome ultimately lead to injury-related multi-organ dysfunction syndrome (MODS) (2). MODS represents a leading cause of late mortality following severe trauma (3, 4). The underlying immunologic disturbance is complex and early activation of the complement cascade plays a crucial role.

Complement activation appears to fuel a vicious cycle of inflammatory damage that exacerbates MODS pathology (5). Growing evidence from our studies (6-14) and those of others (15-20) illustrates that excessive activation of the complement system represents a key mechanism regulating the development of inflammation-mediated MODS after TH. Pronounced early complement activation was identified in civilians sustaining major trauma (19). Elevated concentrations of C3a, C5a, and sC5b-9 were correlated with injury severity and were associated with increased incidence of MODS and mortality (19, 20). Similarly, our recent findings in civilians with trauma or burn injury have revealed robustly elevated plasma levels of C3a; the terminal complement activation (TCA) products C5a/sC5b-9; protein Bb, a product of the

complement alternative pathway; and protein C4d, a derivative of the complement classical/lectin pathways (6). Genetic and pharmacological manipulation of complement levels and complement activation in murine models of ischemia/reperfusion injury, traumatic brain injury (TBI), and TH demonstrate beneficial effects on survival, neuroprotection, inflammation, and tissue damage (17, 21-24). Our previous studies have demonstrated the beneficial effects of pharmacological inhibition of complement activity on increasing survival, improving hemodynamics, reducing fluid requirements, attenuating organ damage, and modulating systemic and local inflammatory responses in rats and pigs in short-term studies (< 6 hours) after TH (9, 11-13).

The latter studies used either a C1 inhibitor (which inhibits the classical and lectin pathways of complement C3 and C5 activation and the contact system that generates bradykinin), or decay accelerating factor (which inhibits the classical, lectin and alternative pathways of complement C3 and C5 activation). There is debate around the best point at which to inhibit complement activation to improve outcomes in trauma. For example, should one inhibit all 3 complement pathways or only one, and should one interrupt formation of all the main effectors (C3a, C5a and C5b-9), only C5a and C5b-9, or only C5a? Since secondary infection is a major risk after blast injury and other forms of trauma, some investigators believe that inhibition of complement activation at the C3 level may be less desirable than TCA-specific blockade, because C3 opsonization has significant antimicrobial function, and C3 and its activation products may have roles in tissue recovery (25, 26). The relative importance of each of the 3 complement activation pathways in trauma is not yet clear and each may have a different degree of importance in different types of injury.

In the current study, we show that the terminal complement is the predominant activated complement pathway early after trauma in a cohort of combat casualties, most of whom had blast-

induced TBI. Based on these clinical findings, we then hypothesized that blocking C5a and C5b-9, the TCA products, may reduce organ tissue damage and increase survival. To evaluate this hypothesis, we tested the effect of C5 inhibition using the clinical-stage complement C5 inhibitor nomacopan in a rat model of TH that recapitulates the immunological responses seen in injured patients.

METHODS AND MATERIALS

Overview of study design

This study was designed 1) to determine the role of TCA-induced morbidity and mortality in military trauma patients with two major mechanisms of injury (69% blast injury and 26% gunshot wounds); and 2) to test the efficacy of a dual specific inhibitor of C5 and leukotriene B4 (LTB4), nomacopan (Akari Therapeutics plc, London, UK), against blast injury/hemorrhage-induced organ damage, and to evaluate whether C5 inhibition provides a survival benefit in a military relevant preclinical rat model (Fig. 1. The primary endpoints were survival and organ damage. Prior to performance of the main study, a pilot study was conducted (Fig. 1). Complement hemolytic 50% activity (CH50) from that study is shown in Fig. S3E. According to these data (drug-treated *vs.* vehicle-treated animals: 89% *vs*. 27% CH50 at 1 hour after injury), a sample size of 10 rats per treatment group was required to meet the expectation of power of 80% and 95% confidence intervals. After recovery (5-7 days) from surgical cannulation and prior to trauma, the animals were randomly assigned to one of two experimental groups (Fig. 1). No data were excluded. Drug and vehicle administration was non-blinded; no bias was applied during husbandry or during tissue harvesting. A randomized blinded code for histological sections was used.

Clinical study

This study was conducted under a protocol reviewed and approved by the US Army Medical Research and Development Command Institutional Review Board and in accordance with the approved protocol (Protocol #MNC1-07021). The study in trauma patients was designed to identify the clinical significance of early complement activation in casualties admitted to a US Army Combat Support Hospital (Role 3) in Baghdad, Iraq over a one-year period (Fig. 1). Foreign nationals, prisoners, enemy combatants, children, and any patient undergoing therapeutic anticoagulation were excluded. Citrated plasma was collected from trauma patients after admission to the emergency department (n=54), and if available, 8 hours (n=23) and 24 hours (n=9) later (Table 1). At these later time points, samples were collected after patients had received appropriate clinical care, including surgery and resuscitation. On admission (45-60 min after injury), the clinical and demographic characteristics of the patients were recorded, including base excess, MAP, blood product transfusion units, and SIRS score during the first 24 hours. Most casualties (n=45) suffered traumatic brain injury from explosions.

Blood collected at the hospital, was processed according to standard clinical practice (27) and the resultant plasma was frozen and transported to the US Army Institute of Surgical Research (USAISR) as described (27) and stored at −80°C until analysis. Ten healthy volunteers were enrolled at the authors' laboratory as reference controls. Volunteers were 18 years or older with no significant medical conditions. Blood samples were drawn once for analysis of selected complement components, coagulation parameters, and the levels of cytokines.

Animal study

Research was conducted in compliance with Animal Welfare Act, the implementing Animal Welfare regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by the AAALAC.

Surgical procedures and injury model in rats

Specific-pathogen-free adult male Sprague-Dawley rats (10-12 weeks old), weighing 350-475 g, were purchased from Charles River Laboratories (Wilmington, MA). Under anesthesia, the carotid artery and jugular vein were cannulated in all rats. The cannulated animals underwent with (main study) or without (pilot study) a recovery period (5-7 day). The blast injury was conducted as described previously (8, 10, 28). Briefly, rats were anesthetized with ketamine/xylazine (60/5 mg/kg body weight) via intra-peritoneal injection, and then placed on a rack holder, which was wheeled into the end of the expansion chamber of a compressed-air-driven shock tube (Applied Research Associates, Inc., Albuquerque, NM) (8). During blast, the animal was immobilized to prevent movement upon impact and subsequent tertiary blast injury. Animals in prone position with head turned to the blast wave were exposed to single mild-moderate blast injury (mean BOP=115.34±0.74 kPa, t+=3.28±0.01ms, I=141.33±0.46kPa-ms, Table S3). 15 minutes after blast exposure, animals were subjected to volume-controlled hemorrhage over 15 minutes. The estimated total blood volume (ETBV) was calculated using the following formula: ETBV (ml) = weight in kg × 65 ml/kg. After hemorrhage, the animals were maintained 30 minutes in shock phase, then received two-times the shed blood volume of Plasma-Lyte A. The animals were monitored under anesthesia 3 hours after hemorrhagic shock (H), then returned to cage and observed for up to 24 hours.

Two doses of nomacopan were administered. The first dose (7.5 mg/kg, intravenously) was given either immediately before blast exposure (NOM_0´), 15 minutes after blast but prior to hemorrhage (NOM_15´), or 60 minutes post-blast (at the end of shock but before fluid resuscitation, NOM_60´), The second dose (also 7.5 mg/kg) was given subcutaneously 10 hours after hemorrhagic shock. The dose of nomacopan via i.v. route of administration has previously demonstrated complete inhibition of serum hemolytic activity in rats with a half-life of 8-12 hours (28). Injured rats not treated with nomacopan received the same volume of saline by the same routes of administration at equivalent times.

In the pilot study, cannulated animals (non-recovery) were allocated to 3 groups (Figs. 8 & S3A and Table S3): (1) B + H (n=6): a single blast injury and 40% hemorrhage with receiving equal volume of normal saline, (2) NOM_0´ (n=3): B + H animals treated with nomacopan, (3) Sham (n=5): the animals underwent the same surgical cannulation, anesthesia, and analgesia but without B + H.

For the main study, the recovered animals post-cannulation were randomly assigned to 4 groups (Figs. 1 & 5A and Table 3S): (1) B + H (n=10): a single blast injury and 52% hemorrhage (7 rats lost 50% of ETBV, and 3 animals lost 57% of ETBV) with receiving equal volume of normal saline, (2) nomacopan_15´ (n=10, 7 rats lost 50% of ETBV, and 3 animals lost 57% of ETBV), (3) NOM_60´ (n=10, 7 rats lost 50% of ETBV, and 3 animals lost 57% of ETBV), and (4) Sham (n=6): the animals underwent all procedures except B + H and subsequent received equal volume of normal saline. During the observation period, the mean arterial pressure was recorded by BIOPAC data acquisition system (BIOPAC Systems, Inc., Goleta, CA). Blood samples were collected before blast, at the end of hemorrhagic shock, then at 1, 3, 10 and 24 hours after shock.

Blood chemistry was analyzed by i-STAT (Abbott Laboratories), and PaO2/Fio2 ratio was based on collected i-STAT data.

Assays

Analysis of complement factors in human/rat plasma

Quantitative levels of complement factors in human plasma, including C3a, C5a, sC5b-9, Bb and C4d were measured by using commercial ELISA kits according to the manufacturer's instructions (Quidel, San Diego, CA). Rat plasma levels of complement C3 and C1q were assessed using ELISA kits (abcam, Cambridge, MA).

Analysis of human cytokines

Human cytokines in the plasma were analyzed by Bio-Plex® Pro Human Cytokine 27-plex Assay (BIO-RAD, Hercules, CA) according to the manufacturer's instructions.

Analysis of cytokines in the lung tissue from rats

Levels of several cytokines (IL-1β, IL-6, TNF-α, and KC/GRO) in homogenates of lung tissue were analyzed with an electrochemical ELISA using the MesoScale Discovery platform (Rockville, MD) (7).

Protein assay

Levels of total protein in plasma were measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Hemolytic complement activity assay

The complement hemolytic 50% activity (CH50) assay was performed to determine the function of the complement classical pathway as described previously (10). Briefly, antibody-sensitized chicken red blood cells (Colorado Serum Company, Denver, CO, catalog #31151) were

incubated for 30 min at 37°C with serial dilutions of rat serum samples in gelatin-veronal buffer (GVB^{++} buffer, Complement Technology, Tyler, TX, catalog #B100). After centrifugation, the supernatant was transferred to a new plate, and the absorbance of supernatant was determined at 405 nm by SpectraMax microplate reader (Molecular Devices). The fold serum dilution inducing 50% of complement hemolytic activity was determined and presented as the CH50 value.

Myeloperoxidase levels in plasma

MPO levels in human/rat plasma were determined by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using kits obtained from Hycult Biotech (Plymouth Meeting, PA), according to the manufacturer's instructions.

Histopathological evaluation

Immunohistochemical (IHC) staining in rat tissues

Lung tissue was processed for IHC staining as described previously (7). Briefly, after 4% paraformaldehyde fixation for 24 hours, the tissues were transferred to 20% sucrose (w/v) in PBS overnight at 4 °C, followed by freezing in the Tissue-Tek OCT mounting medium (Sakura, Japan). Frozen-tissue sections were then cut at 5-μm thickness with a cryostat and mounted onto glass slides. The slides were fixed in cold acetone or 4% paraformaldehyde for 20 min followed by permeabilization with 0.2% Triton X-100 in PBS for a further 10 min. Next, sections were blocked by 10% normal goat serum and incubated with primary antibodies, including anti-C5b-9 (Hycult Biotech, Plymouth Meeting, PA), anti-C3/C3a, anti-MPO, and anti-ICAM-1 (Abcam, Cambridge, MA) overnight at 4°C. Following extensive washing, sections were incubated with secondary antibodies labeled with Alexa Fluor 488 (Green) or 594 (Red) (Abcam, Cambridge, MA) for 1 hour at room temperature (RT). Subsequently, after washing, sections were mounted with ProLong

Gold Antifade solution containing 4',6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA) for staining the nuclear DNA and then visualized under a fluorescence microscope (Nikon eclipse Ti). Experiments with negative controls were conducted by substituting the primary antibodies with corresponding immunoglobulin isotypes.

Quantification of IHC staining

The numbers of positively stained cells and the total numbers of cells in a given section were determined on the basis of particle size by using ImageJ software (ImageJ 1.50b). Three to four animals in each group were analyzed.

Tissue pathological evaluation and semi-quantitative scoring

Histological images for each individual rat tissue were recorded with 10x objective under a slide scanner (Axio Scan. Z1 v1.0, Zeiss, Germany), and representative images of each group were presented (magnification, 400x). Semi-quantitative scoring of lung, brain and liver tissues was performed by a pathologist blinded to the treatment information, and the criteria for the evaluation of histological injury scores are as follows.

For the lung injury score, four parameters (alveolar fibrin edema, alveolar hemorrhage, septal thickening, and intra-alveolar inflammatory cells) were scored on each hematoxylin and eosin (H&E) stained slide based on: 1) severity (0: absent; 1, 2, 3 and 4 for increasingly severe changes); and 2) the extent of injury (0: absent; 1: <25%; 2: 25–50%; 3: 50-75%; 4 >75%). Total injury score for each slide was calculated as the sum of the severity plus the extent of injury (12).

For the scoring brain injury score, we undertook the approach previously described (10). Two parts of the brain tissue were scored, including the frontal cortex and hippocampus. Damage was assessed using 5 distinct morphological parameters: neuronal morphological changes

(shrinkage of the cell body, pyknosis of the nucleus, disappearance of the nucleolus, and loss of Nissl substance, with intense eosinophilia of the cytoplasm), neuronal loss, cytotoxic edema, vasogenic edema, and inflammatory cell infiltration in the brain cortex. The changes were scored according to their extent (score 0, 1, 2, 3, and 4 for an extent of 0%, < 25%, 25–50%, 50–75%, and 75–100%, respectively) and the severity of the injury (score 0 = normal histology, score 1 = slight, 2 = mild, 3 = moderate, and 4 = severe alterations).

For the hepatic injury score, four parameters, including vascular congestion, hepatocyte death, degeneration, and inflammation were considered (12), and these parameters were assayed for severity (score 0 for no change, score 1, 2, 3 and 4 for more severe changes) and for the extent of injury (0: absent; 1: <25%; 2: 25-50%; 3: 50-75%; 4: >75%). The injury score represents the sum of the extent and the severity of injury.

For the jejunum, each slide was scored according to the following scale: 0, normal villi; 1, villi with tip distortion; 2, villi lacking goblet cells and containing Guggenheim's spaces; 3, villi with patch disruption of the epithelial cells: 4, villi with exposed but intact lamina propria and epithelial cell sloughing; 5, villi in which the lamina propria was exuding; and 6, hemorrhaged or denuded villi.

Statistical analysis

Demographic data are presented as interquartile ranges (IQR) or percentages as appropriate. Other data are presented as mean ± SEM. Intergroup comparisons for complement factors and inflammatory mediators in patients or animals were assessed using Mann-Whitney U test, or unpaired *t*-test with Welch's correction. Correlation analyses were analyzed using Spearman's correlation test. For animal survival analysis, Kaplan-Meier plot and log-rank test

were performed. Two-way ANOVA was performed to compare the animal groups on particular variables**.** The receiver operating characteristic curve (ROC) and area under the ROC surve (AUC) were performed using univariate logistic regression. The optimal cutoff values were obtained by ROC curves analysis with Youden index. P <0.05 was considered significant. All data were included and none were treated as outliers. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).

RESULTS

Patient demographics and clinical data

Patient demographics and clinical data are shown in Table 1. Most patients were male (98%) with median (IQR) age of 25 (22-30) years, and injury severity score of 16 (9-24). The predominant cause of injury was blast (69%). Forty-five of the 54 patients (83%) had TBI, of whom most sustained mild TBI (80%). During treatment in the hospital, mechanical ventilation was used in 7 patients (13%), and 5 patients (9%) died. All patients received operative care and intravenous resuscitation fluids. Ten healthy controls, 6 male and 4 female with a median (IRQ) age of 36 (28-41) years were sampled once, and served as controls. No clinical data were collected for healthy controls.

Early complement activation in casualties

To avoid misinterpretation of the measured complement components (C3a, C4d, C5a, sC5b-9, and Bb) (Fig. 2) by the dilution effects of early fluid infusion, data were normalized to total plasma protein levels (19). Levels of activated complement factors, including C5a, sC5b-9, and C4d, were significantly higher in injured patients on admission compared with healthy

controls. These factors remained elevated at 8 and 24 hours after admission. The mean values for C5a, sC5b-9, and C4d at 24 hours increased by 3.2-, 3.6-, and 7.0-fold, respectively, compared to controls (Fig. 2A-D). There was a strong positive correlation between complement C5a and sC5b-9 with Bb levels (Fig. 2E, F) on admission. However, C3a was not significantly changed during the observation period (Fig. S1A, B). Patients with blast injuries showed a peak in activated complement factors at 8 hours after admission among 24 hours observation period (Fig. S1C-F). Patients with gunshot-wound injuries generally had elevated levels of C5a, sC5b-9, Bb, and C4d throughout the 24 hours after admission (Fig. S1C-F), although Bb levels clearly peaked at 8 hours (Fig. S1E). This early Bb elevation indicates that the alternative complement pathway (AP), unlike the classical or mannose-binding lectin (MBL) complement pathways, contributed to early complement activation in these patients.

Early complement activation and early systemic inflammatory response in casualties

We measured the plasma levels of inflammatory mediators and determined the relationship between plasma concentrations of complement activation products (C3a, C5a, sC5b-9, Bb, C4d) and inflammatory mediators at admission after trauma. We found that the levels of pro-inflammatory mediators (TNF-α, INF-γ, IL-1β, IL-6, IL-8, MCP-1, MPO, GM-CSF) (Fig. S2A-H), anti-inflammatory cytokines, including IL-4, IL-10 and G-CSF (Fig. S2I, J, L), and regulatory cytokines such as IL-2, IL-7, IL-12, and MIP-1β (Fig. S2M-P) were significantly elevated on admission and at 8 hours when compared with the healthy controls. Most of these mediators declined and returned to their baseline levels within 24 hours. The ratios of the pro-inflammatory factors (IL-6, MCP-1, or GM-CSF) to IL-10, a classical anti-inflammatory cytokine, were significantly higher than in the controls (Fig. S2S).

At admission, blood levels of IL-6 and MPO strongly correlated with C5a, sC5b-9, and Bb levels (Fig. 3). MCP-1 and MIP-1β clearly correlated with the blood levels of C3a, C5a, sC5b-9, and fragment Bb but not with C4d (Fig. 3 and Table. S1). Complement protein C4d, a degradation product of the classical/lectin pathways negatively correlated with the pro-inflammatory cytokines IL-1β, IL-17, and TNF-α (Fig. 3J, K, L).

Clinical outcomes and early complement activation in casualties

There was a positive correlation between admission plasma levels of C5a/sC5b-9 (Fig. 4A-H) or Bb (Fig. 4I-L) and ISS, international normalized ratio (INR), severity of TBI, and crystalloid resuscitation requirements. However, we found an inverse correlation between plasma concentrations of C4d at admission and blood transfusion requirements (Table S2). No significant correlation was found between C4d plasma levels at admission and ISS, TBI and crystalloid resuscitation requirements (Table S2). We next tested the accuracy of admission plasma concentrations of C5a and Bb for distinguishing TBI patients from non-TBI patients, or distinguishing ventilated patients from non-ventilated patients using ROC analysis (Fig. 4M-N, Q). Admission plasma levels of C5a ≥0.185 μg/mg plasma protein (AUC=0.73, specificity=68%, sensitivity=71%,) (Fig. 4M&Q) and Bb ≥0.015 μg/mg plasma protein (AUC=0.69, specificity=65%, sensitivity=71%) (Fig. 4N&Q) were associated with the presence of TBI. Admission plasma C5a ≥0.195 μg/mg plasma protein was also as a fair predictor of mechanical ventilation requirements (AUC=0.75, specificity=71%, sensitivity=86%, Fig. 4O&Q). However, patients who survived had higher levels of C4d than patients who did not survive, and admission plasma C4d ≥0.025 μg/mg plasma protein was as a fair predictor of mortality (AUC=0.83, specificity=78%, sensitivity=80%) (Fig. 4P&Q). These data suggest that inhibition of complement

terminal pathway or alternative pathway, but not classical/lectin pathways, may be the optimal therapeutic approach to improve clinical outcomes after TH.

Having shown that complement is activated in combat casualties and that the alternative pathway activation and terminal activation pathway are associated with inflammatory mediators that may drive organ damage, we investigated the effect of inhibiting terminal complement activation in a rat model of TH.

Pilot study results

We first carried out a pilot study with the following goals: 1) test the effect of surgical cannulation on complement activation; 2) evaluate blast overpressure intensity; 3) measure TH-induced complement activation; 4) determine an effective therapeutic regimen and 5) assess efficacy of the therapeutic regimen on organ damage and survival. We showed that surgical cannulation alone triggered complement cascade activation, reducing functional classical pathway complement activity by >50% (Fig. S3B and Table S3); and that TH further rapidly activated the complement system (Fig. S3B-D). Complement activation peaked about 1-2 hours after blast injury (or after the shock phase in hemorrhaged rats) and then gradually recovered. In injured rats, low serum levels of C1q (a subunit of the C1 enzyme complex) (Fig. S3C) and of C3 (Fig. S3D) also indicated that early consumption of complement factors was driving the decrease in CH50. Pretreatment prior to blast with nomacopan (Fig. S3E), increased the base excess (Fig. S3F), mean arterial pressure (MAP) (Fig. S3G), and survival (Fig. S3H). It also improved organ-damage scores on histology including those of lung, brain and liver (Fig. S3I). We then decided to address the problem of surgery-induced complement activation by introducing a recovery period between surgery and the injury/treatment study.

Nomacopan improved hemodynamics, respiration, and blood chemistries

We used the data generated from the pilot study to formulate the design of the main study in which we: 1) allowed the animals to recover full complement activity by adding a post-surgical recovery period of 5-7 days before study; 2) increased hemorrhage volume (from 40% to 52%); and 3) adjusted the treatment window to administer the study drug after blast but before hemorrhage to be consistent with early battlefield use (Fig. 5A).

In this main study (Fig. 5A and Table S3), the MAP in the injured animals was 97.27 (± 2.93) mmHg at baseline (Fig. 5B). MAP decreased to 30.35 (± 2.55) mmHg at the end of hemorrhage. In the injured/treated group, the MAP baseline was 93.96 (± 1.19) mmHg and decreased to 26.57 (± 1.71) mmHg at the end of hemorrhage. However, injured/treated animals showed significantly increased MAP in the first 30 minutes of the resuscitation phase when compared with injured/untreated rats. CH50 in the blood of injured rats started to decrease 15 minutes after blast injury due to complement consumption, reached its lowest level one hour after shock, and then returned to baseline level at 10 hours after the end of hemorrhagic shock (Fig. 5C). CH50 was even higher than 100% in injured/untreated rats at the end of the observation period. In the injured/treated rats, CH50 was significantly lower than in the injured/untreated group and was almost completely inhibited at the end of hemorrhagic shock, approximately 45 minutes after the drug administration. In the injured/treated group, CH50 remained at a very low level until the end of the experiment.

Nomacopan treatment also improved blood chemistries in injured/treated rats (Table S4). The pH value in injured/untreated animals changed from baseline of 7.44 (± 0.02) to 7.07 (±0.37) at 4 hours after blast, while the pH in injured/treated animals had almost returned to the baseline

level at that point (7.41 ± 0.16 to 7.44 ± 0.03). The PaO_2/FiO_2 ratio in the injured/treated rats was clearly higher at the end of shock period and throughout first 4 hours when compared with injured/untreated rats (Fig. 5D). There was a significant difference in the lactate level (Fig. 5E), but not the base excess (Fig. 5F). Injured/treated animals had significantly lower potassium levels at the end of the observation period in comparison to injured/untreated rats (Fig. 5G).

Nomacopan reduced systemic and local inflammatory responses

Next, we measured inflammatory cytokines in the plasma. The high mobility group box 1 (HMGB1) protein level increased after TH, reaching a peak 4 hours after injury. Nomacopan treatment significantly reduced HMGB1 levels at 4 hours (Fig. 6A) after blast. MPO gradually increased after TH, and nomacopan treatment significantly slowed this increase (Fig. 6B).

In lung homogenates, we found significantly increased levels of IL-1β, IL-6, TNF-α, and KC/GRO in rats subjected to TH compared to the sham group (Fig. 6C-F). These tissue cytokine levels trended lower with nomacopan treatment, but the reduction was not significant.

Immunohistochemical (IHC) images demonstrated that the MPO level was increased in the lung tissue after TH. However, after treatment with nomacopan, MPO expression was significantly reduced (Fig. 6G, I). ICAM-1, a marker of endothelial injury, was expressed in the lung tissue of injured/untreated rats. Treatment with nomacopan significantly reduced ICAM-1 expression in the lung tissue (Fig. 6G, I). It also reduced C5b-9 and complement C3 deposition in lung tissue (Fig. 6H and 6I). These findings indicate that treatment with nomacopan reduced both systemic and local inflammatory responses.

Effect of nomacopan on MODS and survival

Histological evaluation revealed that nomacopan treatment reduced MODS severity following TH. After TH, extensive cellular inflammatory infiltrates were present in the pulmonary tissue; the majority of these were neutrophils and macrophages. Lung edema was severe after TH but nomacopan treatment alleviated this phenomenon (Fig. 7A). Typical neuronal apoptosis, neuronal loss, and neuronal degeneration were seen in the cerebral cortex and hippocampus, which were significantly reduced by nomacopan treatment (Fig. 7A). TH induced hepatic tissue damage characterized by hepatic cell apoptosis or necrosis and severe thrombosis. Nomacopan treatment alleviated this damage (Fig. 7A). TH also induced intestinal mucosal injury. Photomicrographs of jejunal tissue show that TH denuded the villi to the level of the lamina propria with inflammatory cell infiltration while nomacopan clearly helped to preserve villus structure and improved the recovery of jejunal mucosa (Fig. 7A). Semi-quantitative scoring of injury severity on histology further validated these observations (Fig. 7B).

We tested the efficacy of 2 treatment windows of nomacopan in the rats. Early treatment with nomacopan 15 minutes after blast but immediately before hemorrhage significantly improved survival by 50% (Fig. 8), whereas later treatment with nomacopan during the resuscitation phase immediately after TH did not improve survival compared to the vehicle control (Fig. S4B). Nomacopan's beneficial effect on survival depended on its administration time and resulting early complement inhibition (Fig. S4A and S4B).

DISCUSSION

Hemorrhage and/or TBI are the leading cause of deaths in the prehospital phase care (29); the most deaths occur within a few hours after trauma despite recent advances in trauma care (29-31); approximately 90% of battlefield casualties die in the prehospital environment; 85% of these

prehospital deaths are due to bleeding, and of those about 25% are deemed potentially survivable (29, 32). Furthermore, although patients with TH may survive the initial injury by means of hemorrhage control and resuscitation, they may still succumb to a complex series of inflammatory events ending in multi-organ failure that largely contributes to late mortality (15, 33-36). Thus, the current primary goal of early care is to keep patients alive long enough and maintain organ functional while awaiting transport to higher echelons of care for definitive treatment.

In this study, using a translational medicine approach, we identified a complement therapeutic target (C5) in a cohort of military casualties, selected a clinical drug candidate (nomacopan) with desirable properties (a bifunctional anti-inflammatory protein binding highly specifically to both C5 and LTB4, thermostability, easy-to-transport/store/reconstitution, amenable-to-manufacture in single use dual chamber autopen, and multiple routes of administration) that make it suitable for battlefield/prehospital use, and evaluated its efficacy in a clinically relevant animal model of blast injury and hemorrhagic shock.

We first investigated the relationship between complement activation, cytokine production, and clinical variables in casualties. The study revealed that multiple complement factors (C5a, sC5b-9, Bb and C4d) were significantly increased on admission to the emergency department of a Combat Support Hospital, suggesting that injury induces rapid complement activation in patients. This is consistent with previous findings that complement was activated in animal models of trauma (10, 14) and in both civilian burn (6) and non-burn trauma patients (19, 20). We observed that the activation of the terminal complement and the alternative pathways was associated with ISS, TBI, coagulopathy, the requirements of crystalloid fluid resuscitation, and the need of mechanical ventilation. Ganter et al. showed that activation of these pathways in civilian trauma patients was directly related to clinical outcomes, including acute lung injury, renal injury, TBI,

and mortality (19, 20, 37). These observations are consistent with our previous publications demonstrating that complement blockade by decay-accelerating factor or C1 inhibitor mitigated organ damage and increased survival of rats and pigs subjected to blast injury and hemorrhagic shock (9-13, 38). Demonstration that mice deficient in factor B gene had significantly decreased neuronal cell death after experimental brain injury (21) also indicates the involvement of the alternative pathway in the body's response to trauma. Further highlighting the importance of complement, De Blasio et al. (36) reported that complement system activation in human brain contusions depends on the lectin pathway and likely on amplification via the alternative pathway. Increased levels of MASP-2, a driving enzyme of lectin pathway activation, were associated with TBI severity. Moreover, complement activation is elicited not only by the traumatic event itself, but also by secondary insults after trauma (37) and therapeutic interventions such as extracorporeal life support, mechanical ventilation, damage-control surgery, instrumentation, volume resuscitation, and blood transfusion (26, 39, 40). Immunomodulatory approaches aim to rebalance trauma-induced complement activation and thus may change trauma management procedures to improve outcome.

Huber-Lang et al reported that thrombin functions as a C5 convertase and can independently activate complement terminal pathway in C3-null mice (41). Unlike TCA in this study, circulating C3a was not significantly altered in the military casualties during the observation period, indicating that extrinsic/common pathway-induced TCA is the major pathway of complement activation after trauma (20, 42).

Complement is a critical component of innate immunity and a major initiator of the inflammatory reaction. In this study, we found that increased complement factors C3a, C5a, sC5b-9, and Bb correlated with the levels of multiple cytokines, suggesting an interplay between the

complement system and other immune systems. The complement system also functions as a bridge between innate and adaptive immunities (43, 44). Specifically, complement's role in the regulation of the inflammatory response may be through activation of innate immune cells (neutrophils, monocytes, mast cells, macrophages and dendritic cells) and adaptive immune cells (Th1, Th2, Th17, natural Treg, and B cells) via a synergistic interaction between C3a/C5a-C3aR/C5aR-MAPK-NF-κ-NLRP3 inflammasome and HMGB1-TLR4-MAPK-NF-κ-NLRP3 inflammasome in response to trauma (35, 45). Indeed, our data showed that increased plasma levels of C5a, sC5b-9 and Bb were positively correlated with blood levels of MPO, IL-6, MCP-1 and MIP-1β, and with INR. The positive correlation between activated complement products (C5a, sC5b-9, Bb) and INR in military casualties indicates that coagulation pathways may contribute to early complement activation. Furthermore, increased concentrations of MPO and IL-1β in plasma, and prolonged PT/increased INR, were associated with clinical variables like blood transfusion requirements, SIRS and hypotension after trauma. This suggests that an interplay between these signaling pathways may synergistically increase morbidity and mortality after trauma.

Typically, C4d is increased after trauma in animals and patients as reported earlier (20). It is interesting to note that the C4d level at admission was significantly higher in surviving trauma patients when compared with those who died. The C4d level inversely correlated with many clinical outcomes, suggesting that classical and/or lectin complement pathway activation may play a role in protecting against damage to the host. Indeed, genetic manipulation of complement levels and activation in murine models demonstrated that the classical and/or lectin complement pathways play a crucial role in clearance of damaged cells and in host defense against infection (46, 47).

Nomacopan is a complement C5 inhibitor in Phase 3 clinical development that is equally potent in man and other mammals including rodents. Other C5 inhibitors such as the monoclonal antibody eculizumab are available but are not equally active in rodents. Furthermore, nomacopan not only inhibits TCA but also specifically sequesters LTB4 (48, 49) thereby blocking multiple inflammatory cascades as well as suppressing thrombogenicity (49) in mice, rats, pigs and humans (34, 50). LTB4 is an inflammatory mediator that induces the adhesion and activation of leukocytes on endothelium, allowing them to bind to and cross into tissue. Interestingly, compelling evidence suggests that C5a/C5aR1-β2 integrin-LTB4/BLT1 and/or sublytic doses of C5b-9-LTB4 axis is as a major effector mechanism for leukocyte tissue swarming, and thus participates in the pathogenesis of inflammatory diseases (51). Early increase in local and systemic LTB4 levels was found and high LTB4 concentration was associated with pulmonary complication development in multiple traumatized patients (52). Early administration of nomacopan 15 minutes after TBI in mice clearly improved neurologic recovery (53). Therefore, nomacopan may constitute the most effective therapeutic principle for preclinical and clinical development for the treatment of trauma.

Several studies reported that the treatment with nomacopan improved morbidity and mortality in bacterial infectious disease and sepsis models particularly when used together with an anti-CD14 inhibitor (25, 54-56), but no information has been published on the efficacy of nomacopan in TH. We showed that early nomacopan treatment significantly increased survival in rats after TH. Nomacopan promptly abolished TCA within 45 minutes of drug administration, as measured by CH50. Nomacopan also improved hemodynamics, blood chemistry, and significantly reduced systemic and local inflammatory responses. In addition, nomacopan prevented MODS as substantially less tissue damage was observed in the lungs, brain, small intestine, and liver tissues.

The survival of rats subjected to TH was significantly better in those that received nomacopan compared to rats receiving the saline control.

Complement activation is the early innate immune response activated soon after traumatic injury (18). We previously showed that the complement activation appears as early as 3 hours after blast injury in rats (10, 14) and by 3 hours after hemorrhagic shock in swine (13). Here, we found that the complement was quickly activated even after surgical cannulation, with TCA as measured by CH50 reduced more than 50% immediately after surgery in the pilot study. To avoid the surgical cannulation interfering with our experimental goals, we allowed the animals to recover full complement activity over 5-7 days before exposing the animals to trauma and hemorrhagic shock.

In injured animals, we observed that the TCA as measured by CH50 was reduced by about 65% one hour after hemorrhagic shock. This quick decrease of CH50 indicated a rapid complement activation after injury. In addition, complement deposition, including C5b-9 and C3, were soon detected in the lung tissue. These findings suggest that both systemic and local complement activation occurred in the injured animals. Our rodent model of traumatic hemorrhagic shock closely mimics the clinical setting, as a typical early complementopathy is observed after trauma (19).

Traumatic injury, including blast and hemorrhage, triggers a complex cascade of post-traumatic events that are related to inflammatory and immune responses. Excessive or maladaptive activation of inflammatory pathway is considered to contribute to MODS, and eventually death (57, 58). We found increased TCA measured by CH50 (up to 150%) in injured but untreated rats at 25 hours, the end of the observation period. We explain this phenomenon by referring to the systemic acute-phase immune response that includes pro-inflammatory cytokines and increased C3 (59). Thus, this study correlates with previous literature, who also observed the elevation of

multiple inflammatory factors and cytokines, such as MPO, NF-κB, TNF-α, IL-1β, IL-6, IL-12, IL-13 and IL-18, both systemically and locally after blast and hemorrhagic shock in rats and swine (7, 8, 10, 14, 60-62). Synchronous activation of pro-inflammatory and anti-inflammatory cytokines were also observed in battlefield trauma patients as presented in this study. The inflammatory response seen in battlefield trauma patients correlated with clinical variables. Modulation of trauma-induced inflammation may therefore be important in improving clinical outcomes. In this study, we found that nomacopan significantly attenuated both systemic and local levels of HMGB and MPO and therefore, systemic and local inflammatory responses.

Nomacopan may alter the inflammatory cytokine milieu in several ways. First, nomacopan inhibits TCA (C5a and C5b-9) and consequently prevents infiltration of inflammatory cells via C5a, a critical neutrophil and monocyte chemotactic factor (63, 64). Accordingly, we found that inflammatory cell infiltration in the lung, brain and liver was reduced after nomacopan treatment. Second, nomacopan may inhibit DAMP-induced inflammatory responses. After trauma, tissue debris, ATP, mtDNA, potassium, heme, reactive oxygen species (ROS), F-actin, HMGB1 and other DAMPs are released. These can induce a rapid inflammatory response via multiple signaling pathways, including TLR-MyD88-MAPK-NF-κB-inflammasome and ATP-P2X7-inflammasome pathways (45, 65, 66), with complement functioning as an initial and amplifying mediator (7, 45, 67, 68). Indeed, systemic and local levels of inflammatory mediators (HMGB1, MPO, IL-1β, IL-6, TNF-α, KC, ICAM-1, C3, sC5b-9) were significantly reduced in our rodent trauma model after nomacopan treatment. Unexpectedly, decreased lung C3 deposition in nomacopan-treated animals was noted, indicating that C5b-9-induced tissue damage and/or C5a-C5aR-mediated cellular infiltration/activation may have triggered complement activation, neo-synthesis and subsequent C3 deposition/expression (34).

We observed that nomacopan treatment clearly improved hemodynamic and blood chemistry variables. During the resuscitation period, nomacopan significantly increased the mean arterial pressure (MAP) in injured rats when compared to placebo. This finding is consistent with the previous report that administration of an anti-C5 antibody reduced fluid requirements and improved responsiveness to fluid resuscitation in a rat model of hemorrhagic shock. It is reported that complement activation products directly or indirectly alter vascular tone after trauma and hemorrhagic shock (17). Thus, C5a induces a reversible decrease of the MAP and an increase of central venous pressure (CVP) when used *in vivo* in rabbits (69). Nomacopan may reverse the C5b-9-induced damage to the endothelial barrier, and reduce vascular leakage caused by blast and hemorrhage (70). Moreover, the significantly lower plasma level of potassium in nomacopan-treated rats implicates C5b-9-mediated K^+ efflux (68). C5a-C5aR-induced electrolyte imbalance (17, 71), and/or tissue damage may have contributed, at least partially, to hyperkalemia-induced early death after TH.

Because of unbound nomacopan's short half-life, Ort et al. suggested daily subcutaneous injections to maintain complement inhibition (72). In an experimental setting, Barratt-Due et al. applied a continuous infusion of the drug in order to inhibit newly synthesized C5 (49). Our treatment with nomacopan was performed 15 minutes after blast injury and immediately before hemorrhage. When treatment was delayed until 60 minutes after TH, nomacopan showed no effect on survival at 24 hours. This finding is aligned with a report that administration of nomacopan 15 minutes after TBI in mice reduced neuropathology and improved neurological performance, whereas delaying treatment to 30 minutes after TBI was not beneficial (53). These observations indicate that there is an optimal therapeutic window for timely and effective intervention after TH.

The observational study in patients has limitations. The limited data set at 8 and 24 hours might have been biased by a number of factors related to patient evacuation as well as hospital treatments that may negatively impact the profiles of complement and cytokines. Consequently, we focused on the data acquired on admission and performed correlation analysis only using these data. Blood samples from injured service members were obtained, shipped frozen to the U.S. and stored, whereas samples from the healthy controls were collected and assayed the same day. The impact of storage time on the observed differences is unknown, but it is assumed that storage may reduce rather than increase such levels (73, 74).

Taken together, our clinical and preclinical findings demonstrate that TCA plays a pivotal role in the pathogenesis of TH, and that nomacopan as a pro-survival and organ-protective drug, may be a promising pharmacological solution for the treatment of severely injured patients on the battlefield and in pre-hospital environments.

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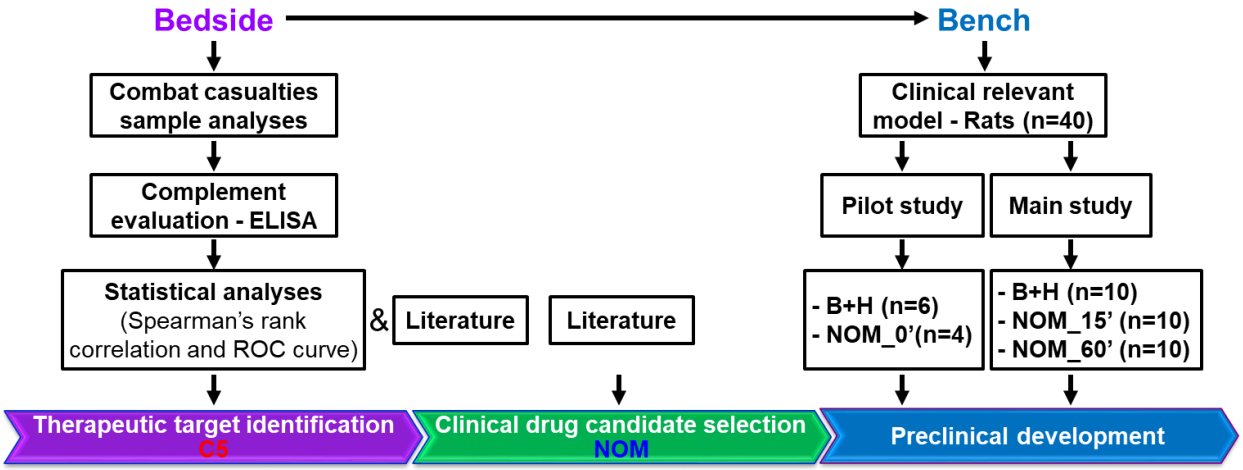
Main Text _ Table & Figures

	Injured Service-members (n=54)	Healthy Controls (n=10)
Age in years, median (IQR)	25 (22-30)	36 (28-41)
Gender, n (%)		
Male	53 (98)	6 (60)
Female	1 (2)	4 (40)
Combat theater		
OIF, n	54	N/A
Mortality, n (%)	5 (9)	N/A
Injury severity score, median (IQR)	16 (9-24)	N/A
Mechanism of injury, n (%)		
Blast	37 (69)	N/A
GSW	14 (26)	N/A
Burn	2 (4)	N/A
MVA	1 (2)	N/A
Traumatic brain injury, n (%)		
Yes	45 (83)	N/A
No	4 (7)	N/A
Unknown	5 (9)	N/A
Glasgow Coma Scale, median (IQR)	15 (14-15)	N/A

Table 1: Demographics and Characteristics of Trauma Patients and Healthy Controls. Abbreviations: IQR, interquartile range; OIF, Operation Iraqi Freedom; GSW, gunshot wound; MVA, motor vehicle accidents. Data are presented as number (percentage) of patients unless otherwise indicated.

Fig. 1



953

954 **Fig. 1. Workflow of translational study design.** Blood plasma from 54 casualties on admission,
955 8 and 24 hours after admission to a hospital and 10 civilian volunteers was used for analysis of
956 the complement activation. On the base of complement activation products in the casualties'
957 plasma, complement component C5 was identified as a reasonable therapeutic target.
958 Prophylactic and therapeutic effects of nomacopan, an inhibitor of C5 were tested in injured rats.
959 Abbreviation: NOM, nomacopan.

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Fig. 2

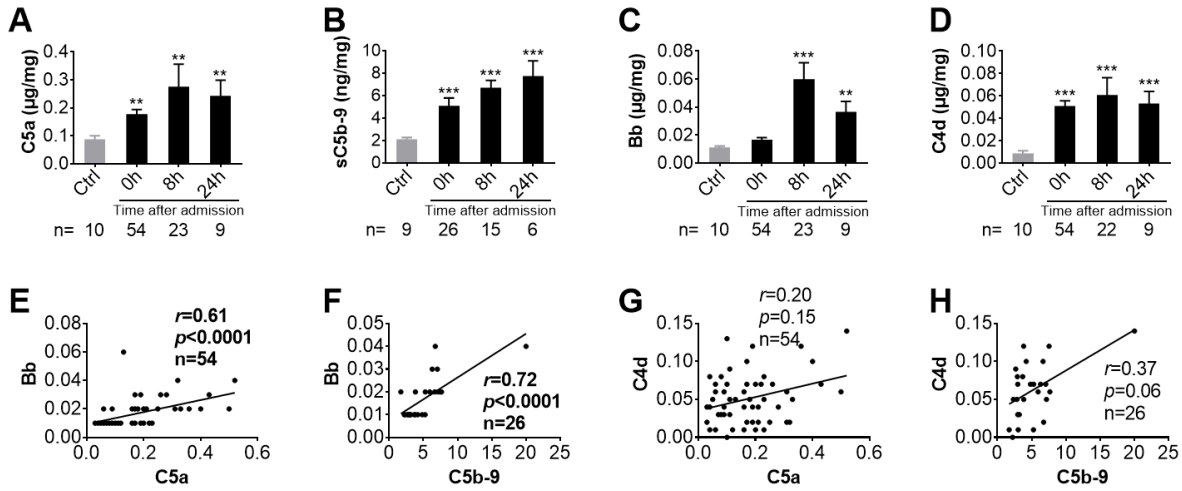
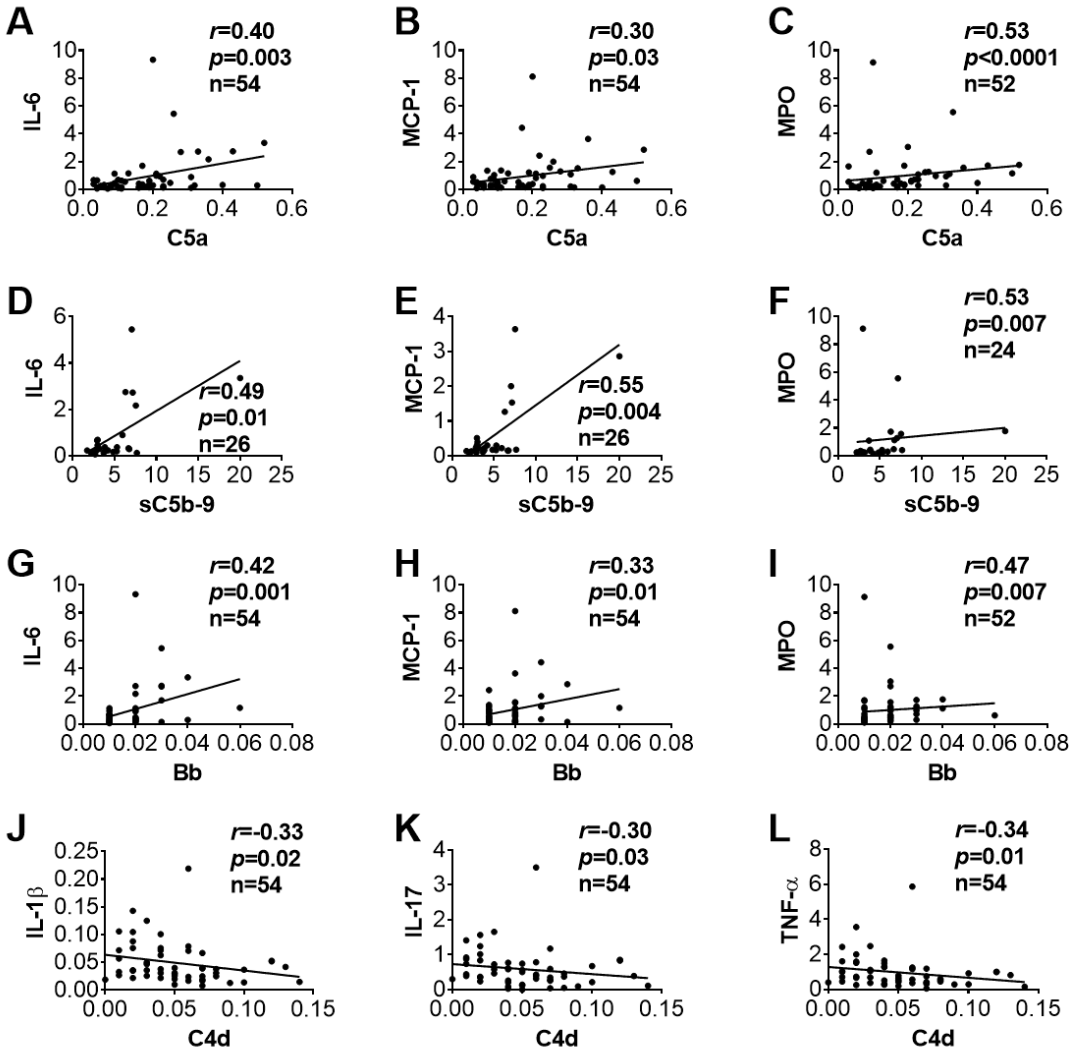


Fig. 2. Early activation of complement terminal and alternative pathways after trauma in military casualties. Plasma levels of C5a **(A**), sC5b-9 **(B**), Bb **(C**) and C4d (**D**) were measured to determine activation of terminal complement (C5a and sC5b9), alternative pathway (Bb), and classical and lectin pathways (C4d) by ELISA in healthy donors, and trauma patients on admission to hospital, 8 and 24 hours after admission. The data were presented as mean ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* Healthy (by Mann-Whitney U test). (**E-H**) Showing correlation of Bb and C4d with either C5a or sC5b-9 in the injured patients at admission. Correlation analysis between complement factors Bb or C4d and C5a or sC5b-9 were performed using Spearman's rank correlation, and the data are presented with coefficient (r_s) and *p*-values. Significant correlations (p <0.05) are indicated by boldface type.

Fig. 3

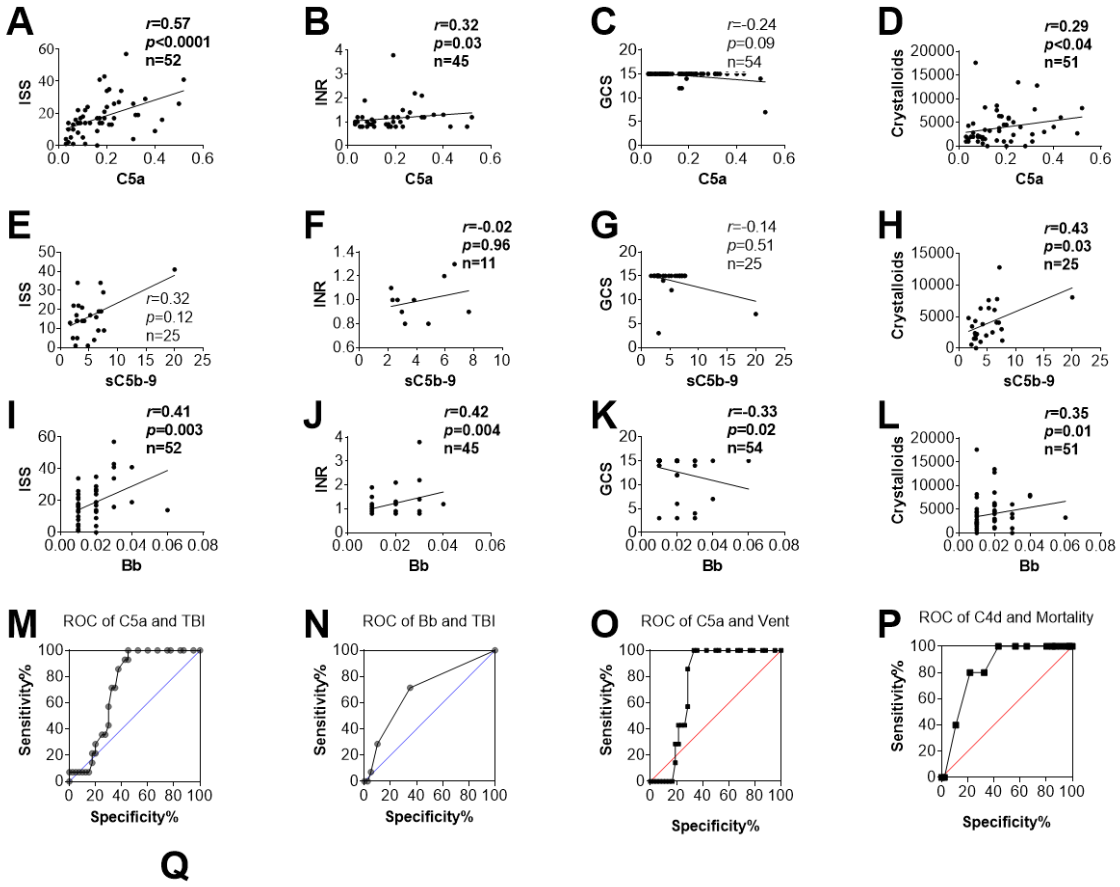


978

979 **Fig. 3. Activation of complement terminal and alternative pathways is related to systemic**
980 **inflammatory response after trauma in battlefield casualties.** Inflammatory factors and
981 cytokines were measured by ELISA and by Bio-Plex Kits, respectively. Positive correlation
982 between plasma concentrations of C5a in the trauma patients and IL-6 (**A**), MCP-1 (**B**), and MPO
983 (**C**) in the blood plasma of the patients on admission. Positive correlation of plasma levels of
984 C5b-9 on the admission with IL-6 (**D**), MCP-1(**E**) and MPO (**F**) in the injured patients on
985 admission. Plasma concentration of Bb on the admission positively correlated with IL-6 (**G**),
986 MCP-1 (**H**), and MPO (**I**), whereas the plasma levels of C4d on admission inversely correlated

with IL-1β (**J**), IL-17 (**K**), and TNF-α (**L**). The data were expressed as microgram per milligram plasma protein except for C5b-9, which is nanogram per milligram plasma protein. Correlation analysis between complement factors (C5a, C5b-9, Bb and C4d) and inflammatory factors/cytokines were performed by using Spearman's rank correlation, and the data are presented with coefficient (r_s) and *p*-values. Significant correlations ($p < 0.05$) are indicated by boldface type.

Fig. 4



Q

ROC	AUC	Cut-off	Sensitivity	Specificity
C5a with TBI	0.73	0.185 μg/mg	71.43	67.50
Bb with TBI	0.69	0.015 μg/mg	71.43	65.00
C5a with Vent	0.75	0.195 μg/mg	85.71	71.43
C4d with Mortality	0.83	0.025 μg/mg	80.00	78.26

Fig. 4. Complement activation correlated with clinical outcomes in trauma patients on admission. A-D, correlation of C5a plasma levels with clinical scores, infused fluid and INR, a standard coagulation test; **E-H**, correlation of C5b-9 with clinical scores, infused fluid and INR; I-L, correlation of fragment Bb with clinical scores, infused fluid and INR; **M-N**, the receiver-operator characteristic curve (ROC) analysis tested diagnostic ability of C5a and fragment Bb in identifying traumatic brain injury (TBI); the cut-off value, specificity and sensitivity are shown (**Q**); **O**, patients on mechanical ventilation had significantly higher plasma levels of C5a in comparison to those not-ventilated; **P**, ROC analysis showed that the C4d plasma levels had a

strong predictive value for the survival; the cut-off value, specificity and sensitivity are presented (**Q**). The plasma levels of complement components and inflammatory agents are expressed per milligram of plasma proteins. *=$p<0.05$, ***=$p<0.001$.

Fig. 5

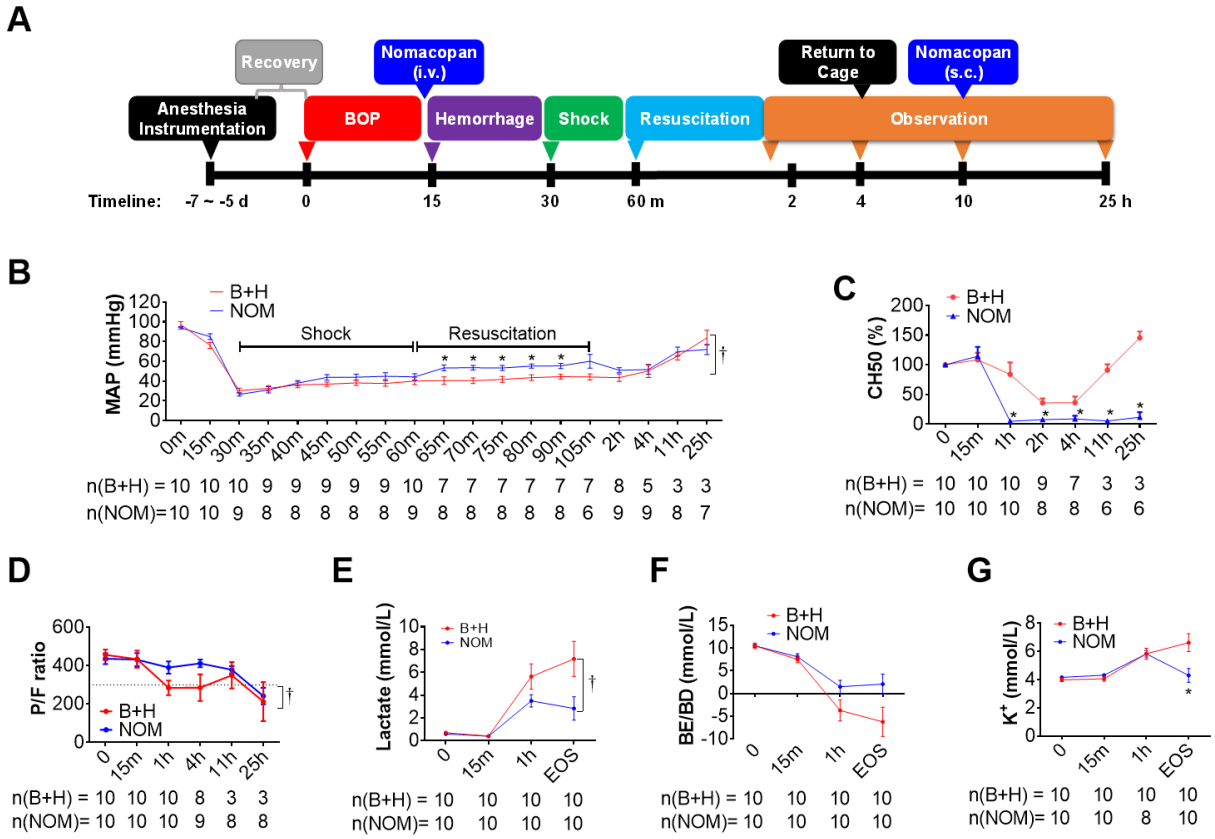


Fig. 5. Effects of nomacopan treatment early after blast injury on MAP, CH50, and blood chemistry changes in a clinically relevant rat model of traumatic hemorrhagic shock. A, experimental design; **B**, changes of MAP were monitored via the carotid artery using the BIOPAC system. During shock and resuscitation period the MAP was recorded every 5 minutes. Data are presented as mean ± SEM; **C**, hemolytic TCA of sera was measured by CH50 and normalized to baseline level, which was pre-blast injury; the percentages of baseline are shown; **D**, PaO_2/FiO_2 ratio (PFR) following injuries. The PFR was calculated at each time point based on the artery i-STAT data. A PFR less than 300 (dashed line) is suggestive of acute respiratory distress syndrome (ARDS). **E-G**, the blood chemistry of lactate, BE/BD and K^+ in injured and treated animals were measured by Istat and presented. *= $p<0.05$, the individual time points were

compared by unpaired *t*-test with Welch's correction, and †= $p<0.05$, comparison of the groups was performed by two-way ANOVA. **Labels:** BOP = blast overpressure; B+H = blast + hemorrhagic shock; NOM = nomacopan given to injured rats; MAP= mean arterial pressure; EOS = end of the study.

Fig. 6

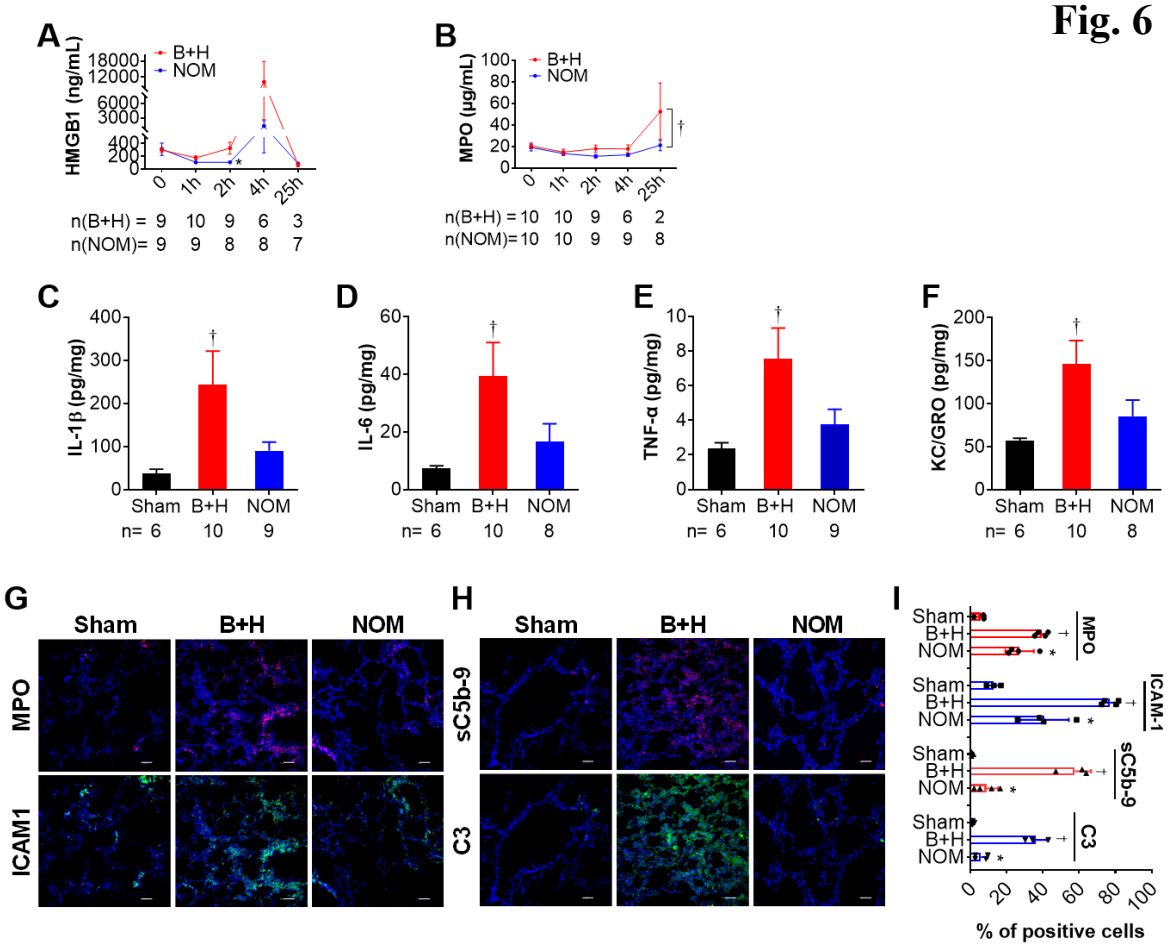


Fig. 6. Effect of nomacopan treatment early after injury on systemic and local inflammatory response in rats after blast injury and hemorrhage. A and **B**, inflammatory mediators HMGB1and MPO were analyzed by ELISA. Comparison between the vehicle (saline) and nomacopan group was performed by two-way ANOVA; **C-F**, cytokines/chemokines were measured in the lung homogenates; **G**, the lung tissue at necropsy or at the end of study were collected, fixed by PFA, and stained by IHC. The representative images of immunostaining of MPO (red) and ICAM1 (green) are shown; **H**, antibodies for detecting of C5b-9 (red) and C3 (green) were used to detect complement deposition in the lung tissue. Representative images (**G and H**) are shown (original magnification, 200X), and semi-quantitative analysis (**I**) of the positive stained cells to total cells are presented (n=3 to 4 animals per group). † =p<0.05 vehicle vs. sham,

and *= $p<0.05$ nomacopan vs. vehicle, analyzed by unpaired *t*-test with Welch's correction. Scale bar=50 µm. Labels: B+H = blast + hemorrhagic shock; NOM = nomacopan given to injured rats; MPO = myeloperoxidase.

Fig. 7

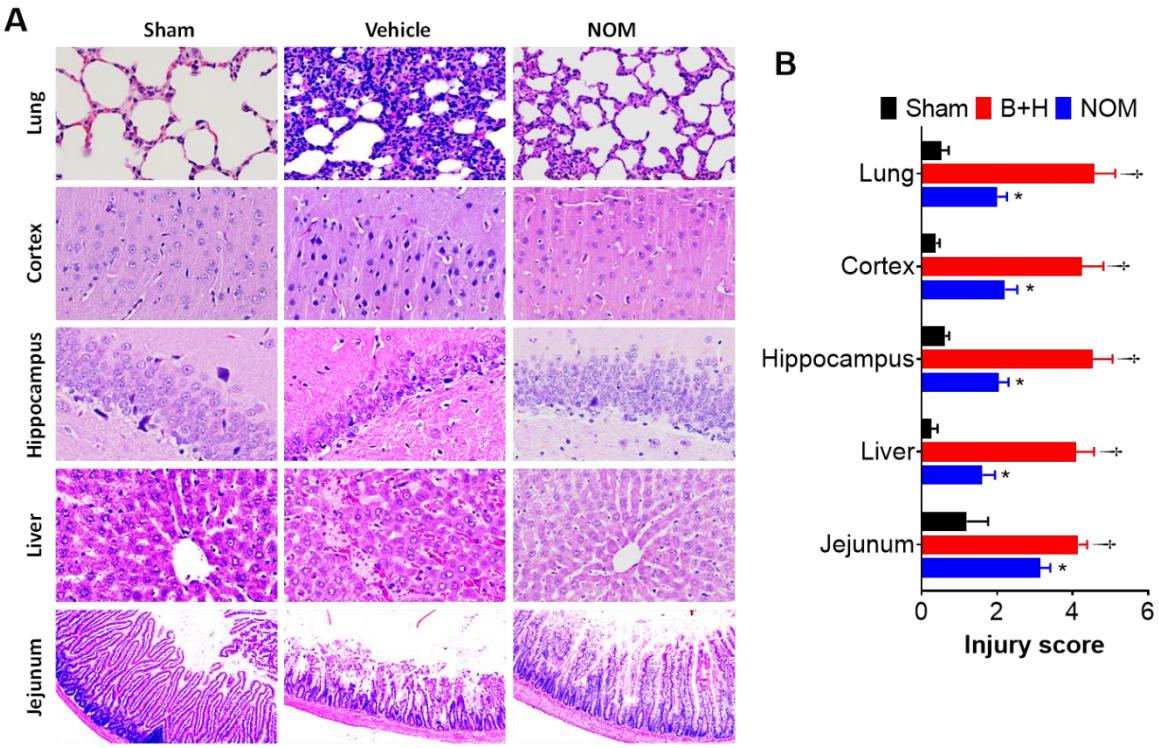


Fig. 7. Effect of nomacopan treatment on histological changes in rats after blast injury and hemorrhage. A and **B**: Representative H&E photomicrographs of organs harvested at the time of necropsy (**A**), and organ injury scored based on the criteria described in the Materials and Methods (**B**). The data are presented as mean ± SEM, *= $p<0.05$ *vs.* B + H, †=$p<0.05$, nomacopan *vs.* B + H (by the Mann-Whitney U test).

Fig. 8

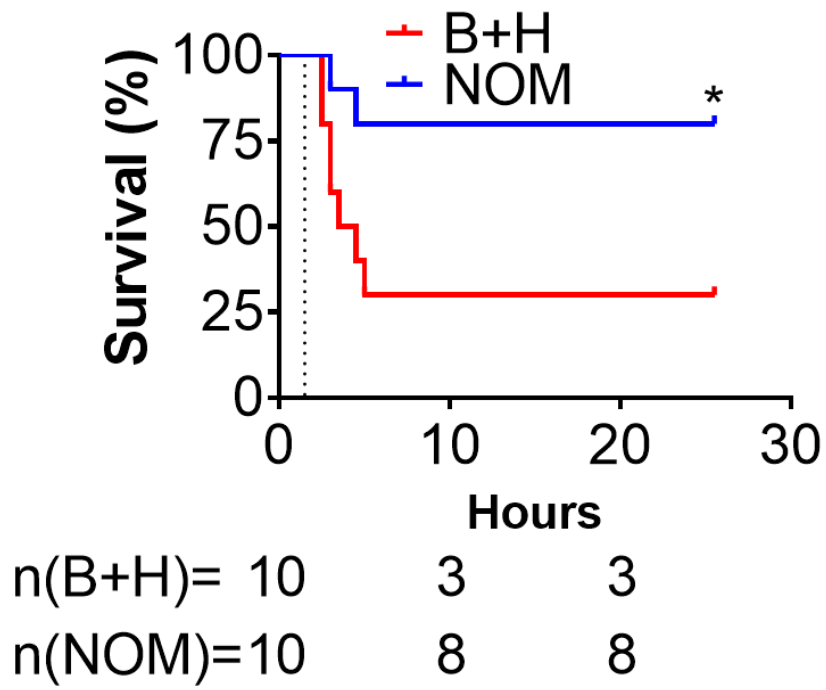


Fig. 8. Effect of nomacopan on injury survival. All rats were subjected to blast and severe hemorrhage (B + H), treated with vehicle control (saline) or nomacopan (NOM), and monitored for survival up to 24 hours after traumatic hemorrhagic shock. Survival distribution of these two groups was determined by using the log-rank Mantel-Cox test. *=$p < 0.05$.

Supplementary Materials:

Fig. S1. Systemic activation of the complement pathways in battlefield trauma.

Fig. S2. Systemic inflammatory response to trauma.

Fig. S3. Preventive effects of nomacopan treatment on the acidosis, hemodynamics, tissue damage and survival after blast injury and hemorrhage in rats (Pilot study).

Fig. S4. Complement hemolytic activity and nomacopan effect on survival dependent on its administration time.

Table S1. Correlations Complement to Inflammatory Mediators/Cytokines/Chemokines.

Table S2. Correlations Between Complement/Inflammatory Cytokines/Chemokines and Clinical Outcomes.

Table S3. Blast wave parameters from pilot and main (treatment) studies.

Table S4. Blood chemistry changes in control and nomacopan treatment (NOM_30') groups.

Supplementary Materials:

Refer to Web version on PubMed Central for supplementary material.

Supplementary Materials and Methods

1. Analysis of coagulation parameters in patients
2. The receiver-operator characteristic curve analysis

Analysis of coagulation parameters: Prothrombin time (PT) and platelet count were measured according to standard clinical assays.

The receiver-operator characteristic (ROC) curve analysis: ROC curve analysis was used to evaluate some complement components and inflammatory agents in diagnostic testing, also complement components in predictive models.

Supplementary Figures

Fig. S1

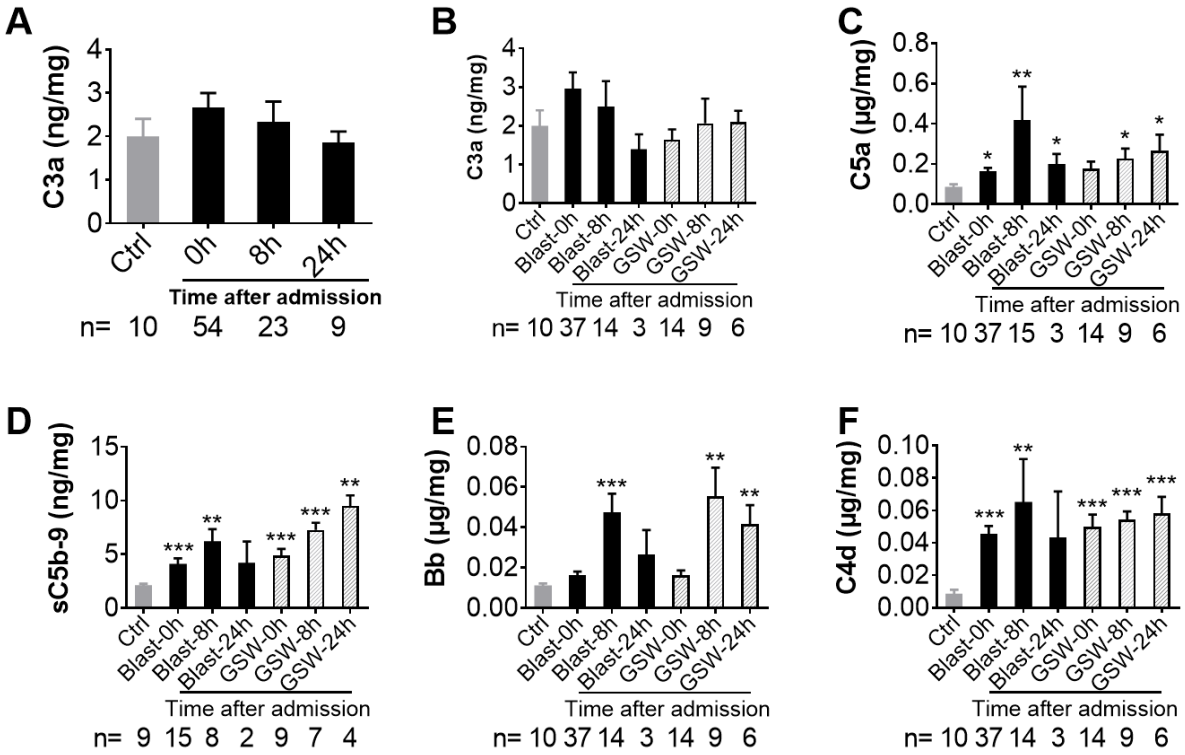
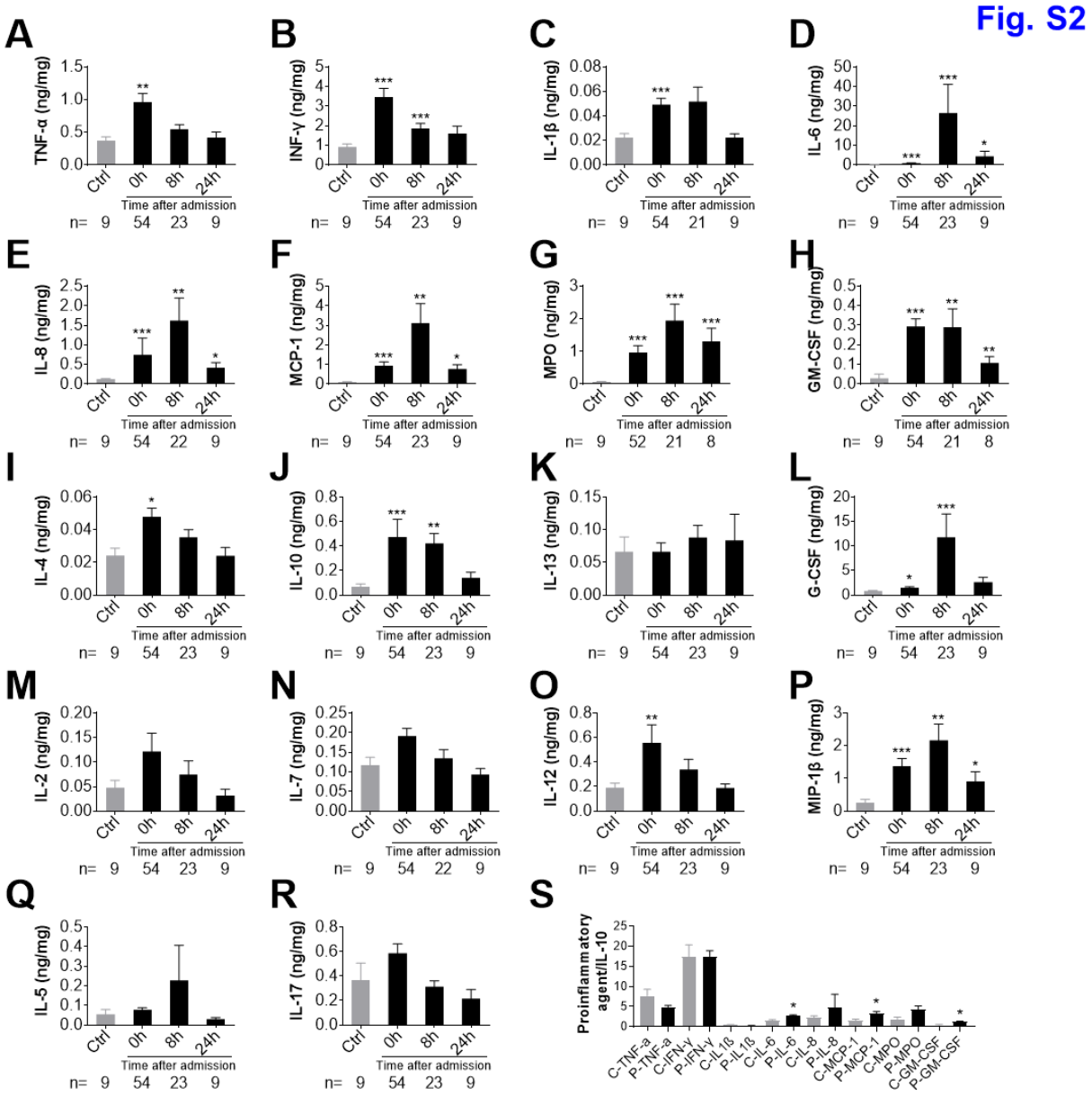


Fig. S1. Systemic activation of the complement pathways in battlefield trauma. **A-F**, plasma levels of complement factors (C3a, C5a, sC5b-9, Bb, and C4d) were measured by ELISA in healthy donors and trauma patients at admission to hospital (0h), and at 8 and 24 hours after admission. **A**, plasma levels of C3a for all admitted patients; **B-F**, plasma levels of complement components in patients with two major mechanisms of injury - blast or gunshot wounds. The data are expressed as nanograms per milligram of total plasma proteins and presented as mean ± SEM, *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ *vs.* Healthy.

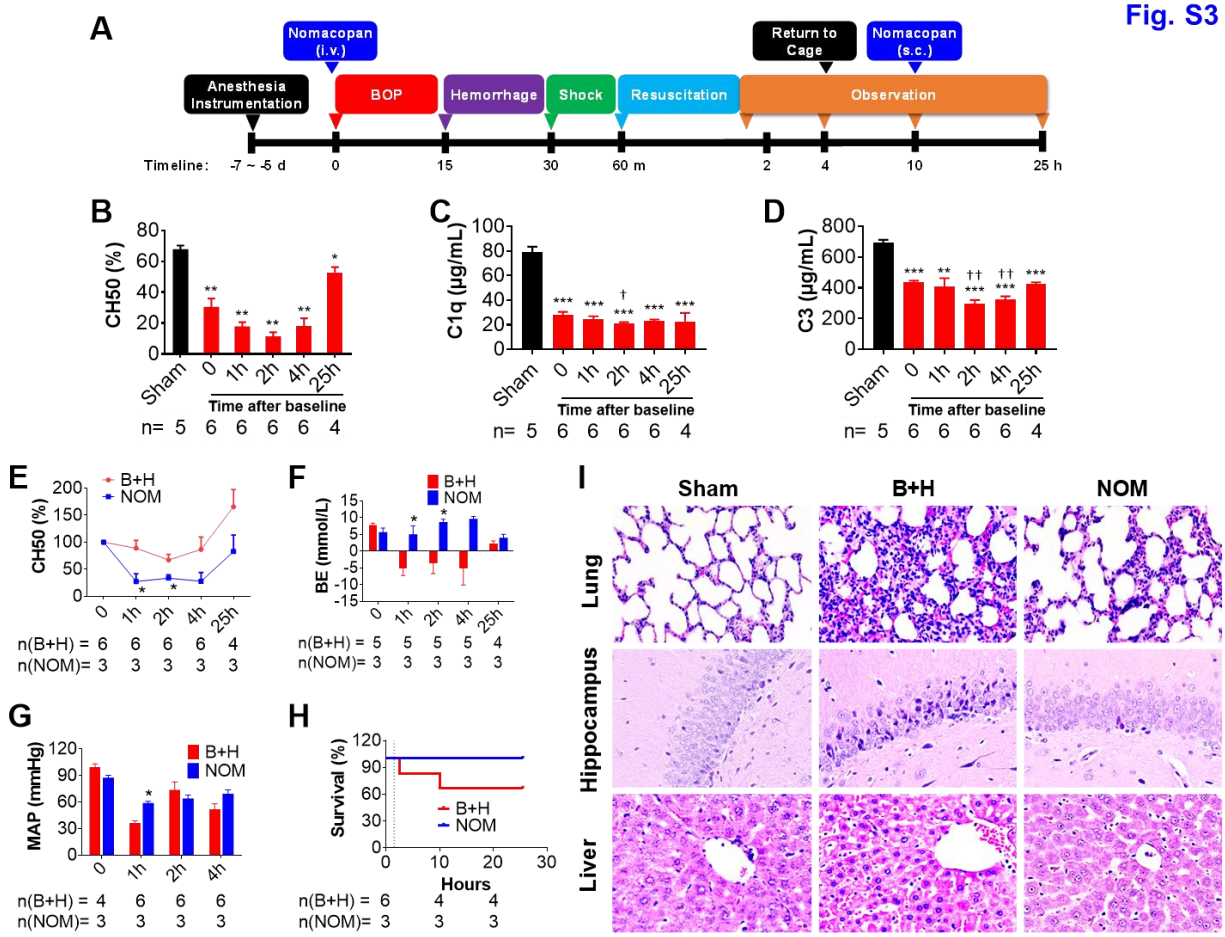


1180

1181 **Fig. S2. Systemic inflammatory response to trauma.** Inflammatory factors and cytokines were
1182 measured by ELISA and by Bio-Plex Kits, respectively. Pro-inflammatory factors/cytokines (**A-**
1183 **H**), anti-inflammatory cytokines (**I-L**) and regulatory cytokines (**M-R**) from healthy donors
1184 (n=10) and trauma patients on admission (n=54), and at 8 (n=23) and 24 hours (n=9) after
1185 admission were presented. The data are expressed as nanogram per milligram plasma protein and

presented as mean ± SEM, *= p<0.05, **=p<0.01, *** = p<0.001 vs. Healthy. Pro-inflammatory versus anti-inflammatory response (**S**). Ratio of systemic inflammatory factors (TNF-α, IFN-γ, IL-1β, IL-6, IL-8, MCP-1, MPO, and GM-CSF) to IL-10 on admission is given. C, Healthy controls (n=10); P, trauma patients (n=54). *= p < 0.05 vs. respective control (Unpaired t- test with Welch's correction).

Fig. S3



1204

1205 **Fig. S3. Preventive effects of nomacopan treatment on the acidosis, hemodynamics, tissue**
1206 **damage and survival after blast injury and hemorrhage in rats (Pilot study).** (**A**)
1207 Experimental design. Anesthetized male rats were subjected to a moderate blast overpressure
1208 (BOP = 111.65 ± 2 kPa, t_+ = 3.16 ± 0.03 ms, impulse = 143 ± 2.26 kPa-ms) and a controlled
1209 hemorrhage (40% blood volume). After 30 min of shock, animals were resuscitated with Plasma-
1210 Lyte A (2× shed blood volume). Animals were randomized to three study arms: nomacopan (15
1211 mg/kg, n=3), B + H (saline, n=6) and Sham (no injury, n=4). First dose of nomacopan (7.5 mg/kg,
1212 i.v.) and a repeated dose of nomacopan (7.5 mg/kg, s.c.) were given immediately before blast
1213 injury and at 11 hours after blast injury, respectively. Blood pressure was monitored and recorded

with the BIOPAC MP160 Data Acquisition and Analysis Systems via the carotid arterial catheter. Blood and tissue samples were collected for blood complement/chemistry analysis and histopathological evaluation, respectively. (**B-D**) Bar graphs showing serum CH50, and plasma concentrations of C1q and C3, respectively. The data were presented as mean ± SEM, *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ *vs.* Sham, †= $p<0.05$, ††= $p<0.01$ *vs.* baseline (0 hour; by Mann-Whitney U test). (**E-G**) Bar graphs displaying effect of nomacopan on CH50, BE, and MAP, respectively. The data were presented as mean ± SEM, *= $p<0.05$ *vs.* B + H (by Mann-Whitney U test). (**H**) Effect of nomacopan treatment on survival. (**I**) Representative H & E images showing effect of nomacopan on histological changes of the organs. **Labels**: **B**+**H** = blast overpressure (BOP) + hemorrhage; NOM = nomacopan *i.v.* + BOP + hemorrhage + nomacopan *s.c.* ; MAP = mean arterial pressure; BE= base excess/base deficit.

Fig. S4

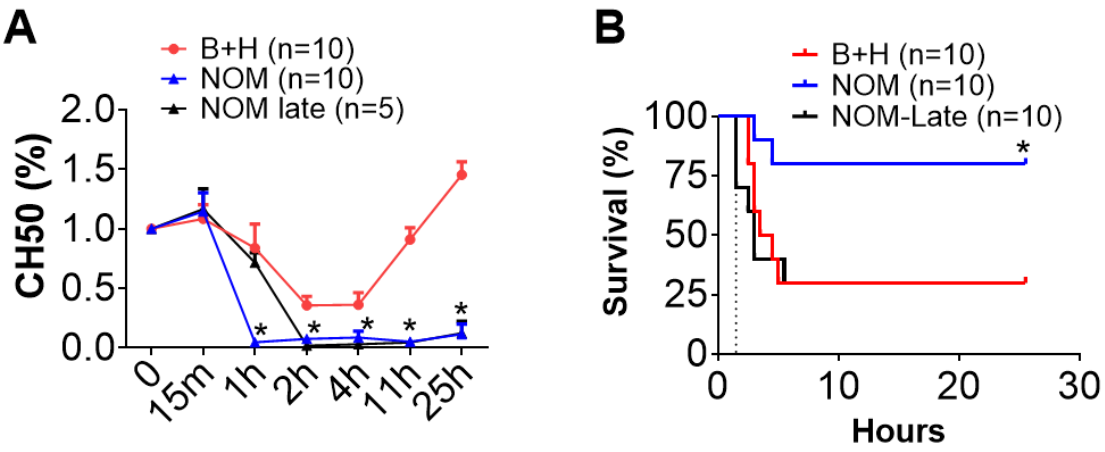


Fig. S4. Complement hemolytic activity and nomacopan effect on survival dependent on its administration time. Experimental groups: All animals were subjected to blast and hemorrhage, and treated with vehicle (saline, B+H group) or with nomacopan**; B** + **H** = blast + hemorrhage; **NOM** = blast + nomacopan *i.v.* + hemorrhage + nomacopan *s.c.* in resuscitation phase; **NOM-Late** = nomacopan *i.v.*, with first dose was infused at the end of hemorrhagic shock, immediately before fluid resuscitation + nopmacopan *s.c.* with second dose was given in the resuscitation phase; *i.v.* = intravenous; *s.c.*= subcutaneous. **A**, the CH50 test data throughout the observation period; **B**, the survival distribution for three experimental groups was compared using the log-rank Mantel-Cox test; *= p <0.05. NOM, nomacopan.

Supplementary Tables

Table S1

	C3a		C5a		C5b-9		Bb		C4d	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
Eotaxin	-0.13	0.76	0.18	0.70	0.16	0.73	-0.18	0.48	-0.03	0.89
FGF basic	-0.57	0.20	0.11	0.84	-0.07	0.91	-0.06	0.74	-0.27	0.53
G-CSF	-0.07	0.62	-0.09	0.52	-0.14	0.49	-0.11	0.43	-0.07	0.60
GM-CSF	0.13	0.35	0.09	0.50	-0.04	0.85	0.00	0.99	-0.22	0.12
IFN-γ	-0.09	0.51	-0.05	0.75	-0.10	0.64	-0.13	0.36	-0.25	0.07
IL-1β	0.05	0.71	-0.14	0.32	-0.25	0.22	-0.09	0.50	n/a	n/a
IL-1ra	0.54	0.24	-0.54	0.24	-0.04	0.96	-0.42	0.23	0.69	0.10
IL-2	0.07	0.62	0.06	0.67	-0.22	0.29	-0.09	0.54	-0.02	0.86
IL-4	-0.11	0.43	-0.04	0.80	-0.24	0.23	-0.17	0.21	-0.21	0.13
IL-5	-0.14	0.33	-0.01	0.96	-0.17	0.39	-0.09	0.51	**-0.27**	**0.05**
IL-6	**0.45**	**0.00**	n/a	n/a	n/a	n/a	n/a	n/a	0.17	0.23
IL-7	-0.12	0.37	-0.20	0.14	-0.22	0.28	-0.22	0.11	-0.25	0.06
IL-8	0.09	0.54	0.08	0.54	0.18	0.39	0.08	0.56	-0.09	0.51
IL-9	-0.43	0.35	0.07	0.91	0.00	> 0.9999	-0.06	0.74	-0.22	0.61
IL-10	**0.35**	**0.01**	**0.40**	**0.00**	0.34	0.09	**0.31**	**0.02**	0.00	0.97
IL-12	-0.23	0.09	-0.01	0.96	0.06	0.75	-0.15	0.29	-0.21	0.12
IL-13	0.08	0.57	0.10	0.48	-0.02	0.93	0.18	0.19	-0.26	0.06
IL-15	0.11	0.84	-0.18	0.71	0.04	0.96	-0.30	0.40	0.18	0.70
IL-17	-0.16	0.25	-0.05	0.70	-0.26	0.20	-0.16	0.25	n/a	n/a
IP-10	-0.25	0.59	0.36	0.44	0.18	0.71	-0.36	0.29	-0.27	0.53
MCP-1	**0.29**	**0.03**	n/a	n/a	n/a	n/a	n/a	n/a	-0.02	0.89
MPO	**0.34**	**0.01**	n/a	n/a	n/a	n/a	n/a	n/a	0.00	0.97
MIP-1α	-0.64	0.14	0.39	0.40	0.14	0.78	0.30	0.55	-0.45	0.29
MIP-1β	0.26	0.06	0.23	0.10	**0.52**	**0.01**	**0.29**	**0.03**	0.00	0.97
PDGF-bb	0.11	0.84	0.04	0.96	0.18	0.71	-0.42	0.23	0.36	0.42
RANTES	0.57	0.20	0.32	0.50	0.71	0.09	0.18	0.74	0.31	0.50
TNF-α	-0.06	0.66	-0.12	0.37	-0.23	0.25	-0.13	0.33	n/a	n/a
VEGF	-0.11	0.84	0.32	0.50	0.39	0.40	0.18	0.74	-0.11	0.79

Table S1. Correlations Complement to Inflammatory Mediators/Cytokines/Chemokines.

Abbreviations: FGF basic, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; MIP, macrophage inflammatory protein; PDGF-bb, platelet derived growth factor-BB; RANTES, regulated on activation, normal

T cell expressed and Secreted; VEGF, vascular endothelial growth factor. n/a, not applicable. The correlation analyses were performed by Spearman's rank correlation. Significant correlation (p<0.05) is indicated by boldface type.

Table S2

	Base Deficit		GCS		ISS		MAP		SIRS score		RBC		PLT units		FFP units		Crystalloids	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
C4d	-0.15	0.49	0.24	0.085	-0.135	0.340	0.205	0.136	-0.040	0.801	**-0.287**	**0.042**	**-0.28**	**0.046**	**-0.348**	**0.01**	0.03	0.83
MPO	0.03	0.89	-0.19	0.20	**0.33**	**0.02**	-0.03	0.83	**0.37**	**0.02**	**0.47**	**0.00**	0.17	0.24	**0.36**	**0.01**	**0.35**	**0.01**
Eotaxin	-0.80	0.33	-0.45	0.10	-0.18	0.67	-0.27	0.53	-0.50	0.33	0.15	0.73	**-0.41**	**< 0.0001**	0.02	0.92	-0.40	0.36
FGF basic	-1.00	0.08	**-0.76**	**< 0.0001**	0.21	0.66	-0.54	0.24	-0.11	0.83	0.67	0.12	**-0.61**	**< 0.0001**	0.58	0.19	-0.21	0.66
G-CSF	0.22	0.30	**-0.29**	**0.04**	**0.30**	**0.03**	-0.10	0.47	0.03	0.83	**0.29**	**0.04**	0.08	0.56	0.06	0.65	0.03	0.82
GM-CSF	0.35	0.09	**-0.48**	**0.00**	**0.28**	**0.04**	-0.23	0.09	0.01	0.95	**0.34**	**0.02**	0.10	0.46	**0.34**	**0.01**	-0.09	0.54
IFN-γ	0.29	0.16	**-0.28**	**0.05**	0.18	0.19	**-0.31**	**0.02**	-0.09	0.55	0.21	0.13	0.06	0.68	0.11	0.42	-0.07	0.62
IL-1β	0.36	0.08	**-0.39**	**0.00**	0.27	0.05	**-0.27**	**0.05**	-0.09	0.57	**0.39**	**0.00**	0.21	0.14	**0.27**	**0.05**	0.01	0.92
IL-1ra	-0.40	0.75	-0.27	0.29	-0.39	0.40	-0.61	0.17	0.63	0.50	0.16	0.73	**-0.61**	**< 0.0001**	0.58	0.19	-0.21	0.66
IL-2	-0.24	0.27	-0.09	0.55	0.15	0.29	-0.12	0.38	-0.01	0.96	0.14	0.34	-0.01	0.93	-0.07	0.60	0.10	0.50
IL-4	0.11	0.62	**-0.32**	**0.02**	0.27	0.05	**-0.35**	**0.01**	0.09	0.55	**0.32**	**0.02**	0.15	0.28	0.12	0.37	0.00	0.99
IL-5	0.20	0.36	**-0.33**	**0.02**	**0.30**	**0.03**	**-0.33**	**0.02**	0.03	0.85	**0.31**	**0.03**	0.14	0.32	0.11	0.45	-0.03	0.83
IL-6	0.25	0.25	-0.26	0.07	**0.35**	**0.01**	-0.03	0.83	0.20	0.20	**0.42**	**0.00**	0.12	0.37	**0.30**	**0.03**	**0.31**	**0.03**
IL-7	0.33	0.11	-0.26	0.06	0.21	0.13	**-0.38**	**0.00**	-0.05	0.73	0.20	0.17	0.15	0.29	0.06	0.66	-0.13	0.36
IL-8	0.21	0.33	**-0.36**	**0.01**	**0.41**	**0.00**	**-0.28**	**0.04**	0.18	0.25	**0.41**	**0.00**	0.20	0.15	0.25	0.07	0.11	0.46
IL-9	-1.00	0.08	**-0.76**	**< 0.0001**	0.04	0.96	-0.50	0.27	0.11	> 0.9999	0.67	0.12	**-0.61**	**< 0.0001**	0.58	0.19	-0.25	0.59
IL-10	0.17	0.42	**-0.33**	**0.02**	**0.37**	**0.01**	-0.10	0.46	0.15	0.35	**0.43**	**0.00**	0.07	0.62	**0.30**	**0.03**	0.26	0.07
IL-12	0.28	0.18	-0.20	0.15	0.14	0.31	**-0.32**	**0.02**	-0.18	0.26	0.15	0.31	0.15	0.27	0.01	0.97	0.00	0.99
IL-13	0.01	0.97	**-0.32**	**0.02**	0.22	0.12	0.03	0.83	-0.09	0.59	**0.36**	**0.01**	0.13	0.35	0.24	0.08	0.18	0.21
IL-15	-1.00	0.08	-0.45	0.14	-0.43	0.35	-0.36	0.44	0.32	0.67	0.23	0.61	**-0.61**	**< 0.0001**	0.32	0.49	-0.43	0.35
IL-17	0.08	0.69	**-0.38**	**0.01**	**0.33**	**0.02**	**-0.40**	**0.00**	0.12	0.44	**0.36**	**0.01**	0.14	0.30	0.17	0.22	0.07	0.64
IP-10	-0.80	0.33	**-0.67**	**< 0.0001**	0.21	0.66	-0.39	0.40	-0.74	0.17	0.22	0.64	**-0.41**	**< 0.0001**	0.22	0.63	-0.64	0.14
MCP-1	0.22	0.31	**-0.33**	**0.02**	**0.40**	**0.00**	-0.16	0.24	0.05	0.74	**0.45**	**0.00**	0.24	0.08	**0.43**	**0.00**	0.18	0.21
MIP-1α	-0.80	0.33	**-0.76**	**< 0.0001**	0.50	0.27	-0.46	0.30	0.21	0.83	**0.85**	**0.03**	**-0.20**	**< 0.0001**	0.58	0.19	0.14	0.78
MIP-1β	0.35	0.09	-0.22	0.12	0.18	0.21	-0.19	0.16	-0.02	0.91	**0.29**	**0.04**	0.14	0.31	**0.36**	**0.01**	0.20	0.17
PDGF-bb	-0.80	0.33	**-0.58**	**0.05**	-0.04	0.96	-0.71	0.09	-0.74	0.17	0.18	0.70	**-0.61**	**< 0.0001**	0.30	0.52	-0.43	0.35
RANTES	0.40	0.75	-0.27	0.29	-0.32	0.50	-0.21	0.66	0.32	0.67	0.18	0.70	0.20	0.57	-0.02	0.88	0.07	0.91
TNF-α	0.27	0.20	**-0.31**	**0.03**	0.21	0.13	**-0.28**	**0.04**	-0.07	0.67	**0.29**	**0.04**	0.11	0.41	0.14	0.32	0.00	0.99
VEGF	-0.80	0.33	**-0.58**	**0.05**	-0.18	0.71	-0.29	0.56	-0.21	0.67	0.50	0.25	**-0.20**	**< 0.0001**	0.19	0.70	-0.07	0.91

Table S2. Correlations Between Complement/Inflammatory Cytokines/Chemokines and Clinical Outcomes. Abbreviations: MPO, myeloperoxidase; FGF basic, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor. MCP-1, monocyte chemoattractant protein-1 (also known as CCL2); MIP, macrophage inflammatory protein (also known as CCL3); PDGF-bb, platelet derived growth factor-BB; RANTES, regulated on activation, normal T cell expressed and Secreted (also known as CCL5); VEGF, vascular endothelial growth factor. n/a, not applicable. The correlation analyses were performed by Spearman's rank correlation. Significant correlation ($p<0.05$) is indicated by boldface type.

Table S3

	Reference			Overpressure			Reflected		
	P0 (kPa)	t+ (ms)	I (kPa-ms)	P0 (kPa)	t+ (ms)	I (kPa-ms)	P0 (kPa)	t+ (ms)	I (kPa-ms)
Pilot study									
B+H (n=6)	101.20 ± 1.13	3.31 ± 0.03	136.37 ± 1.11	108.49 ± 1.22	3.29 ± 0.03	137.46 ± 1.12	153.13 ± 2.45	3.45 ± 0.01	171.17 ± 1.64
NOM_0' (n=3)	111.70 ± 0.65	3.33 ± 0.02	144.13 ± 0.65	119.75 ± 0.70	3.31 ± 0.02	145.29 ± 0.65	166.37 ± 5.00	3.48 ± 0.01	180.37 ± 0.95
Main study									
B+H (n=10)	108.93 ± 1.22	3.32 ± 0.01	140.23 ± 0.77	116.78 ± 1.31	3.30 ± 0.01	141.35± 0.78	161.17 ± 1.87	3.48 ± 0.03	178.51 ± 1.21
NOM_15' (n=10)	107.96 ± 1.11	3.27 ± 0.02	140.54 ± 0.70	115.74 ± 1.19	3.25 ± 0.02	141.67 ± 0.70	162.84 ± 1.36	3.49 ± 0.03	178.82 ± 1.07
NOM_60' (n=10)	108.47 ± 1.01	3.30 ± 0.03	141.00 ± 0.55	116.29 ± 1.09	3.28 ± 0.03	142.13 ± 0.56	158.48 ± 2.16	3.49 ± 0.03	178.34 ± 1.07

Table S3. Blast wave parameters from pilot and main (treatment) studies. Legend: B + H group = blast + hemorrhage; NOM group = nomacopan *i.v.* + blast + hemorrhage + nomacopan *s.c.*; **P0** (peak pressure) in **kPa** (the kilopascal, a unit of pressure); **t+** [the positive-pressure phase duration in milliseconds (**ms**)]; **I** [impulse (kPa-ms)].

Table S4

Parameters		0m	15m	1hs	4hs	11hs	25hs/EOS
Arterial pH	Vehicle (n=10)	7.44 ± 0.02	7.25 ± 0.07#	7.30 ± 0.16#	7.07 ± 0.37#	7.44 ± 0.06	7.40 ± 0.03
	nomacopan (n=10)	7.44 ± 0.03	7.33 ± 0.08#	7.38 ± 0.16	7.41 ± 0.16*	7.44 ± 0.10	7.47 ± 0.09
Arterial pCO2 (mmHg)	Vehicle (n=10)	51.56 ± 4.49	79.16 ± 14.07#	51.71 ± 17.51	68.24 ± 24.35	54.17 ± 7.54	50.30 ± 8.90
	nomacopan (n=10)	51.06 ± 4.20	64.75 ± 10.90#*	46.82 ± 18.49#	50.00 ± 12.21	52.06 ± 6.27	40.64 ± 8.56#
Arterial HCO3 (mmHg)	Vehicle (n=10)	34.72 ± 2.00	34.57 ± 1.89	23.30 ± 5.55#	32.25 ± 7.26	36.27 ± 0.60	24.98 ± 8.65#
	nomacopan (n=10)	34.59 ± 1.32	34.07 ± 1.34	26.10 ± 2.28#	31.13 ± 5.09#	33.45 ± 2.46	28.88 ± 2.43#
Chloride (mmol/L)	Vehicle (n=10)	100.20 ± 1.32	100.70 ± 1.34	103.60 ± 2.59#	104.38 ± 3.07#	103.00 ± 4.69	103.00 ± 2.65
	nomacopan (n=10)	101.56 ± 1.67	99.78 ± 1.86	101.38 ± 3.54	102.67 ± 2.40	101.57 ± 2.51	105.71 ± 2.36#
iCa (mmol/L)	Vehicle (n=10)	1.37 ± 0.03	1.44 ± 0.05#	1.35 ± 0.04	1.25 ± 0.10#	1.34 ± 0.02	1.38 ± 0.07
	nomacopan (n=10)	1.35 ± 0.04	1.42 ± 0.06#	1.35 ± 0.05	1.29 ± 0.05#	1.34 ± 0.05	1.37 ± 0.05
Glucose (mg/dL)	Vehicle (n=10)	188.10 ± 20.95	269.00 ± 50.14#	375.56 ± 98.61#	131.71 ± 93.87	157.00 ± 6.08#	250.33 ± 41.49#
	nomacopan (n=10)	180.00 ± 27.87	263.80 ± 51.39#	366.88 ± 127.97#	170.44 ± 52.22	147.75 ± 19.00#	153.38 ± 24.33#*

Table S4. Blood chemistry changes in control and nomacopan treatment (NOM_30') groups. Legend: Data are expressed as mean ±SD; statistical analyses were performed by Mann-Whitney U test; *=p <0.05 vs. the vehicle (saline); #=p <0.05 vs. baseline (0 hour).