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Translational immunology in trauma - to provide new insights for improving outcomes

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Translational immunology in trauma - to provide new insights for improving outcomes

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Editorial: Translational immunology in trauma - To provide new insights for improving outcomes

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Editorial on the Research Topic
Translational immunology in trauma - To provide new insights for improving outcomes

As the importance of the post-traumatic and/or post-surgical immune response for outcomes is beyond any doubt, numerous pre-clinical experiments have focused on the underlying mechanisms. Thereby, both detrimental but also regenerative roles of immunological mediators have been described under these conditions. Both the complexity and unrevealed interactions of the immunologic response after trauma and surgery require interprofessional scientific exchange to share and connect new findings. Therefore, the articles included in this Research Topic reflect the current knowledge from latest basic science and clinical studies as well as reviews written by experts in their field.

Focusing on the central nervous system (CNS), Dobson et al. in their review outlined the molecular drivers of secondary injury, which include damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and other immune-modifying agents that activate the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic stress response. Despite the potential limitations of previous studies [i.e., heterogeneity of humans, poorly designed trials, inappropriate use of specific pathogen-free (SPF) animals, ignoring sex-specific differences, and the flawed practice of single-nodal targeting] in studying drugs that target specific inflammatory pathways, the authors conclude that especially CNS protection and control of cardiovascular function maintain mitochondrial energetics, thereby resolving inflammation and minimizing immune dysfunction (Dobson et al.). Also focusing on the CNS, Ning et al. performed a single-center, prospective, randomized, and double-blind study on scalp nerve

blockade (SNB) in pediatric patients undergoing craniotomy to investigate the effect on postoperative pain, intraoperative hemodynamic stability, and narcotic consumption under general anesthesia. The authors found adequate postoperative pain control and good intraoperative hemodynamic stability during noxious events in the intervention group compared to the control group (Ning et al.).

Focusing on immune dysfunction after trauma, Mizugaki et al. investigated the role of different neutrophil phenotypes in a murine burn-associated sepsis model. The group reported on higher levels of transforming growth factor-beta 1 and lower concentrations of cytokines in the burned compared to the sham group. Moreover, diverse neutrophil phenotypes (DC172a, CD68, CD11b) were observed to be differently expressed between the groups, suggesting that neutrophils are significantly involved in the immune imbalance following trauma (Mizugaki et al.). Wan et al. investigated the role and molecular mechanisms of Bruton's tyrosine kinase (BTK), a member of the Tec family in burn sepsis-induced intestinal injury. The authors found that LFM-A13 administration significantly inhibited p-BTK expression in the intestine. LFM-A13 also reduced the histopathological changes and cellular apoptosis in intestinal tissues, inhibited the release of pro- and anti-inflammatory cytokines (IL-4, IL-6, and TNF-alpha) in serum and intestinal tissues, and significantly inhibited the increase in intestinal MPO activity induced by burn-associated sepsis. The authors concluded that BTK activation is one important aspect of the signaling event that may mediate the release of pro- and anti-inflammatory cytokines, oxidative stress, and intestinal cell apoptosis and thus might contribute to burn sepsis-induced intestinal injury (Wan et al.).

The posttraumatic immune response after multiple trauma without an associated burn injury was investigated by Vollrath et al. The authors focused on the pathogen-specific reaction of phagocytes taken from a porcine polytrauma model at different time points, stimulated by different pathogens (i.e., *E. coli*, *S. aureus*, or *S. cerevisiae*). Throughout the observation phase of 72 h, the phagocytic activity and capacity of granulocytes and monocytes followed a significantly different pattern, depending on the pathogen strain (Vollrath et al.).

Xu et al. reports on Indoleamine 2,3-dioxygenase (IDO), its immune suppressive impact and potential in the emerging field of isolated liver injuries. As IDO is one of the initial rate-limiting enzymes of the kynurenine pathway (KP), causing immune suppression and the induction of T cell anergy, it is well-known to be associated with the imbalance of immune homeostasis and liver dysfunction (Xu et al.). In a comprehensive review, the authors give an overview of its function, impact, current research directions, and possible ways to use IDO in patients with hepatic damage. As liver dysfunction furthermore contributes negatively to the patients' coagulation system, Egea-Guerrero and Quintana-Diaz outline the role of prothrombin complex concentrate (PCC) in the severely injured patient and indicates careful application of PCC in the traumatized patient. Therefore, the authors point out that fibrinolysis must be controlled simultaneously to balance management of patients with hemorrhagic shock or trauma induced coagulopathy (Egea-Guerrero and Quintana-Diaz).

Cell function and characteristics are of highest importance to understand their involvement in the development of infectious complications (de Fraiture et al.). Analysis of neutrophil phenotypes and their function as complex biomarkers has become accessible for point-of-care decision making after trauma. Thus, potential neutrophil based cellular diagnostics may help to recognize patients at risk for infectious complications when presented in the trauma bay. These patients display increased numbers of neutrophil subsets, decreased responsiveness to fMLF and/or increased CD64 expression. According to these possibilities, de Fraiture et al. emphasizes measuring these biomarkers over time in patients at risk of infectious complications. This might help to guide an individual treatment strategy regarding the timing, extent of surgery and administration of (preventive) antibiotics (de Fraiture et al.). As complications significantly influence patient outcomes over the clinical course, Schindler et al. examined microbiological aspects of anti-infective treatment in trauma patients. Of 114 trauma patients, 45 suffered from post-traumatic infections during the first 10 days of hospitalization. Severely injured patients with concomitant traumatic brain injury (PT + TBI) showed the highest rate of post-traumatic infection. The leading entity of infection was pneumonia, followed by infections of the urinary tract. Although 67.5% of all trauma patients received single-shot antibiosis during initial care in the trauma bay, the development of secondary colonization was not relevantly positively correlated with single-shot antibiosis and prophylactically calculated antibiotic administration. Due to these findings, the authors concluded that one-time antibiotic and calculated prophylactic use of antibiotics, like cephalosporins, must be critically discussed in terms of their role in the development of post-traumatic infections and microbial selection (Schindler et al.).

In conclusion, this Research Topic presents the newest scientific insights on immunological and cellular reactions as well as tissue-related and circulatory responses after severe trauma or during the further clinical course of critically ill patients. This highly relevant data will deepen the understanding of the pathomechanistic influence of several *in vivo* and *in vitro* elaborated mediators and thus has the potential to improve the treatment of patients after a traumatic or surgical impact. We thank all the authors and the Publication Committee of *Frontiers in Medicine* for the privilege of publishing this Research Topic.

Author contributions

KH, IM, LL, and FH defined the topic of this Research Topic. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Alterations of Phagocytic Activity and Capacity in Granulocytes and Monocytes Depend on the Pathogen Strain in Porcine Polytrauma

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Background: Polytraumatized patients undergo a strong immunological stress upon insult. Phagocytes (granulocytes and monocytes) play a substantial role in immunological defense against bacteria, fungi and yeast, and in the clearance of cellular debris after tissue injury. We have reported a reduced monocytes phagocytic activity early after porcine polytrauma before. However, it is unknown if both phagocyte types undergo those functional alterations, and if there is a pathogen-specific phagocytic behavior. We characterized the phagocytic activity and capacity of granulocytes and monocytes after polytrauma.

Methods: Eight pigs (*Sus scrofa*) underwent polytrauma consisting of lung contusion, liver laceration, tibial fracture and hemorrhagic shock with fluid resuscitation and fracture fixation with external fixator. Intensive care treatment including mechanical ventilation for 72 h followed. Phagocytic activity and capacity were investigated using an *in vitro ex vivo* whole blood stimulation phagocytosis assays before trauma, after surgery, 24, 48, and 72 h after trauma. Blood samples were stimulated with Phorbol-12-myristate-13-acetate and incubated with FITC-labeled *E. coli*, *S. aureus* or *S. cerevisiae* for phagocytosis assessment by flow cytometry.

Results: Early polytrauma-induced significant increase of granulocytes and monocytes declined to baseline values within 24 h. Percentage of *E. coli*-phagocytizing granulocytes significantly decreased after polytrauma and during further intensive care treatment, while their capacity significantly increased. Interestingly, both granulocytic phagocytic activity and capacity of *S. aureus* significantly decreased after trauma, although a recovery was observed after 24 h and yet was followed by another decrease. The percentage of *S. cerevisiae*-phagocytizing granulocytes significantly increased after 24 h, while their impaired capacity after surgery and 72 h later was detected. Monocytic *E. coli*-phagocytizing percentage did not change, while their capacity increased after 24–72 h. After a significant decrease in *S. aureus*-phagocytizing monocytes after surgery,

a significant increase after 24 and 48 h was observed without capacity alterations. No significant changes in *S. cerevisiae*-phagocytizing monocytes occurred, but their capacity dropped 48 and 72 h.

Conclusion: Phagocytic activity and capacity of granulocytes and monocytes follow a different pattern and significantly change within 72 h after polytrauma. Both phagocytic activity and capacity show significantly different alterations depending on the pathogen strain, thus potentially indicating at certain and possibly more relevant infection causes after polytrauma.

Keywords: phagocytes, inflammation, infection, trauma, pathogen

INTRODUCTION

Trauma is responsible for around 5 million deaths per year worldwide with more than a quarter (29%) of these deaths following road traffic injuries (1). Polytraumatized patients surviving the initial phase after injury are at great risk to die from late-occurring complications like sepsis, lung failure or multi organ failure (MOF) (2, 3). Although clinical management of polytraumatized patients has significantly improved over the past decades leading to a reduced mortality, these advances are somewhat mitigated when the trauma patient succumbs to postinjury sepsis, which increases mortality from 7.6 to 23.1% (4–7). Polytraumatized patients are at risk of infections at least partly due to the disruption of immune system homeostasis (7). Thus, it is critical to investigate immunological changes after trauma to identify patients at risk for posttraumatic infections as well as possible therapeutic targets. Neutrophilic granulocytes are the first line of defense against rapidly dividing bacteria, fungi, and yeast and, thus, are instrumental in promoting resistance to infection (8). Elimination of pathogens by neutrophils can be achieved either by phagocytosis and subsequent killing using reactive oxygen species or antibacterial proteins (cathepsins, defensins, lactoferrin and lysozyme), or by degranulation of antibacterial proteins into the extracellular milieu and/or by NETosis (9, 10). Monocytes as another major type of circulating phagocytes eventually migrate into damaged or traumatized tissues where they accomplish their terminal differentiation into macrophages (11). Monocytes/macrophages contribute substantially to the adaptive immunity *via* antigen presentation to T cells and a general orchestration of the immune reactions (11).

The most frequent nosocomial infections of the lower respiratory tract (28%), the urinary tract (24%) or surgical wound infections (18%) after polytrauma are often caused by *S. aureus* and *E. coli* (12, 13). When the human body is confronted with invasive pathogens such as bacteria or fungi, these can be cleared from infection sites by professional phagocytes like monocytes and granulocytes (14). Phagocytosis thereby is an important contributor to the first line of defense against infections (14). With regard to development of infections during the clinical course after trauma divergent results on phagocytic activity of circulating neutrophils and monocytes have been reported (15–17). Spittler et al. compared the phagocytic capacity of monocytes from patients with sepsis with low or high IL-6 serum concentrations and observed significantly increased phagocytic properties as well as worse outcomes in patients with high IL-6 levels (17). Reduced phagocytic activity of granulocytes and monocytes has been reported in septic patients after trauma or surgery and reduced phagocytic activity of neutrophils in the first 24 h of sepsis has been a negative predictor for survival (15, 16). In our study group we investigated the phagocytic activity of granulocytes and monocytes after severe trauma and observed early decreased activity of granulocytes followed by significant increase from day 2 till the end of the study period (18). Phagocytic activity of monocytes returned to values comparable to healthy volunteers after 2 days (18). Furthermore, we observed decreased phagocytic activity and capability of monocytes for *S. aureus* early after trauma in the porcine polytrauma model (19). The porcine polytrauma model offers great opportunity to study posttraumatic immunological changes under standardized conditions. Thus, we investigated the phagocytic activity and capacity of granulocytes and monocytes for three different pathogen strains in order to further characterize immunological changes after polytrauma, and to potentially get closer to answering the urgent question what mechanisms put patients at risk for developing infectious complications after polytrauma.

MATERIALS AND METHODS

Ethics

All experiments were performed in compliance with the federal German law with regards to the protection of animals, institutional guidelines and the criteria in "Guide for the Care and Use of Laboratory Animals" (20). During the whole study animals were consistently handled in accordance with the ARRIVE guidelines (21) and experiments were authorized by the responsible government authority ("Landesamt für Natur-, Umwelt- und Verbraucherschutz": LANUV-NRW, Germany, AZ: 81.02.04.2018.A113). All Animal experiments were performed at the Institute of Laboratory Animal Science & Experimental Surgery, RWTH Aachen University, Germany.

Animals

A total of eight male German landrace pigs (*Sus scrofa*, 3-month-old, 30 ± 5 kg) from a disease-free barrier breeding facility were included in this study. Prior to experimentation the animals were fasted over night with free access to water. All animals were

housed in air-conditioned rooms, allowed to acclimatize to their surroundings for at least 7 days before surgery and underwent examination by a veterinarian before experimentation. The large animal polytrauma model has been described before (22).

Experimental Model

Eight animals underwent polytrauma (PT) with a standardized tibia fracture, liver laceration, unilateral blunt chest trauma, hemorrhagic shock (40 ± 5 mm Hg for 90 min), ensuing resuscitation and surgical fracture fixation via external fixation. Before experimentation all animals were pre-medicated with an intramuscular application of azaperone (Stressni™, Janssen, Germany) in a dose of 4 mg/kg. Prophylactic antibiotic treatment with cefuroxime 1.5 g i.v. (Fresenius Kabi, Bad Homburg, Germany) was administered before surgery and every 24 h for the whole experiment. Anesthesia was induced with an intravenous injection of propofol (3 mg/kg, Propofol Claris 2% MCT, Pharmore GmbH, Ibbenbüren, Germany) followed by an orotracheal intubation (7.5 ch tube, Hi-Lo Lanz™). During the whole study period of 72 h general anesthesia was maintained with intravenous injection of propofol, fentanyl (Rotexmedica, Trittau, Germany) and midazolam (Rotexmedica, Trittau, Germany) at a sufficient level in order to prevent any pain or awareness. The animals were ventilated with volume control mode (Draeger, Evita 4, Lübeck, Germany) at a tidal volume setting of 6–8 mL/kg, positive end expiratory pressure (PEEP) of 8 mm Hg (plateau pressure < 28 mm Hg) and a pCO_2 of 35–45 mm Hg. Central venous catheter (4-Lumen Catheter, 8,5 Fr, ArrowCatheter, Teleflex Medical, Germany) was aseptically inserted into the right external jugular vein for anesthesia and fluid administration and continuous monitoring of the central venous pressure. In order to induce hemorrhage a two-lumen hemodialysis catheter (Arrow International, Teleflex Medical, Germany) was aseptically inserted into the left femoral vein. An arterial pulse contour cardiac output (PiCCO, Pulsion Medical Systems, Germany) catheter was aseptically inserted into the right femoral artery to monitor blood pressure and for blood gas analysis. A urinary catheter was inserted into the bladder (12.0 Fr, Cystofix, Braun, Melsungen, Germany). The baseline measurements were acquired after an equilibration period of 1 h. The polytrauma was induced as previously described with slight modifications (22). Before trauma induction the fraction of inspired oxygen (FiO_2) was set to 21% as this depicts the conditions during trauma more accurately. Fluid administration was reduced to 10 mL/h. Warming with forced-air warming systems to maintain normothermia was stopped and animals were allowed to descend into hypothermic state after hemorrhagic shock as this mimics the preclinical scenario. After placing the animal on the left side, tibial fracture was induced using a bolt gun (Blitz-Kerner, turbocut JOBB GmbH, Germany; ammunition: 9 × 17, RUAG Ammotec GmbH, Fürth, Germany). After that animals were placed on the right side and blunt thoracic trauma was induced with a bolt shot to the left dorsal lower thorax. Then a midline-laparotomy and uncontrolled bleeding for 30 s after crosswise incision of the caudal lobe of the liver (4.5 × 4.5 cm) was induced followed by packing with five sterile gauze-compresses (10 × 10 cm). Hemorrhagic shock was induced via exsanguination via the femoral venous catheter until a mean arterial blood pressure (MAP) of 40 ± 5 mm Hg was reached and maintained for 90 min. Resuscitation was managed by adjusting the FiO_2 to baseline again and re-infusing the withdrawn blood and additional crystalloid fluids (4 mL/kg body weight/h). Rewarming was performed using forced-air warming system until normothermia was reached (38.7–39.8°C). After induction of trauma and resuscitation clinical treatment of the tibia fracture was performed using external fixation according to established trauma guidelines. The intensive care treatment as well as the management of complications followed the standardized clinical protocols according to the latest recommendations of the European Resuscitation Council and the Advanced Trauma Life Support (ATLS®) (23, 24). After 72 h animals were euthanized using potassium chloride. The experimental design is shown in **Figure 1**.

Blood Sampling and Processing

Blood samples for *ex vivo in vitro* phagocytosis assay were obtained immediately after implementation of the central venous catheter (before trauma), after surgery and after 24, 48, and 72 h in heparin tubes (S-Monovette® Lithium-Heparin, Sarstedt, Nümbrecht, Germany), kept at room temperature and immediately processed for further analysis via FACS.

Ex vivo in vitro Whole Blood Stimulation Phagocytosis Assay

Blood samples (200 μL) were transferred to polystyrene FACS tubes (BD Biosciences, Franklin Lakes, USA) and 5 μL *phorbol 12-myristate 13-acetate* (PMA, Merck, Darmstadt, Germany) with a final PMA concentration of 0.1 μg/mL were added to the samples followed by incubation at 37°C with 5% CO_2 and protected from light for 30 min. Then, 2 mL FACS buffer (2,5 g bovine serum albumine in 500 mL phosphate-buffered saline) were added and samples were centrifuged at 1,500 RPM for 7 min at room temperature. After removal of the supernatant cells were resuspended in 200 μL FACS buffer and 40 μL were transferred to new polystyrene FACS tubes. These samples then were incubated with *Escherichia coli* (K-12 strain) BioParticles™ (Cat. nr.: #E-2861, Thermo Fisher, Waltham, USA), *Staphylococcus aureus* (Wood strain without protein A) BioParticles™ (Cat. nr.: #S-2851, Thermo Fisher), *Zymosan A S. cerevisiae* BioParticles™ (Cat. nr.: #Z-2841, Thermo Fisher) or nothing serving as controls at 37°C with 5% CO_2 for 30 min and protected from light according to the manufacturer's instructions. Approximately 10 bioparticles per leukocyte were added and all were labeled with fluorescein isothiocyanate (FITC). Subsequently 2 mL FACS buffer were added and samples were centrifuged at 1,500 RPM for 5 min at room temperature followed by incubation in 0.5 mL of BD FACS Lysing Solution at room temperature and protected from light for 10 min. Then, 2 mL of FACS buffer was added and samples were centrifuged at 1,500 RPM for 7 min at room temperature. This step was repeated one more time and cells were diluted in 90 μL FACS buffer and stored on ice until measurement. From each sample a minimum of 50.000 cells was measured, which were subsequently analyzed. Monocyte and granulocyte populations were defined by gating

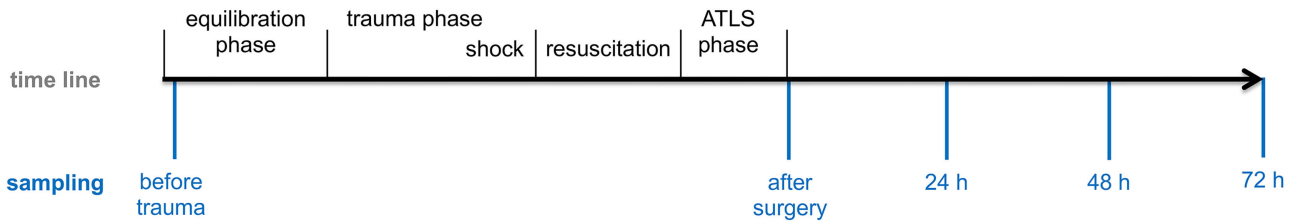

FIGURE 1 | Experimental design. Pigs underwent polytrauma consisting of lung contusion, liver laceration, tibial fracture and hemorrhagic shock followed by fluid resuscitation and fracture fixation with external fixator. Blood sampling was performed at the beginning after implementation of the central venous catheter (before trauma), after ATLS phase (after surgery) and after 24, 48 and 72 h.

the corresponding forward and side scatter scan and the duplets were excluded by plotting the height against the area for forward scatter. The percentage and the mean fluorescent units of FITC-labeled bioparticles engulfed by monocytes or granulocytes were assessed. The measurement was performed by flow cytometric analyses using a BD FACS Canto 2™ and FACS DIVA™ software (BD Biosciences, Franklin Lakes, USA).

Statistical Analyses

All analyses were performed using GraphPad Prism 6 (Graphpad Software Inc., San Diego, USA). Data are presented as mean ± standard error of the mean. Based on the D'Agostino-Pearson normality test differences between the groups were determined by the non-parametric Kruskal-Wallis test followed by Dunn's *post hoc* test for the correction of multiple comparison. A p-value <0.05 was considered to be statistically significant.

RESULTS

Granulocytes and Monocytes in Peripheral Blood

The total proportion of granulocytes and monocytes out of viable leukocytes in peripheral blood was measured before trauma, after trauma and after 24, 48, and 72 h. Both granulocytes and monocytes showed a significant polytrauma-induced increase after surgery which declined to initial values again after 24 h (**Figure 2**).

Phagocytizing Granulocytes and Their Phagocytic Capacity After Polytrauma

E. coli-phagocytizing granulocytes significantly decreased after polytrauma and during further intensive care treatment. The capacity of granulocytes to phagocytize *E. coli* significantly increased in parallel during the intensive care treatment (**Figures 3A**, **4A**). Interestingly granulocytes showed a different phagocytosis behavior for *S. aureus*. Both phagocytic activity and capacity of *S. aureus* significantly decreased after trauma. After 24 h a complete recovery was observed which was followed by another significant decrease, in both the number of phagocytizing granulocytes as well as their phagocytic capacity, after 48 and 72 h (**Figures 3A**, **4B**). The percentage of *S. cerevisiae*-phagocytizing granulocytes significantly increased after 24 h and for the rest of the observation period. In contrast, a significant decrease of the *S. cerevisiae* phagocytic capacity was observed after surgery and 72 h (**Figures 3A**, **4C**).

Phagocytizing Monocytes and Their Phagocytic Capacity After Polytrauma

E. coli-phagocytizing monocytes did not change during the observation period while their capacity to phagocytize *E. coli* significantly decreases after surgery followed by a distinct and significant increase after 24 and 48 h (**Figures 3B**, **5A**). The number of *S. aureus*-phagocytizing monocytes shows a significant decrease after surgery followed by a significant increase which was higher after 24 and 48 h compared to the initial values. The capacity of monocytes to phagocytize *S. aureus* did not change during the first three days after polytrauma (**Figures 3B**, **5B**). The percentage of *S. cerevisiae*-phagocytizing monocytes did not show any significant changes during the study period while the capacity of monocytes to phagocytize *S. cerevisiae* significantly dropped after 48 and 72 h (**Figures 3B**, **5C**).

DISCUSSION

Polytraumatized patients who survive the initial phase after injury remain at high risk to die from late-occurring infectious complications like sepsis, organ or multiple organ failure (MOF) (2, 3, 25, 26). Regardless of an infection or septic complication, trauma itself leads to a leukocytosis (27). We observed early leukocytosis in our porcine trauma model showing a significant peak of the number of granulocytes and monocytes immediately after surgery (**Figures 2A,B**). After 24 h in both cell types the levels turned to normal again possibly due to emigration of the leukocytes to the damaged tissue. Disturbed phagocytosis of neutrophils and/or monocytes has been linked to development of posttraumatic or postoperative sepsis (16). Therefore, we investigated phagocytic activity and capacity of granulocytes and monocytes in a standardized porcine polytrauma model during a prolonged observational period of 72 h after trauma. Interestingly, the phagocytic activity and capacity showed significantly different alterations depending on the pathogen strain (*E. coli, S. aureus* or *S. cerevisiae*).

S. aureus is one of the most common causative pathogens of hospital-acquired pneumonia (HAP)/ventilator-acquired pneumonia (VAP) and wound infections (12, 28). In our

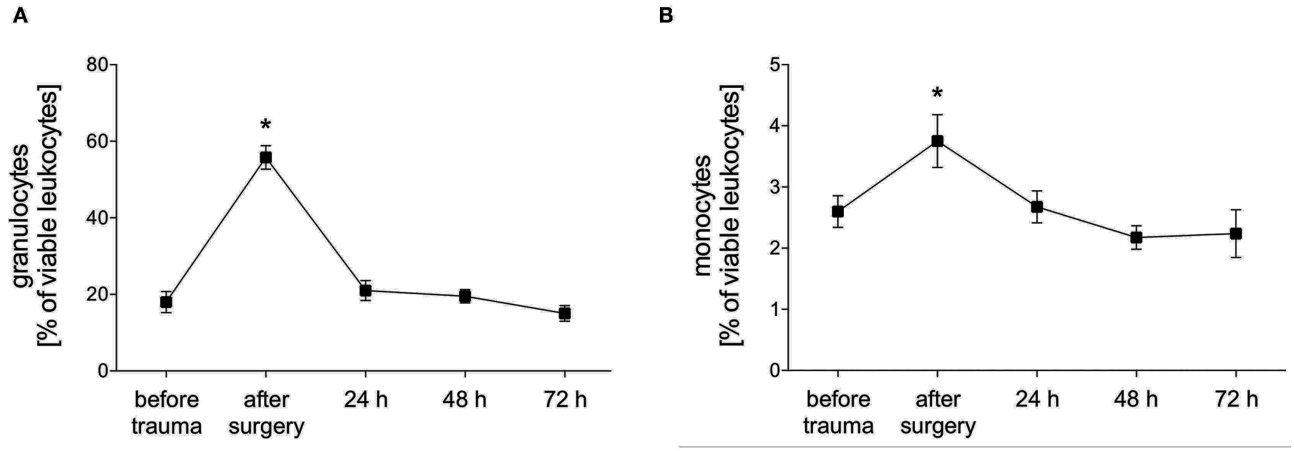

FIGURE 2 | Percentage of granulocytes and monocytes of viable leukocytes. The percentage of granulocytes **(A)** and monocytes **(B)** of viable leukocytes was measured with FACS-analysis before trauma, after trauma and after 24, 48 and 72 h. Data are given as mean ± standard error of the mean, $^*p < 0.05$ vs. baseline, $n = 8$.

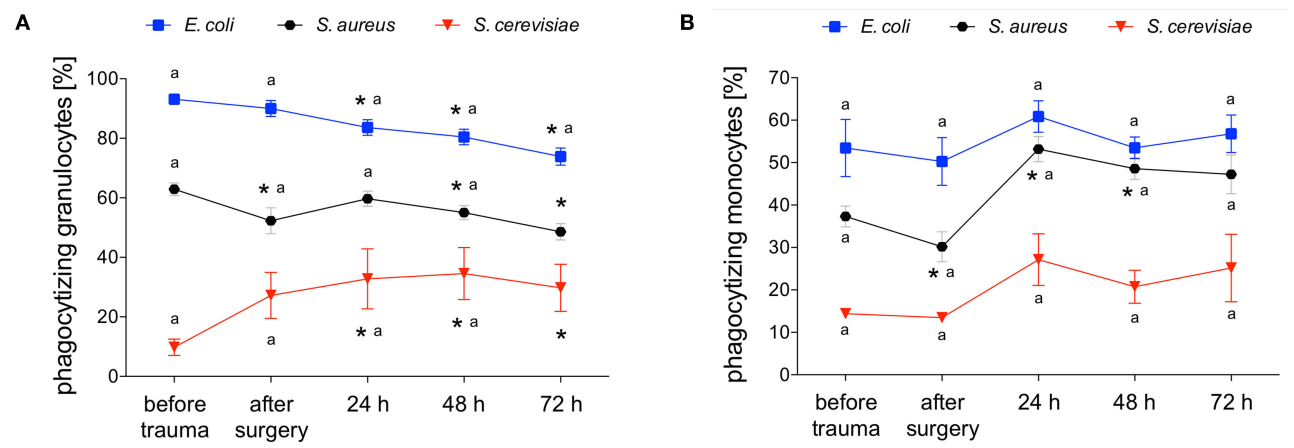

FIGURE 3 | Percentage of *E. coli*-, *S. aureus*- or *S. cerevisiae*-phagocytizing granulocytes and monocytes. The percentage of *E. coli*-, *S. aureus*- or *S. cerevisiae*-phagocytizing granulocytes **(A)** and monocytes **(B)** was measured with FACS-analysis before trauma, after trauma and after 24, 48 and 72 h. Data are given as mean ± standard error of the mean, $^*p < 0.05$ vs. baseline, $n = 8$.

study, we observed a distinct and significant decrease in the total number of *S. aureus*-phagocytizing granulocytes after 48 and 72 h while their capacity to phagocytize *S. aureus* did not increase. Since granulocytes are not able to clear the bacterial load it remains to be clarified in further studies if a decreased number of *S. aureus*-phagocytizing granulocytes post-trauma may be a risk factor for developing pneumonia or wound infections. In contrast to our results it was shown that the percentage of *S. aureus*-phagocytizing granulocytes in polytraumatized patients increased significantly from day two till the end of the study period (10 days) (18). After severe injury granulocytes are replaced through (immature) bone marrow granulocyte which are rapidly released to the blood stream (29). This process is known as 'emergency myelopoiesis' (29). A possible reason for the initially decreased number of *S. aureus*-phagocytizing granulocytes observed in our study therefore might be their maturation state as immature granulocytes may not be able to perform phagocytosis. However, due to a study period of 72 h we can only speculate whether the number of *S. aureus*-phagocytizing granulocytes will return to "normal" or even higher numbers after three days or later will further decrease. Possibly we would also observe the same increase after three days that Sturm et al. already observed after two days. In line with our results phagocytic activity of granulocytes was impaired three days after traumatic brain injury (TBI) in mice (8). The authors also investigated the long term effect after TBI and showed impaired phagocytic activity of granulocytes still after 60 days (8). However, despite differences in immune response among different species, the posttraumatic response after brain injury and non-brain injury are not completely comparable. To date the changes in phagocytic behavior of granulocytes and monocytes after

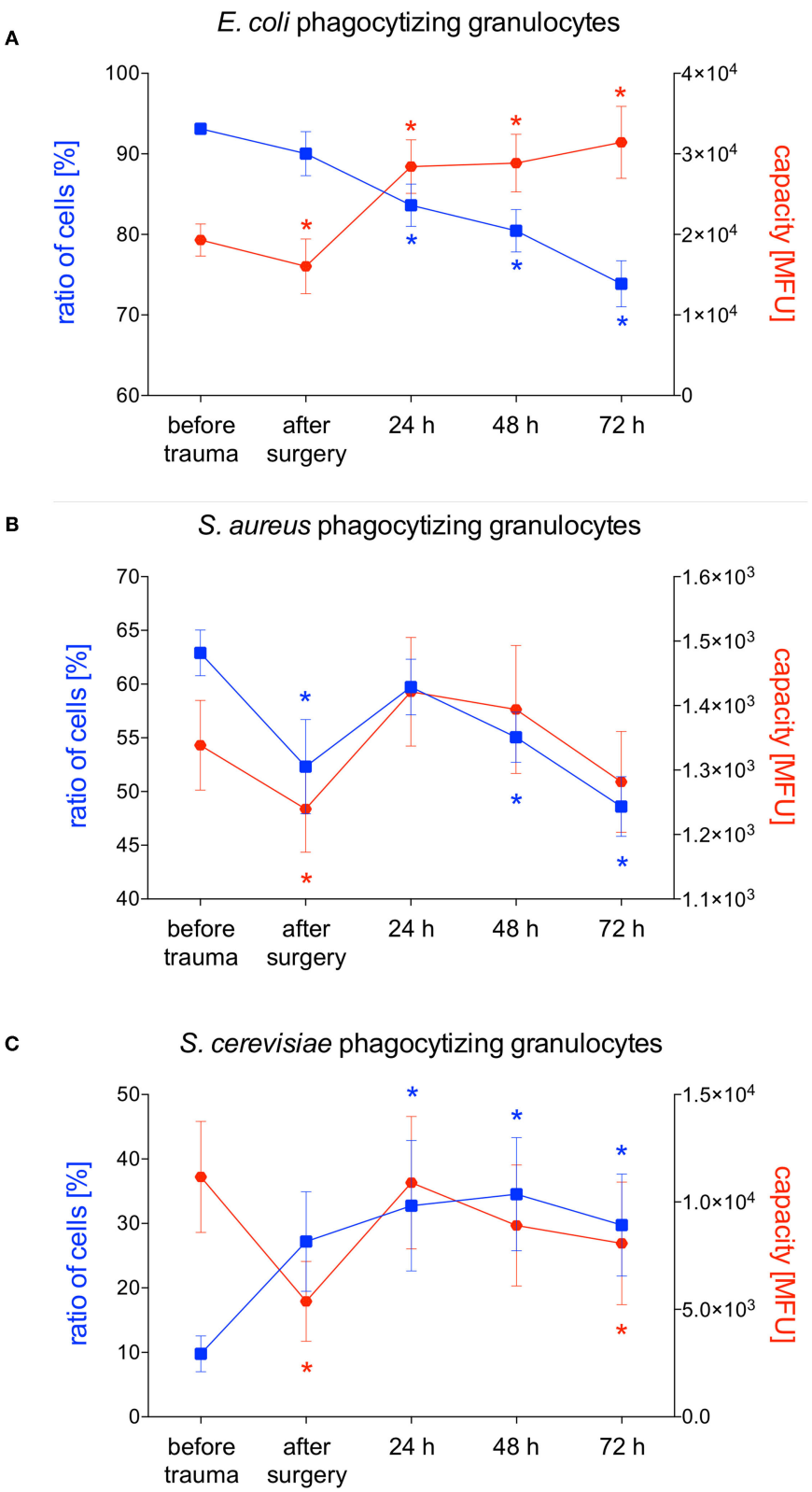

FIGURE 4 | Percentage of phagocytizing granulocytes and phagocytic capacity for *E. coli*, *S. aureus* or *S. cerevisiae*. The percentage of phagocytizing granulocytes and their capacity to phagocytize *E. coli* **(A)**, *S. aureus* **(B)** or *S. cerevisiae* **(C)** was measured with FACS-analysis before trauma, after trauma and after 24, 48 and 72 h. Data are given as mean ± standard error of the mean, $^*p < 0.05$ vs. baseline, $n = 8$.

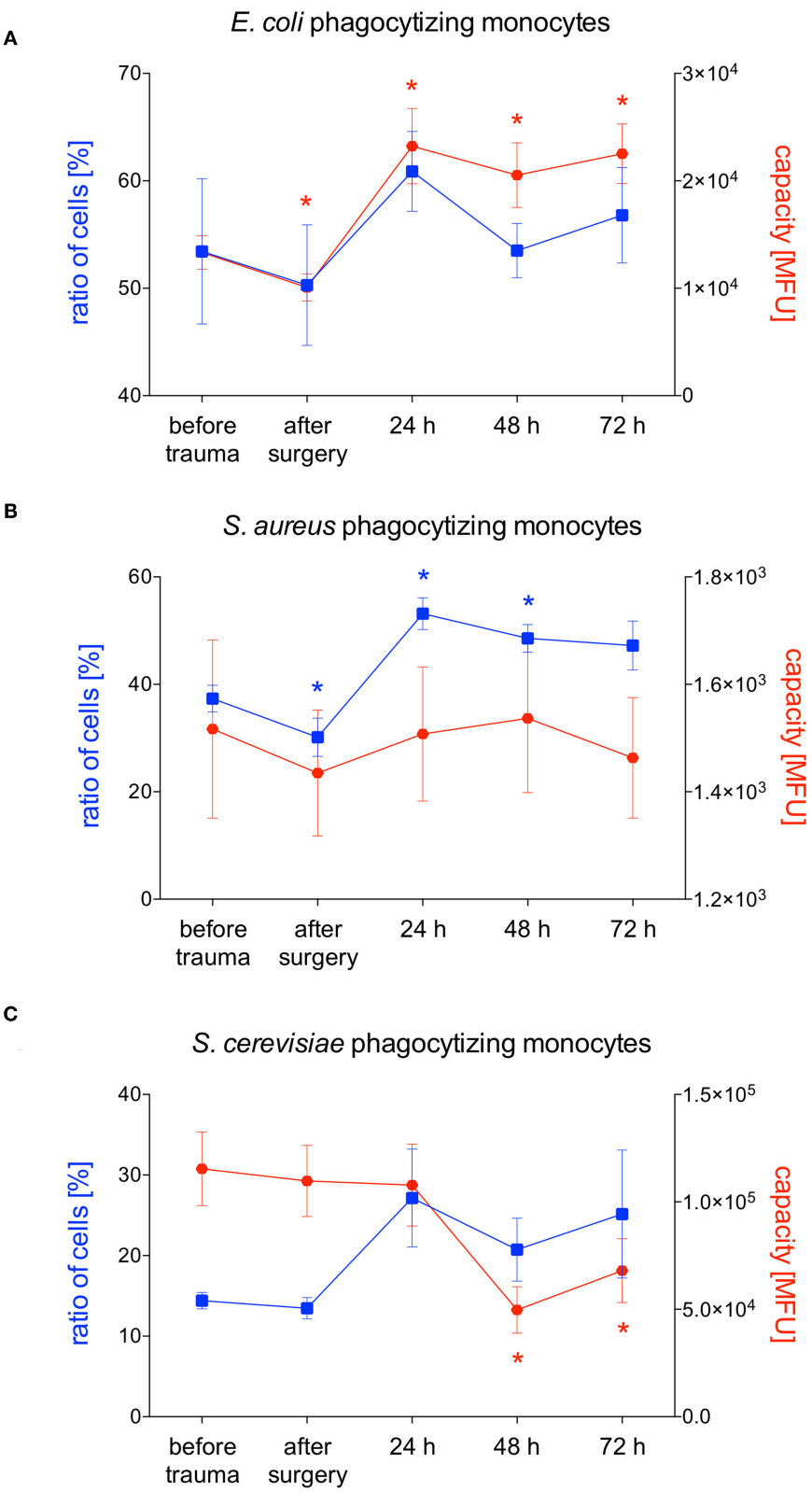

FIGURE 5 | Percentage of phagocytizing monocytes and phagocytic capacity for *E. coli*, *S. aureus* or *S. cerevisiae*. The percentage of phagocytizing monocytes and their capacity to phagocytize *E. coli* **(A)**, *S. aureus* **(B)** *S. cerevisiae* **(C)** was measured with FACS-analysis before trauma, after trauma and after 24, 48 and 72 h. Data are given as mean ± standard error of the mean, $^*p < 0.05$ vs. baseline, $n = 8$.

polytrauma are not completely characterized, otherwise it would be tempting to speculate about therapeutic options. Decreased phagocytic activity might be restored by medications activating phagocytosis (30–32). On the other hand, overwhelming phagocytosis might be reduced by inhibiting medications (33, 34). However, septic complications after trauma do not necessarily have to be caused by reduced or overstimulated phagocytosis itself. Phagosomal acidification, another critical part of neutrophil function, has been shown to be altered in polytraumatized patients developing septic complications (35). Nevertheless, it must be mentioned that these investigations have been made in a relatively small patient cohort of only 15 patients (35).

In our study, we observed early decreased phagocytic activity of monocytes for *S. aureus* while phagocytic activity for *E. coli* and *S. cerevisiae* were not altered. Schimunek et al. investigated the number of phagocytes as well as their capacity to phagocytize *S. aureus* in the porcine polytrauma model. In line with our results, an early decreased phagocytic activity of monocytes was observed (19). Furthermore, the authors showed reduced phagocytic capability for *S. aureus* after surgery (19). Both the phagocytic activity and capability recovered to baseline after 24 h (19). In the underlying study, we also observed a significant decrease of monocytic phagocytic activity after surgery but in contrast to the study by Schimunek et al. the phagocytic activity increased to higher values compared to the baseline after 24 and 48 h. In line with this a significantly increased phagocytic activity of monocytes in mice 3 days after TBI was reported (8). In polytraumatized patients the number of *S. aureus* phagocytizing monocytes was also diminished early after trauma and recovered to baseline after 2 days (18). Obviously, the majority of studies report early decreased monocytic phagocytic activity followed by return to normal or even increased activity. Possibly after trauma monocytes leave blood stream by migrating into the traumatized tissue and are replaced by immature cells from the bone marrow that need time to bring phagocytic activity to "normal" or even higher state again. The capacity to phagocytize *S. aureus* did not significantly change in our study but we observed an overall higher rate in the phagocytic capacity of *S. aureus* compared to Schimunek et al. (19). Possible differences may have been observed due to the use of heparin blood samples instead of EDTA blood in the underlying study as compared to Schimunek et al. (36). In contrast, Seshadri et al. incubated monocytes with (FITC)-labeled *S. aureus* and opsonizing reagent to evaluate phagocytosis and did not see any changes in monocyte phagocytosis after injury (7). Pérez-Bárcena et al. compared TLR2 and TLR4 functionality in trauma patients receiving nutrition with or without glutamine supplement as use of glutamine as a dietary supplement is associated with a reduced risk of infection and observed no changes in phagocytic capability of monocytes between the two groups (37).

Besides pneumonia, infections of the urinary tract are a common complication during clinical course after trauma (12). These infections are often caused by *E. coli* which is why we also investigated the phagocytic activity and capacity of granulocytes and monocytes for *E. coli* in the underlying porcine trauma model. Interestingly we observed a different pattern than for phagocytosis of *S. aureus*. The percentage of *E. coli*-phagocytizing granulocytes significantly decreased early after trauma with a continuous decrease till the end of the study period. Probably as a compensatory mechanism the capacity of granulocytes significantly increased after 24 h, thus, among several others, maybe giving a gentle hint why infections with *E. coli* are less frequent than infections with *S. aureus*. In line with our results Liao et al. observed significantly decreased percentage of *E. coli*-phagocytizing granulocytes at 24, 48 and 72 h in trauma patients compared to healthy controls (38). Furthermore, in TBI patients *E. coli*-phagocytizing activity of granulocytes was decreased during the whole study period of 2 weeks (38). No difference in the percentage of neutrophils able to do phagocytosis but a significantly lower ability of neutrophils to phagocytose fluorescent *E. coli* were observed in patients with spinal cord injury (39). The above mentioned decline in phagocytosis has been suggested to represent a compensatory mechanism, where reduced phagocytosis offsets the deleterious effects that the increase in ROS generation, that has been described especially in patients with TBI and SCI but also with blunt or penetrating trauma, would have on bystander tissue (38, 40). Nevertheless, given that granulocytes are the first-line of defense against invading microbes, a reduced phagocytosis may also be detrimental to the host and therefore at least in part explains the high incidence of nosocomial infections reported amongst patients following neurological trauma (40, 41). In line with these observations, we also observed reduced phagocytic activity of granulocytes for *S. aureus* and *E. coli*, two pathogens frequently seen in nosocomial infections. Furthermore, a reduced phagocytosis of neutrophils in the first 24 h of sepsis and a reduced phagocytic activity of granulocytes and monocytes in septic patients after trauma or surgery have been reported (15, 16). On the other hand, Spittler et al. compared the phagocytic capacity of monocytes from ten patients with sepsis with low IL-6 serum concentrations and eight patients with sepsis with high IL-6 plasma concentrations and observed significantly increased phagocytic properties as well as worse outcomes in patients with high IL-6 levels (17).

Invasive fungal infections are rare but serious complications of traumatic injury with an increasing incidence of approximately 0.43–1.7 cases per million persons (42). In line with our reported results for phagocytosis of *S. cerevisiae* granulocytes in the group of multiple traumatized patients showed significantly higher yeast-phagocytizing activity on the first and third day after injury compared to the control group (43). This higher phagocytic activity of granulocytes might, among others, be a reason for the lower frequency of invasive fungal infections.

In this study, we observed significant changes in phagocytic activity and capacity of granulocytes and monocytes after polytrauma. It still has to be further elucidated whether increased phagocytic activity or capacity is a "beneficial" inflammatory state and if their reduced phagocytic activity, as seen for *S. aureus* and *E. coli*, puts polytraumatized patients at risk to developing septic complications. On the other hand, reduced

phagocytic activity might also be a "beneficial" mechanism to reduce overwhelming inflammatory response which would lead to further tissue damage. In future studies it remains important to investigate the phagocytic behavior of leukocytes which have emigrated from blood to tissue since it has been shown that the surrounding milieu has an impact on the phagocytic behavior (43). This study has several limitations of which the most important one is the limited sample size. Moreover, it remains to be elaborated, if the above described changes in phagocytic activity and capacity will influence the outcome after trauma beyond the observational period of 72 h, as our findings cannot be linked to defined outcomes like infections, sepsis, length of stay on ICU or requirement for ventilation. The interspecies variability in innate immune response has to be taken into consideration when results of animal experiments are transferred to human situation (44). Furthermore, no data of samples without PMA-stimulation could be provided.

Taken together, our data indicate that phagocytic activity and capacity of granulocytes and monocytes follow a different pattern and significantly change within 72 h after polytrauma. Furthermore, the phagocytic activity and capacity show significantly different alterations depending on the pathogen strain.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Experiments were authorized by the responsible government authority (Landesamt für Natur-, Umwelt- und Verbraucherschutz: LANUV-NRW, Germany). The approval number is: AZ: 81.02.04.2018.A113.

AUTHOR CONTRIBUTIONS

BR: conceptualization and supervision. JV, FK, FB, JG, EB, AJ, ID, and BR: methodology. JV: validation, data curation, and writing-original draft preparation. BR and JV: formal analysis and visualization. JV, FB, JG, and EB: investigation. JV, FK, FB, JG, EB, AJ, ID, FH, IM, and BR: writing-review and editing. JV, FB, and BR: project administration. BR and FH: funding acquisition. All authors: contributed to the article and approved the submitted version.

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REFERENCES

1. World Health Organization. *Global Health Estimates 2016: Disease Burden by Cause, Age, Sex, by Country and by Region, 2000-2016*. Geneva: World Health Organization (2018).
2. Nast-Kolb D, Aufmkolk M, Rucholtz S, Obertacke U, Waydhas C. Multiple organ failure still a major cause of morbidity but not mortality in blunt multiple trauma. *J Trauma.* (2001) 51:835–41. doi: 10.1097/00005373-200111000-00003
3. Relja B, Land WG. Damage-associated molecular patterns in trauma. *Eur J Trauma Emerg Surg.* (2019) 324:1–25. doi: 10.1007/s00068-019-01235-w
4. Dutton RP, Stansbury LG, Leone S, Kramer E, Hess JR, Scalea TM. Trauma mortality in mature trauma systems: are we doing better? An analysis of trauma mortality patterns, 1997-2008. *J Trauma.* (2010) 69:620–6. doi: 10.1097/TA.0b013e3181bbfe2a
5. Gerber LM, Chiu Y-L, Carney N, Härtl R, Ghajar J. Marked reduction in mortality in patients with severe traumatic brain injury. *J Neurosurg.* (2013) 119:1583–90. doi: 10.3171/2013.8.JNS13276
6. Osborn TM, Tracy JK, Dunne JR, Pasquale M, Napolitano LM. Epidemiology of sepsis in patients with traumatic injury. *Crit Care Med.* (2004) 32:2234–40. doi: 10.1097/01.CCM.0000145586.23276.0F
7. Seshadri A, Brat GA, Yorkgitis BK, Keegan J, Dolan J, Salim A, et al. Phenotyping the immune response to trauma. *Crit Care Med.* (2017) 45:1523–30. doi: 10.1097/CCM.0000000000002577
8. Ritzel RM, Doran SJ, Barrett JP, Henry RJ, Ma EL, Faden AI, et al. Chronic alterations in systemic immune function after traumatic brain injury. *J Neurotrauma.* (2018) 35:1419–36. doi: 10.1089/neu.2017.5399
9. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol.* (2013) 13:159–75. doi: 10.1038/nri3399
10. Winterbourn CC, Kettle AJ, Hampton MB. Reactive oxygen species and neutrophil function. *Annu Rev Biochem.* (2016) 85:765–92. doi: 10.1146/annurev-biochem-060815-014442
11. Lambert C, Preijers FWMB, Yanikkaya Demirel G, Sack U. Monocytes and macrophages in flow: an ESCCA initiative on advanced analyses of monocyte lineage using flow cytometry. *Cytometry B Clin Cytom.* (2017) 92:180–8. doi: 10.1002/cyto.b.21280
12. Kerwat K, Graf J, Wulf H. Krankenhaushygiene – nosokomiale infektionen. *Anästhesiol Intensivmed Notfallmed Schmerzther.* (2010) 45:30–1. doi: 10.1055/s-0029-1243375
13. Papia G, McLellan BA, El-Helou P, Louie M, Rachlis A, Szalai JP, et al. Infection in hospitalized trauma patients: incidence, risk factors, and complications. *J Trauma.* (1999) 47:923–7. doi: 10.1097/00005373-199911000-00018
14. Flannagan RS, Jaumouillé V, Grinstein S. The cell biology of phagocytosis. *Annu Rev Pathol.* (2012) 7:61–98. doi: 10.1146/annurev-pathol-011811-132445
15. Danikas DD, Karakantza M, Theodorou GL, Sakellaropoulos GC, Gogos CA. Prognostic value of phagocytic activity of neutrophils and monocytes in sepsis. Correlation to CD64 and CD14 antigen expression. *Clin. Exp. Immunol.* (2008) 154:87–97. doi: 10.1111/j.1365-2249.2008.03737.x
16. Hirsh M, Mahamid E, Bashenko Y, Hirsh I, Krausz MM. Overexpression of the high-affinity Fcgamma receptor (CD64) is associated with leukocyte dysfunction in sepsis. *Shock.* (2001) 16:102–8. doi: 10.1097/00024382-200116020-00003
17. Spittler A, Razenberger M, Kupper H, Kaul M, Hackl W, Boltz-Nitulescu G, et al. Relationship between interleukin-6 plasma concentration in patients with sepsis, monocyte phenotype, monocyte phagocytic properties, and cytokine production. *Clin Infect Dis.* (2000) 31:1338–42. doi: 10.1086/317499

18. Sturm R, Heftrig D, Mörs K, Wagner N, Kontradowitz K, Jurida K, et al. Phagocytizing activity of PMN from severe trauma patients in different post-traumatic phases during the 10-days post-injury course. *Immunobiology.* (2017) 222:301–7. doi: 10.1016/j.imbio.2016.09.010
19. Schimunek L, Serve R, Teuben MPJ, Störmann P, Auner B, Woschek M, et al. Early decreased TLR2 expression on monocytes is associated with their reduced phagocytic activity and impaired maturation in a porcine polytrauma model. *PLoS ONE.* (2017) 12:e0187404. doi: 10.1371/journal.pone.0187404
20. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the Care and Use of Laboratory Animals.* Washington, DC: National Academies Press (2011).
21. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *Osteoarthr Cartil.* (2012) 20:256–60. doi: 10.1016/j.joca.2012.02.010
22. Horst K, Simon TP, Pfeifer R, Teuben M, Almahmoud K, Zhi Q, et al. Characterization of blunt chest trauma in a long-term porcine model of severe multiple trauma. *Sci Rep.* (2016) 6:39659. doi: 10.1038/srep39659
23. ATLS Subcommittee, American College of Surgeons' Committee on Trauma, International ATLS working group. Advanced trauma life support (ATLS®): the ninth edition. *J Trauma Acute Care Surg.* (2013) 74:1363–6. doi: 10.1097/01586154-201305000-00026
24. Nikolaou NI, Welsford M, Beygui F, Bossaert L, Ghaemmaghami C, Nonogi H, et al. Part 5: Acute coronary syndromes: 2015 International consensus on cardiopulmonary resuscitation and emergency cardiovascular care science with treatment recommendations. *Resuscitation.* (2015) 95:e121–46. doi: 10.1016/j.resuscitation.2015.07.043
25. Pfeifer R, Tarkin IS, Rocos B, Pape H-C. Patterns of mortality and causes of death in polytrauma patients–has anything changed? *Injury.* (2009) 40:907–11. doi: 10.1016/j.injury.2009.05.006
26. Relja B, Mörs K, Marzi I. Danger signals in trauma. *Eur J Trauma Emerg Surg.* (2018) 44:301–16. doi: 10.1007/s00068-018-0962-3
27. Riley LK, Rupert J. Evaluation of patients with leukocytosis. *Am Fam Physician.* (2015) 92:1004–11.
28. Barbier F, Andremont A, Wolff M, Bouadma L. Hospital-acquired pneumonia and ventilator-associated pneumonia. *Curr Opin Pulm Med.* (2013) 19:216–28. doi: 10.1097/MCP.0b013e32835f27be
29. Nacionales DC, Szpila B, Ungaro R, Lopez MC, Zhang J, Gentile LF, et al. A detailed characterization of the dysfunctional immunity and abnormal myelopoiesis induced by severe shock and trauma in the aged. *J Immunol.* (2015) 195:2396–407. doi: 10.4049/jimmunol.1500984
30. Alsharif KF, Thomas MR, Judge HM, Khan H, Prince LR, Sabroe I, et al. Ticagrelor potentiates adenosine-induced stimulation of neutrophil chemotaxis and phagocytosis. *Vascul Pharmacol.* (2015) 71:201–7. doi: 10.1016/j.vph.2015.02.006
31. Chen M-L, Wu S, Tsai T-C, Wang L-K, Tsai F-M. Regulation of neutrophil phagocytosis of *Escherichia coli* by antipsychotic drugs. *Int Immunopharmacol.* (2014) 23:550–7. doi: 10.1016/j.intimp.2014.09.030
32. Pinder EM, Rostron AJ, Hellyer TP, Ruchaud-Sparagano M-H, Scott J, Macfarlane JG, et al. Randomised controlled trial of GM-CSF in critically ill patients with impaired neutrophil phagocytosis. *Thorax.* (2018) 73:918–25. doi: 10.1136/thoraxjnl-2017-211323
33. Mancuso P, Nana-Sinkam P, Peters-Golden M. Leukotriene B4 augments neutrophil phagocytosis of Klebsiella pneumoniae. *Infect Immun.* (2001) 69:2011–6. doi: 10.1128/IAI.69.4.2011-2016.2001
34. Ries F, Alflen A, Aranda Lopez P, Beckert H, Theobald M, Schild H, et al. Antifungal drugs influence neutrophil effector functions. *Antimicrob Agents Chemother.* (2019) 63:1435. doi: 10.1128/AAC.02409-18
35. Hesselink L, Spijkerman R, de Fraiture E, Bongers S, Van Wessem KJP, Vrisekoop N, et al. New automated analysis to monitor neutrophil function point-of-care in the intensive care unit after trauma. *Intensive Care Med Exp.* (2020) 8:12–12. doi: 10.1186/s40635-020-0299-1
36. Ducusin RJ, Sarashina T, Uzuka Y, Tanabe S, Ohtani M. Phagocytic response of bovine polymorphonuclear leukocytes to different incubation conditions and following exposure to some effectors of phagocytosis and different anticoagulants *in vitro*. *Can J Vet Res.* (2001) 65:38–44.
37. Perez-Barcena J, Crespi C, Regueiro V, Marsé P, Raurich JM, Ibáñez J, et al. Lack of effect of glutamine administration to boost the innate immune system response in trauma patients in the intensive care unit. *Crit Care.* (2010) 14:1–11. doi: 10.1186/cc9388
38. Liao Y, Liu P, Guo F, Zhang Z-Y, Zhang Z. Oxidative burst of circulating neutrophils following traumatic brain injury in human. *PLoS ONE.* (2013) 8:e68963–12. doi: 10.1371/journal.pone.0068963
39. Kanyilmaz S, Hepguler S, Atamaz FC, Gokmen NM, Ardeniz O, Sin A. Phagocytic and oxidative burst activity of neutrophils in patients with spinal cord injury. *Arch Phys Med Rehabil.* (2013) 94:369–74. doi: 10.1016/j.apmr.2012.09.015
40. Hazeldine J, Hampson P, Lord JM. The impact of trauma on neutrophil function. *Injury.* (2014) 45:1824–33. doi: 10.1016/j.injury.2014.06.021
41. Boque MC, Bodi M, Rello J. Trauma, head injury, and neurosurgery infections. *Semin Respir Infect.* (2000) 15:280–6. doi: 10.1053/srin.2000.20935
42. Kronen R, Liang SY, Bochicchio G, Bochicchio K, Powderly WG, Spec A. Invasive fungal infections secondary to traumatic injury. *Int J Infect Dis.* (2017) 62:102–11. doi: 10.1016/j.ijid.2017.07.002
43. Pap G, Furész J, Fennt J, Kovács GC, Nagy L, Hamar J. Self-regulation of neutrophils during phagocytosis is modified after severe tissue injury. *Int J Mol Med.* (2006) 17:649–54. doi: 10.3892/ijmm.17.4.649
44. Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* (2008) 295:L379–99. doi: 10.1152/ajplung.00010.2008

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frontiers in Medicine

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Influence of Antibiotic Management on Microbial Selection and Infectious Complications After Trauma

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Background: The inflammatory response and post-traumatic complications like infections play an important role in the pathophysiology of severe injuries. This study examines the microbiological aspects in anti-infective treatment of trauma patients and their inflammatory response in post-traumatic infections complications.

Patients and Methods: A retrospective analysis of prospectively collected data in trauma patients (ISS ≥16) over a 1-year period (01/2018 to 12/2018) is provided. Patient population was stratified into severely injured patients without post-traumatic infection (inf-PT), and severely injured patients who developed an infection (inf+PT).

Results: Of 114 trauma patients, 45 suffered from post-traumatic infection during the first 10 days of hospitalization. Severely injured patients with concomitant traumatic brain injury (PT+TBI) showed the highest rate of post-traumatic infection. Pro-inflammatory reaction was tracked by levels of Interleukin (IL-)6 (day 3: inf+T 190.8 ± 359.4 pg/dL > inf-PT 56.2 ± 57.7 pg/mL (mean ± SD); $p = 0.008$) and C-Reactive-Protein (CRP, day 3: inf+PT 15.3 mg/dL > inf-PT 6.7 mg/dL, $p = 0.001$) which were significantly higher in trauma patients who develop an infectious complication and showed a significant positive correlation with the occurrence of infection. The leading entity of infection was pneumonia followed by infections of the urinary tract mainly caused by gram-negative *Enterobacteriaceae*. 67.5% of all trauma patients received single-shot antibiosis during initial care in trauma bay. The development of secondary colonization was not relevant positively correlated with single-shot antibiosis ($r = 0.013$, $p = 0.895$) and prophylactically calculated antibiotic administration ($r = 0.066$, $p = 0.500$).

Conclusion: Severely injured trauma patients have an increased risk for development of infectious complications, which mainly is pneumonia followed by infection of the urinary tract mainly caused by gram-negative *Enterobacteriaceae*. Based on the data in this study, the one-time antibiotic and prophylactic calculated use of antibiotics, like Cephalosporins must be critically discussed in terms of their role in the development of post-traumatic infections and microbial selection.

Keywords: polytrauma, traumatic brain injury, Interleukin-6, severely injured patient, Interleukin-10, inflammation

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INTRODUCTION

Polytrauma (PT) and severe traumatic brain injury (TBI) caused by road traffic accidents and falls are the main causes of death and disability with immense socioeconomic impact through loss of productivity, medical and rehabilitation costs (1, 2). The treatment of patients with multiple organ injuries poses a particular challenge due to different injury patterns and severity, but also due to the complex immune response (3). Post-traumatic exaggerated immunomodulation often leads to postinjury complications, multiple organ failure (MOF) or sepsis and are predictors of mortality in trauma within the first days in Intensive Care Unit (ICU) (4). The mortality rate of septic trauma patients in hospital is still high (19.5–23%). Early prevention of sepsis development can facilitate further treatment of patients and contribute to improved treatment outcomes (5). The prevention of trauma-related infections/sepsis has so far mainly involved infection prevention (e.g., surgical management, prophylactic antibiotics, tetanus vaccination) and the prevention of organ dysfunction (e.g., drugs, temporary intravascular shunts, lung protective strategies) (6). The integrity of the intestinal barrier and the intestinal microbiome are fundamental prerequisites for a functioning immune system and the maintenance of colonization resistance to bacterial pathogens (7). Trauma, perfusion damage, or surgery favor a barrier disorder of the intestinal mucosa, and bacterial translocation and subsequent infections via the bloodstream (8). Dysbiosis and the development of multi-resistant bacteria (MRE) due to the use of antibiotics can lead to severe post-traumatic infections and complications (9).

Detecting the severity of injuries at an early stage, predicting risk factors of post-traumatic complications, and preventing secondary damageare of highest clinical interest. Serum biomarkers of inflammatory reaction are an optimal complement to detect potential complications like infection in an early stage (10–12). Systemic inflammatory reactions can be tracked by leukocytosis and release of acute phase proteins (APPs), such as c-reactive protein (CRP) and cytokines (13–15). Pro- and anti-inflammatory mediators such as Interleukin-6 and -10 (IL-6, IL-10) are released in response to tissue injury and lead to both reactive and restorative inflammatory processes (6, 16, 17).

The aim of this study was to stratify a cohort of trauma patients according to the development of post-trauma infections and to evaluate them with respect to their injury pattern, treatment with anti-infective therapy and bacterial colonization and clinical outcome. We hypothesize that peri-traumatic prophylactic or calculated antibiotic therapy influences the development of post-traumatic infections and secondary bacterial colonization. In addition, we assess the post-traumatic infections by biomarkers of post-traumatic inflammation IL-6, IL-10, CRP, and WBC.

PATIENTS AND METHODS

Ethics

The study was performed at the University Hospital Frankfurt, Goethe University after approval by the Institutional Review Board (89/19) in accordance with the Declaration of Helsinki and following STROBE guidelines (18). Written informed consent was obtained for enrolled patients or their legally authorized representatives in accordance with ethical standards.

Patients and Study Setting

We retrospectively reviewed a cohort of severely injured trauma patients admitted to the emergency department (ED) and level-1-trauma center of the University Hospital Frankfurt from January to December 2018. All clinical data were collected prospectively as part of the quality documentation of our in-house trauma registry. All trauma patients in the ED were treated according to the Advanced Trauma Life Support (ATLS® American College of Surgeons, Chicago, IL, USA) standard and the polytrauma guidelines (19). Injury severity from trauma was calculated using the Injury Severity Score (ISS), the New Injury Severity Score (NISS) and the Abbreviated Injury Scale (AIS) score which assigns each injury a severity level between 1 (mild) and 6 (maximum) in different regions (head, face, thorax, abdomen, extremities, and external injuries) (20–22). To calculate a mortality prognosis RISC II Score (Revised Injury Severity Classification, Version 2) was used (23).

We evaluated all patients with a history of acute trauma with an ISS $\geq$16 admitted to the emergency department during this 12-month period. Participating patients had to be $\geq$18 years of age. Patients who met this inclusion criteria were stratified into 2 cohorts: severely injured patients without post-traumatic infection (inf-PT), and severely injured patients who developed a relevant post-traumatic infection during their in-hospital stay (inf+PT). Relevant infections included respiratory tract infection/pneumonia (Examination material: sputum, tracheal secretion, bronchial lavage), urinary tract infection, soft tissue infection (Examination material: swab, biopsy), catheter-associated infection (Examination material: catheter tip; except urinary catheters), and bacteremia (Examination material: blood culture). In addition, infections were designated as "unknown" if patients had clear symptoms of infection such as fever or laboratory signs of inflammation of unclear origin. Severe open fracture was defined by fractures with an AIS $\geq$2 and a soft tissue injury of type 2 or greater according to the Gustilo-Anderson classification. Recent major surgery describes a major impact by surgical intervention performed early (within 5 days) after trauma, such as osteosyntheses of long bones, spine, or pelvis, hemi or bi-craniotomies or major intra-abdominal or -thoracal interventions. These are intended to represent a possible second hit. We defined relevant post-traumatic disability at the time of hospital discharge. Retrospectively, we evaluated several indicators to assess patient's outcome. These included the Barthel index $\leq$90% as a measure of limitations in self-care and activities of daily living, the Glasgow outcome scale extended (GOSE $\leq$4) for patients with TBI. As well as any impairment in physical function that led to discharge to inpatient rehabilitation, nursing home, or prescription of home care.

Patients with known pre-existing immunological disorders, immunosuppressive medication, burns, concomitant acute myocardial infarction, or thromboembolic events were excluded.

Blood Processing, Serum Marker, and Microbiological Analysis

Serial venous blood samples were obtained from traumatized patients on admittance to the ED for day 1–10 after trauma. Following baseline sample, subsequent blood was collected following the standard hospital procedures in pre-chilled tubes (silicate coated granules and polyacrylester gel; S-Monovette©, Sarstedt Inc., Nümbrecht, NRW, Germany) and stored on ice. Blood was centrifuged at 2,000 × g for 15 min at 4°C and the supernatant (serum) was stored at −80°C until analysis. Frozen serum samples were not thawed for this study before analysis. Interleukin (IL-)6 and Interleukin (IL-)10 concentrations were measured by IL-6/IL-10 Elipair Enzyme-linked Immunosorbent Assay (ELISA) (Diaclone, Hoelzel Diagnostica, Cologne, Germany) according to the manufacturer's instructions.

Levels of C-Reactive Protein (CRP, reference range <0.5 mg/mL), IL-6 (reference range <7 pg/mL), and blood count (leucocytes/white blood cells (WBC), reference range 3.96–10.41/nL) are part of routine diagnostics and were collected retrospectively from patients' medical records. Serial blood analyses for these parameters of clinical routine were not available for each of these patients. Microbiological diagnostics like bronchial lavage (BAL), urinary, and blood culture test were ordered only for patients in which potentially serious infection was suspected. Nasal, pharyngeal, and rectal swabs (Multidrug resistant gram-negative bacteria (MRGN) Screening) routinely taken in our clinic upon admission via the trauma ward, to the intensive care unit and then weekly. Tests were performed by chemical pathology and microbiology laboratories adjacent to the hospital where the trauma center is located. Results (incl. data regarding antibiotic therapy) were extracted retrospectively in chart review. A positive microbial result was defined pathogenic bacterium could be cultured in the laboratory. In classifying a bacterium as pathogenic, we considered the symptoms of the patient as recorded in the patient's medical records, the presence of other potential pathogens from other microbiological tests for the same patient, the frequency with which the bacterial species was isolated, the epidemiology associated with the specific bacterium. In cases where the source of the infection could not be identified, it was recorded as "unknown."

Statistical Analysis

Continuous normally distributed variables were summarized using means ± standard deviation (SD), while categorical or continuous variables with skewed distributions were summarized using medians with interquartile ranges (IQR). The *p*-values for categorical variables were derived from the two-sided Fisher's exact test, and for continuous variables from the Mann–Whitney *U*-test or the Kruskal–Wallis test in case of more than two group comparisons. Significant values were adjusted by the Bonferroni *post-hoc* test. Spearman's rank correlation coefficients were calculated to determine correlations between biomarkers and clinical characteristics. Receiver–operator characteristic (ROC) curves were generated to analyze the variables predictive power for the development of post-traumatic infection. $p < 0.05$ was considered to be statistically significant (* = p <0.05; ** = p <0.01; *** = p <0.001). Values are reported as mean for continuous variables and as percentages for categorical variables. All analyses were performed using the Statistical Package for Social Sciences (SPSS for Mac©), version 26 (SPSS Inc., Chicago, IL).

RESULTS

Demographics and Clinical Injury Characteristics

Table 1 shows the demographic and clinical characteristics stratified by the incidence of post-traumatic infection in trauma patients.

During the 12-month study period, $n = 114$ severely injured patients (ISS ≥16) met the inclusion criteria. Of these, $n = 45$ patients developed any post-traumatic infection (inf+PT cohort) during the first 10 days of hospitalization. For $n = 69$ patients no relevant infection or microbiological findings were documented (inf-PT cohort).

The majority of these trauma patients (inf-PT 72.5% and inf+PT 80.0%) were male. The included patients most suffered blunt injury trauma, whereas the incidence of penetrating trauma (e.g., gunshot or stab wounds) was <8% in both groups. No relevant difference in the incidence of open fractures or major soft tissue injuries (inf-PT 18.6% and inf+PT 25.0%, $p = 0.429$) or TBIs (inf-PT 31.4% and inf+PT 20.5%, $p = 0.190$) could be shown between the two groups. Significant more patients with post-traumatic infections (77.8% > 47.8%, $p < 0.001$) underwent major surgery during their in-hospital stay. No significant difference in median (IQR) age [inf-PT 49 (22–73) years vs. inf+PT 47 (30–69) years; $p = 0.586$] was seen. Median (IQR) ISS of inf-PT cohort was slightly lower [inf-PT: ISS 24 (17–32) pts. < inf+PT: ISS 27 (22–34) pts., $p = 0.044$] but no significant difference was found in NISS ($p = 0.315$). Median (IQR) risk of mortality (RISC II) was <6% ($p = 0.916$) for patients in both groups. The median (IQR) length of stay in intensive care unit (inf-PT 2 (0–6) days < inf+PT 14 (7–20) days, $p < 0.001$) and ventilation time (inf-PT 0 (0–2) < inf+PT 5 (1–5), $p < 0.001$) was significantly extended in inf+PT cohort. There was no relevant difference in indicators of hemorrhagic shock, such as blood hemoglobin fraction ($p = 0.565$), systolic blood pressure (RR_{syst}, $p = 0.128$) and pulse ($p = 0.752$). Patients without post-traumatic infections received significant more blood transfusions [median (IQR) 2.5 (2–5) > 0 (0–2), $p = 0.005$].

Higher Level of Inflammation Markers in Trauma Patients With Posttraumatic Infection

Stratification of the cohorts was supported and post-traumatic infection was tracked by serum biomarkers of pro- and anti-inflammatory response. **Figure 1** shows the expression profile of he markers in trauma patients with (inf+PT) compared to patients without (inf-PT) post-traumatic infection.

Blood count analyses showed that the kinetic profile of the WBCs showed to relevant difference betweenboth cohorts

TABLE 1 | Demographic and injury characteristics stratified by the incidence of post-traumatic infection.

	inf-PT ($n = 69$)	inf+PT ($n = 45$)	
	% of n	*% of n*	*p*-value
Sex (m)	72.5%	80.0%	0.355
Penetrating injury	7.6%	6.7%	0.069
Severe open fracture/Decollement	18.6%	25.0%	0.429
Open TBI	31.4%	20.5%	0.190
Recent major surgery	47.8%	77.8%	<0.001
Outcome			
In-hospital Mortality	33.3%	2.2%	<0.001
Severe Disability	5.7%	43.2%	<0.001
	Median (IQR)	**Median (IQR)**	***p*-value**
Age (y)	49 (22–73)	47 (30–69)	0.586
ISS (pts)	24 (17–32)	27 (22–34)	0.044
NISS (pts)	29 (22–41)	34 (27–43)	0.315
AIS_{head} (pts)	4 (2-4)	3 (0–5)	0.889
AIS_{thorax} (pts)	2 (0–4)	2 (0–3)	0.952
$AIS_{abdomen}$ (pts)	0 (0–0)	0 (0–2)	0.035
$AIS_{extremities}$ (pts)	2 (0–2)	2 (0–3)	0.053
RISC II (%)	4.5 (0.6–67.5)	5.5 (1.0–14.2)	0.916
ETI (d)	0 (0–2)	5 (1–5)	<0.001
ICU (d)	2 (0–6)	14 (7–20)	<0.001
$_{syst}RR_{ER}$ (mmHg)	139 (115–165)	127 (106–160)	0.128
$Puls_{ER}$ (bpm)	85 (75–100)	88 (66–116)	0.752
Blood transfusion (250ml bag)	2.5 (2–5)	0 (0–2)	0.005

M, male, IQR, Interquartile Range; y, years; pts, points; TBI, traumatic brain injury; "recent major surgery": Major surgery on head, long bones, spine, pelvis, thorax, or abdomen within 5 days after trauma; AIS, Abbreviated Injury Scale; ISS, Injury Severity Score; RISC II, Revised Injury Severity Classification, Version 2; ICU, Intensive Care Unit; ETI, Endotracheal Intubation; d, days; $_{syst}$RR, systolic blood pressure; bpm, beats per minute; ER, at admission to Emergency room.

(except day 8, which will most likely be an outlier).CRP level in patients with post-traumatic infection supports the positive microbial findings in this cohort and was significantly higher (day 3: inf+PT 15.3 mg/dL > inf-PT 6.7 mg/dL, $p = 0.001$) comparing trauma patients without infection. Spearman's rank correlation showed significant positive correlation between CRP level and the occurrence of post-traumatic infection from day 2–6, 8, and 9.

As well the IL-6 level was significantly higher in inf+PT group (day 3: inf+PT 190.8 ± 359.4 pg/dL > inf-PT 56.2 ± 57.7 pg/mL, $p = 0.008$). A significant positive correlation between pro-inflammatory IL-6 level and the occurrence of post-traumatic infection from day 1 to 7 (day 3: $r = 0.384$; $p = 0.007$) was shown.

Anti-inflammatory IL-10 levels didn't show significant difference in both study cohorts.

Post-traumatic Infections in Trauma Patients

Table 2 shows the entities and number (n) of post-traumatic infection in trauma patients stratified by injury pattern (PT, PT+TBI, isTBI). **Table 3** shows the spectrum of microorganisms found in the cultures of bronchial lavage, tracheal secretion, urine, intraoperative wound swab, catheter, and blood of trauma patients with post-traumatic infection (inf+PT, $n = 45$).

$N = 45$ of 114 patients suffered from post-traumatic infection during the first 10 days after trauma. Among these, the trauma patients with concomitant traumatic brain injury (PT+TBI) had the highest rate (44.4%) of post-traumatic infections. The leading entity of post-traumatic infection was pneumonia (PT+TBI 36.2% > PT 22.2% > TBI 9.7%), which was followed by infections of the urinary tract (PT+TBI 12.8%, TBI 6.5%) and soft tissue (PT 19.4%).

The most common pathogens detected were gram-negative *Enterobacteriaceae* (62.2%), like *E. coli* (17.8%) and *Klebsiellae* (32.1%). 50% of pneumonia and 90% of urinary tract infections were caused by this gram-negative *Enterobacteriaceae.* 8.9% of inf+PT suffered from an infection with *Pseudomonas aeruginosa*. The microbial spectrum of soft tissue infections was spread over both gram-negative and gram-positive bacteria. Among the gram-positive bacteria mainly *Staphylococcaceae* (17.8%), such as *Staphylococcus aureus and St. epidermidis* were found. A proven bacteremia was only found in PT cohort ($n = 5$), which was most likely catheter-associated ($n = 3$), caused by gram-positive *Staphylococcus* and gram-negative *Enterobacteriaceae*. 6.3% of inf+PT suffered from multiple organ infections (PT+TBI 12.8% > PT 5.5.% > isTBI 3.2%). In 31.1% of inf+PT a polymicrobial coinfection with several pathogenic bacteria was found, including those affecting several organ systems.

Indications and Spectrum of Anti-infective Therapy in Trauma Patients

Table 4 shows antibiotic therapy in trauma patients with and without post-traumatic infections. **Table 5** shows the

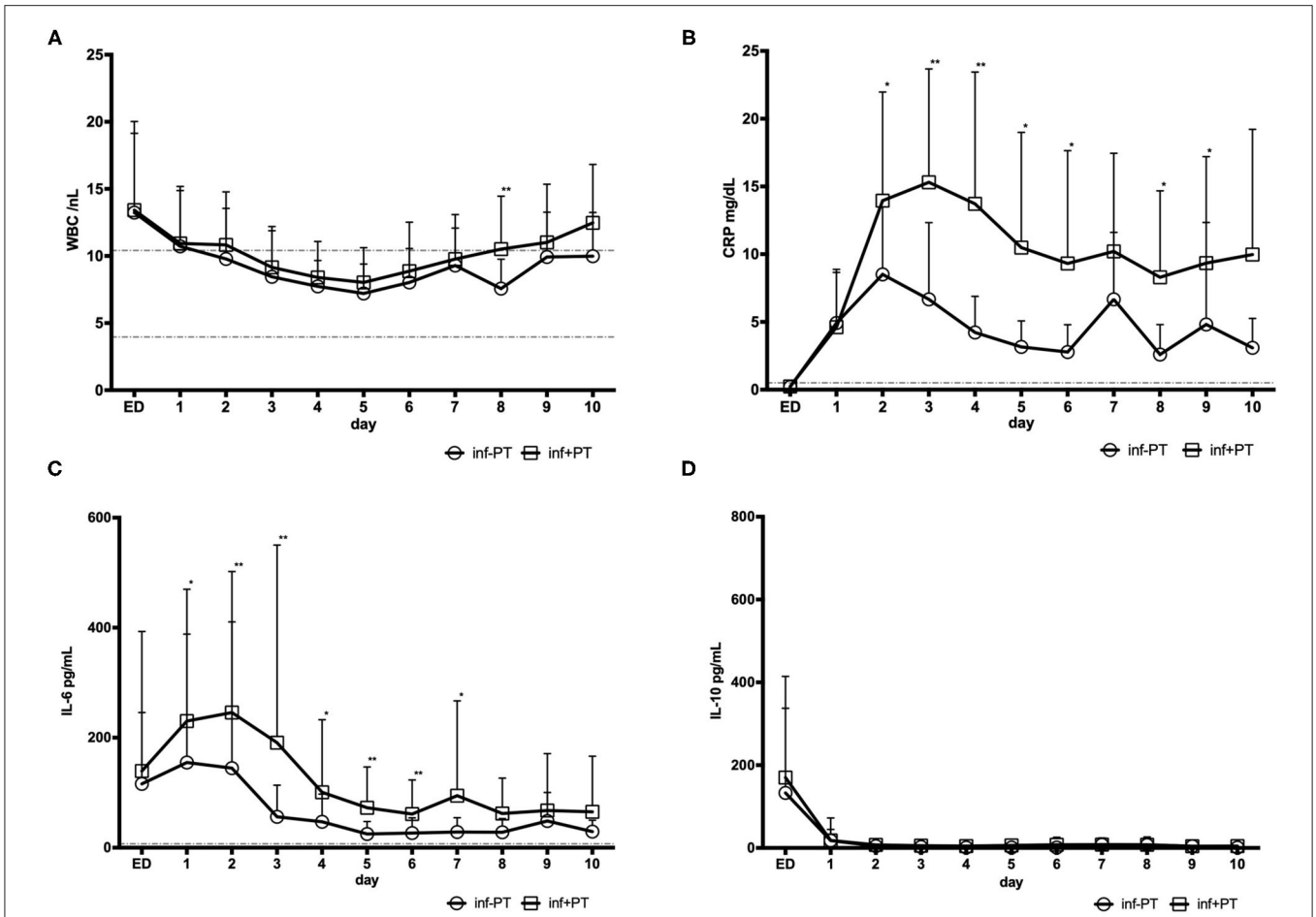

FIGURE 1 | Inflammation markers in trauma patients with (inf+PT) and without (inf-PT) post-traumatic infection **(A)** Leukocytes with reference range 3.96–10.41/ nL **(B)** CRP (C-Reactive Protein) in mg/dL with reference range <0.5 mg/dL **(C)** Interleukine(IL-)6 with reference range <7 pg/mL and **(D)** Interleukine(IL-)10 (no reference range).

TABLE 2 | Post–traumatic infections in trauma patients stratified by injury pattern.

	All		PT		PT+TBI		isTBI	
Entity	***n***	**% of *N***	***n***	**% of *N***	***n***	**% of *N***	***n***	**% of *N***
Infections	45	39.1	16	44.4	24	51.1	5	16.1
Pneumonia	28	19.4	8	22.2	17	36.2	3	9.7
Urinary tract	10	8.8	2	5.5	6	12.8	2	6.5
Soft tissue	7	6.1	7	19.4	0		0	
Intravenous catheter	3	2.6	0		3	6.4	0	
Unknown	5	4.4	1	2.8	3	6.4	1	3.2
Bacteremia	5	4.4	0		5	10.6	0	
Multiple organ infections	9	6.3	2	5.5	6	12.8	1	3.2

All (N = 114) trauma patients with ISS ≥16 stratified by injury pattern: PT (N = 36) = multiple injured patients (polytrauma) without TBI, PT+TBI (N = 47) = multiple injured patients with TBI, isTBI (N = 31) = isolated TBI.

antibiotic spectrum in therapy of peri-traumatic anti-infective management.

67.5% of all 114 trauma patients received single-shot antibiosis during initial care in trauma bay. *Cefuroxime* (inf-PT 26.1% and inf+PT 42.2%) and *Meropenem* (inf-PT 34.8% and inf+PT 26.6%) were the most commonly used antibiotics. Stratified by injury pattern, patients without TBI were mainly medicated with *Cefuroxime* (63.9%) while patients with TBI received

TABLE 3 | Spectrum of microorganisms in trauma patients with post-traumatic infections ($N = 45$).

Microorganisms	*n*	% of *N*	*n* (%) Pneum	*n* (%) Urin	*n* (%) Tiss	*n* (%) Cat	*n* (%) Bac
Gram-negative bacteria							
Enterobacteriaceae	28	62.2	14 (50.0)	9 (90)	3 (42.9)	1 (33.3)	1 (20.0)
Enterobacter aerogenes	3	6.7	1 (3.6)	1 (10)		1 (33.3)	1 (20.0)
Enterobacteriaceae ssp.	6	13.3	4 (14.3)	2 (20)			
Enterococcus faecium (VRE)	2	4.4		1 (10)	1 (14.3)		
Escherichia coli (ESBL/3MRGN)	8	17.8	4 (14.3)	3 (30)	1 (14.3)		
Klebsiella pneumoniae	6	13.3	3 (10.7)	1(10)	1 (14.3)		
Klebsiella ssp.	3	6.7	2 (7.1)	1 (10)			
Haemophilus influenzae	1	2.2	1 (3.6)				
Moraxella catarrhalis	1	2.2	1 (3.6)				
Proteus mirabilis	1	2.2		1 (10)			
Pseudomonas aeruginosa	4	8.9	3 (10.7)		1 (14.3)		
Gram-positive bacteria							
Bacillus cereus	1	2.2			1 (14.3)		
Corynebacterium tuberc.	1	2.2			1 (14.3)		
Propionibacterium acnes	2	4.4			1 (14.3)		1 (20.0)
Staphylococcaceae	8	17.8					
Staphylococcus aureus	3	6.7	3 (10.7)				
Staphylococcus epidermidis	3	6.7				2 (66.7)	3 (60.0)
Staphylococcus ssp.	2	4.4					2 (40.0)
Streptococcus pneumoniae	1	2.2	1 (3.6)				
Polymicrobial coinfections	14	31.1	8 (28.6)	3 (30)	2 (28.6)		1 (20.0)

Pneum, Pneumonia; Urin, Infection of urinary tract; Tiss, soft tissue infection; Cat, Catheter-associated infection; Bac, Bacteremia; ESBL, Extended Spectrum ß-Lactamase; 3MRGN, Multi-Resistant Gram-Negative bacteria; VRE, Vancomycin-Resistant Enterococcus.

TABLE 4 | Indications of antibiotic therapy in trauma patients.

	All		inf-PT		inf+PT	
Indication	*n*	% of *N*	*n*	% of *N*	*n*	% of *N*
Single shot (ER)	77	67.5	45	65.2	32	71.1
Prophylactic/Calculated	63	55.3	32	46.4	31	68.9
Perioperative	5	4.4	4	5.8	1	2.2
Proven infection	38	33.3	0		38	84.4
Multiple antibiotic treatment	25	21.9	2	2.9	23	51.1

All (N = 114) trauma patients ISS ≥16; ER, Emergency room; BLI, Beta Lactamase Inhibitor; PT, Polytrauma patients; infPT, Polytrauma patients with post-traumatic infection.

predominantly *Meropenem* (58.1%). In case of open fracture or extensive soft tissue damage, *Metronidazole* was given additionally (4.8%).

In 55.3% (inf-PT 30.4% and inf+PT 17.9%) the anti-infective therapy was continued prophylactically/calculated to avoid later infection-associated complications or based on the clinical picture of an impending infection. In addition to the above-mentioned indications for the calculated application of *Meropenem* (inf-PT 31.9% and inf+PT 11.1%) and *Cefuroxime* (inf-PT 5.8% and inf+PT 15.5%), *Penicillin + BLI* (inf-PT 5.8% and inf+PT 42.2%) was frequently used. 4.4% of all trauma patients received peri-operative antibiosis. In 33.3% the antibiotic treatment was associated with a proven infection by positive bacterial culture. Drug spectrum is broader in the case of a confirmed infection and, due to the mostly present infection with gram negative anaerobes (shown in **Table 2**), mainly dominated by *Fluoroquinolones* and *Glycopeptide antibiotics*.

Bacterial Carriage in Trauma Patients

Table 6 shows the results of routine nasal, pharyngeal and rectal swabs taken on admission to our trauma bay and during the stay in the intensive care unit.

In $n = 28$ of 114 trauma patients a primary bacteria colonization was found, mainly with *Escherichia coli* (*E. coli*; *ESBL* = Extended Spectrum ß-Lactamase; *3MRGN* = Multi-Resistant Gram-Negative bacteria), followed by *Enterococcus faecium/faecalis* (*VRE* = Enterococcus faecium/faecalis) and *Pseudomonas aeruginosa* in the rectal smear. In $n =$

TABLE 5 | Antibiotic spectrum of anti-infective management in trauma patients.

	All ($N = 114$)		inf-PT ($N = 69$)		inf+PT ($N = 45$)	
Substance	***n***	**% of *N***	***N***	**% of *N***	***n***	**% of *N***
Cephalosporins	45	39.5	20	29.0	25	55.6
1. Generation	3	2.6	1	1.4	2	4.4
2. Generation	38	33.3	19	27.5	19	42.2
3. Generation	4	3.5	0		4	8.9
Nitromidazole (Metronidazol)	12	10.5	4	5.8	8	17.8
Carbapenems	45	39.5	28	40.6	17	37.8
Meropenem	40	35.1	27	39.1	13	28.9
Imipenem	5	4.4	1	1.4	4	8.9
Penicillin + BLI	25	21.9	4	5.8	21	44.4
Lincosamide (Clindamycin)	3	2.6	0		3	6.7
Fluoroquinolone	13	11.4	0		13	28.9
Ciprofloxacin	7	6.1	0		7	15.6
Levofloxacin	6	5.3	0		6	13.3
Glycopetide antibiotics	10	8.8	0		10	22.2
Vancomycin	3	2.6	0		3	6.7
Teicoplanin	7	6.1	0		7	15.6
Cotrimoxazol	4	3.5	0		4	8.9
Ansamycine (Rifampicin)	1	0.9	0		1	2.2
Antibiotics of last resort	3	2.6	0		3	6.7

BLI, Beta Lactamase Inhibitor; inf-PT, Polytrauma patients without post-traumatic infections; inf+PT, Polytrauma patients with post-traumatic infection.

8 trauma patients secondary colonization occurred during hospitalization. The detection of colonization by other bacteria, like *Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter cloacae* and *Proteus mirabilis* was <2%. MRSA (Methicillin-Resistant Staphylococcus aureus) was detected in only $n = 1$ patient. Of these 28 patients with a positive smear, 17 developed a post-traumatic infection.

Of the $n = 8$ patients with secondary colonization, $n = 6$ patients received single-shot antibiotics in trauma bay, $n = 2$ patients received prophylactically calculated antibiotics (because of an open wound). All of them developed a post-traumatic infection during first 10 day after trauma. A statistically significant positive correlation (Spearman's rank correlation, $r = 0.251$; $p = 0.007$) could be shown between a positive screening test result and the occurrence of post-traumatic infection. The development of secondary colonization was not relevant positively correlated with single-shot antibiosis ($r = 0.013$, $p = 0.895$) and prophylactically calculated antibiotic administration ($r = 0.066$, $p = 0.500$).

TABLE 6 | Bacterial carriage in trauma patients ($N = 114$).

Characteristics	***n***	**% of *N***
Primary colonization	28	24.6
Secondary colonization	8	7.0
Microorganisms		
Escherichia coli (ESBL/3MRGN)	15	13.2
Enterococcus faecium/faecalis (VRE)	7	6.1
Pseudomonas aeruginosa	3	2.6
Acinetobacter baumannii	2	1.8
Citrobacter freundii	1	0.9
Enterobacter cloacae	1	0.9
Staphylococcus aureus (MRSA)	1	0.9
Proteus mirabilis	1	0.9

ESBL, Extended Spectrum ß-Lactamase; 3MRGN, Multi-Resistant Gram-Negative bacteria; VRE, c; MRSA, Methicillin-Resistant Staphylococcus Aureus.

DISCUSSION

Risk Factors for Posttraumatic Infections

Of the trauma patients included in this study, the majority were male and under 50 years of age; one-third suffered a post-traumatic infection. Polytrauma is the leading cause of death in Western countries for people up to 45 years of age, in a male-to-female ratio of 2.6:1 (1). Percentage of mortality was significantly higher in the inf-PT group. This initially rather surprising result is most likely explained by the fact that a high percentage of severely injured patients in the inf-PT group died in the early stages after trauma. Thus, they do not suffer post-traumatic infections and bias the mortality rate. One could even assume that the already high infection rate could be even higher. Patients who have extended hospital stay with prolonged periods of coma, immobilization and ventilation are at enormous risk for post-traumatic complications, like pulmonary infections (24). On the other hand, it is quite plausible that patients who have suffered a post-traumatic infection also have

a higher disability rate. Longer periods of respiratory weaning and comorbidities often complicate rehabilitation, including critical illness myopathy, which leads to Intensive care unit acquired weakness (ICUAW) (25). Open fractures and major skin injuries do not appear to have a relevant influence on the development of an infectious post-traumatic complication. One reason for this could be the consistent, calculated antibiotic therapy (Cefuroxime & Metronidazole) applied from the outset for this type of injury. In this study, the impact of a major surgical intervention in a relevant temporal context of 5 days after trauma was investigated. Hereby, all patients were included who were treated according to a dynamic therapy evaluation in the sense of "safe-definitive-surgery." We showed that in patients with post-traumatic infections, surgical interventions were performed significantly more frequently in close temporal relation to the trauma. Early total care (ETC) involves definitive surgical stabilization of all long bone fractures in the early phase of treatment (24–48 h). Damage control surgery (DCS) refers to those maneuvers designed to ensure the survival of the (bleeding) patient with vital-threatening organ injury or long bone and pelvic fractures. The advantage of ETC seemed to be fewer pulmonary complications, shorter duration of ICU, and hospital stay (26). Advances in understanding the pathophysiologic and immunologic mechanisms after severe injury led to a shift from ETC to DCS. Traumatic injury leads to systemic inflammatory response syndrome (SIRS), followed by a recovery phase mediated by a counter-regulatory anti-inflammatory response (CARS). Severe inflammation can lead to acute organ failure and early death after injury. A mild inflammatory response followed by excessive CARS can produce a long-lasting, deleterious immunosuppressed state and promote the development of infections. The initial trauma is referred to as the "first hit" and predisposes the patient to a potential risk of deterioration (27). Major post-trauma interventions in the form of a "second-hit" phenomenon, i.e., due to fat emboli and hypoxic events, can lead to serious complications such as pulmonary dysfunction, coagulopathy, fat or pulmonary embolism, excessive immune reactions and post-traumatic infections. Over the last three decades, extensive research has been published on the "second hit" phenomenon, the pathophysiology of trauma and the consequences of subsequent interventions (28–30). The safe-definitive-surgery (SDS) concept was presented as a dynamic combination of the ETC and DCO care strategies. Regular reevaluation and assessment of the patient's physiology and condition can combine the advantages of the DCO and ETC procedures, which could allow "safe definitive care" (SDS) of the severely injured patient.

Early Tracking of Posttraumatic Infection by Biomarkers Is Efficient

We further analyzed the validity of the routine laboratory and recognized biomarkers of post-traumatic immune response for their value in these patients with post-traumatic infections. For the critically ill trauma patient who survives for more than 3 days, infectious complications are the leading cause of death after serious head injury (31). The standard clinical diagnosis of pneumonia includes fever, leukocytosis, purulent secretions, and new or progressive infiltrates in a chest x-ray (32). Nevertheless, biomarkers are certainly the optimal complement to clinical and radiological findings to assess the course and outcome of the injury and its complications sufficiently (10, 33). We could show that levels of IL-6 and CRP are significantly higher in trauma patients who develop post-traumatic infection. Both markers showed a significant positive correlation with the presence of post-traumatic infection. This result supports our stratification of the patient cohort. Furthermore, the results show that already on day 1 and 2 a differentiation of the markers between PT+Inf and PT-Inf occurs, which in these cases suggests an early enhanced immune response in the PT+Inf group. It was shown before that primary trauma injury triggers an immunomodulatory response that is associated with an increase in CRP (34). Interleukins play a particularly prominent role in the development of post-traumatic complications und and have been widely studied in relation to trauma (15, 16, 35). In the acute phase reaction, IL-6 is known to regulate inflammation and immunity. IL-6 is secreted in increased amounts early after trauma (36, 37). Previous studies described a correlation of IL-6 with the severity of injury (ISS) and mortality, especially during the first post-traumatic hours (15, 36, 38, 39). Additionally, it has potentially predictive value for post-traumatic infection (40, 41). Our results underline the importance of these biomarkers, which can already be early indicators of a complication, while clinical parameters may still be compensated, and microbiological results are pending. However, the early increase of inflammatory markers in PT+Inf basically questions the efficacy of the calculated single shot antibiosis, which was broadly applicated in both groups. Furthermore, the application of calculated prophylactic antibiotic therapy, which was consistently given in the TBI group, which most frequently suffered from pneumonia, remains questionable.

IL-10 is commonly known as an anti-inflammatory cytokine that exerts immunomodulatory functions and is particularly important in the resorption phase (42, 43). However, IL-10 does not appear to have any diagnostic value for the development of post-traumatic infection in this study. WBC analysis is one of the most basic biomarker tracking of post-traumatic complications. Studies showed that leukocytosis can predict the severity of injury in trauma victims (44). On the other hand, Paladino et al. showed that WBC counts are not useful in the diagnostic of trauma, as patients with severe injuries had significantly higher WBC level than those with minor injuries, but both were in the normal range (45). In our study, we saw that after trauma, WBCs are only slightly above normal (especially in the first 24 h). Furthermore, the WBC level of inf+PT does not differ significantly from that of inf-PT and therefore has less diagnostic value in the assessment of a post-traumatic infection.

Post-traumatic Infections Are Mainly Caused by Gram-Negative Enterobacteriaceae

The leading cause of post-traumatic infection was pneumonia mainly caused by gram-negative Enterobacteriaceae. Stratified

by injury pattern severely injured patients with concomitant traumatic brain injury (PT+TBI) shows the highest rate of post-traumatic infection. These findings support Helling et al., who analyzed infectious complications in the severely head injured and found that pulmonary infections occur most commonly, affecting 41% of the patients. The predominantly offending organism was gram-negative bacteria (46). In addition, the urinary tract is also a common source and must always be considered as a relevant differential diagnosis. The trauma patient has an increased risk of infection and the most common cause of late death after trauma is sepsis. Transfusion, hypotension and prolonged ventilatory support are predictive of septic complications. In addition, open wounds, additional surgical procedures, extensive invasion through various tubes and drains, post-traumatic immune modulation, transfusions, medication, and suboptimal nutritional status contribute to an increased risk of infection. Victims of blunt trauma have a significantly higher risk of post-traumatic infection than patients with penetrating trauma (24). Our results do not support the currently discussed idea, that blood transfusions facilitate the development of infections (47). In contrast, it could be concluded that the longer time of endotracheal intubation (ETI) and ICU stay of inf+PT are logically related to the complications of the infection. Longer ventilation times lead to an increased risk of infection after trauma and thus to a longer stay in the intensive care unit (32). However, the higher rate of mortality in the PT group inevitably leads to shorter ICU treatment.

Prophylactic Antibiotic Treatment vs. Microbiome Selection in Trauma Care

The prevention of post-traumatic complications such as infections remains one of the most important challenges in primary as well as intensive care. In the literature, the prophylactic use of antibiotics does not appear to change the development of infections in trauma patients and in many cases, antibiotic therapy is empirical, depending on the patient's condition (31). In our study most patients received prophylactic anti-infective therapy with Cefuroxime. The benefit of a perioperative antibiotic prophylaxis of Cefuroxime has already been proven and is already established in guidelines in Europe (48). The excitation spectrum can vary from place to place. In our institution the most common pathogens are gram-negative Enterobacteriaceae, just like already published in the study by Helling et al. (46). Staphylococcus and other gram-positive bacteria were found to be the main cause of wound infections in inf+PT cohort. Therefore, antibiotics covering the gram-positive and gram-negative bacterial spectrum should be administered as early as possible in infection prophylaxis. However, this does not only affect patients with open injuries. According to our study, however, the general prophylactic administration in all severely injured patients with massive injuries and the risk of reperfusion damage or second hits should also be discussed. A second-generation Cephalosporine is good practice basis. In heavily contaminated wounds this can be supplemented with an Aminoglycoside. If an anaerobic infection is suspected, a combination with e.g., Metronidazole is considerable (49). In our study, patients with TBI mainly received Meropenem. Typical pathogens of acute bacterial meningitis in open traumatic brain injury (TBI) are Staph. aureus, coagulase-negative Staphylococci, aerobic gram-negative bacteria which respond mainly to Meropenem and Cephalosporins (50).

Meropenem is important in the treatment of post-neurosurgical meningitis and penetrates the cerebrospinal fluid (CSF) (51). However, there is currently no evidence-based guideline for the prophylactic administration of antibiotics in trauma patients with or without TBI. Fever without not due to infectious events is common in trauma patients. Reasons for this could be an excessive immune reaction, as described above. In this study we found a high rate of patients receiving calculated Penicillin + beta lactamase inhibitor (BLI). Penicillin is a broad-spectrum antibiotic and often given in connection with unclear infections to cover a possible broad spectrum of pathogens. In the group of inf+PT, prophylactic, and calculated prophylactic antibiotics were administered relatively more frequently, i.e., in the case of already existing risk factors such as open fractures, large wounds or open TBI. Whether or not a single-shot antibiotic was administered did not initially affect the incidence for the occurrence of post-trauma infection. In addition, single-shot antibiotics and prophylactically calculated antibiotic application were not relevant positively correlated with the development of secondary colonization. However, in this study, it appears that prophylactic administration, especially of Cephalosporins, results in selection pressure in favor of gram-negative Enterobacteriaceae.

An important pathophysiological factor in the development of post-traumatic complications is the dysfunction of the external (skin) and internal paracellular blood and organ barriers, including the blood, air, and intestinal blood barriers in the brain (BBB), leading to the flooding of the tissue with immune cells and microbial invasion (52). IL-6 modulates the expression of tight junction proteins and the release of adhesion molecules in the plasma of polytrauma patients (ISS $\geq$18) correlates with disease severity and organ dysfunction (53). In addition, hemorrhagic shock is associated with intestinal barrier dysfunction and the development of MODS after trauma (53). Additional selection pressure favoring pathogenic and multiresistant bacteria further increases the risk of serious infectious complications.

Based on the data in this study, the one-time antibiotic and prophylactic calculated use of antibiotics, like Cephalosporins must be critically discussed in terms of their role in the development of post-traumatic infections by microbial selection (1, 2, 4, 10, 14–17, 31–36, 38–41, 51, 52, 54, 55). The treatment of patients with multiple organ injuries poses a particular challenged (45).

Limitations of the Study

The most important limitation is the retrospective nature of the data analysis; however, all clinical data were acquired in a prospective manner, and only the cytokines were measured later-on. The variance of the individual values is large, specifically of markers, which is due to potential confounders such as volume administration, blood products, shock, which are difficult to standardize. In addition, the values for CRP and WBC are part of routine diagnostics and were collected retrospectively from the patients' medical records. As a result, a complete number $n = 114$ could not be collected for each day over time, which

may have resulted in higher standard deviations with lower significance. The sample of this single center study came from an ICU facility specializing in traumatology. This may have a limiting influence on the generalizability of our study results, and it is possible that these results are not applicable to all trauma situations.

CONCLUSION

Severely injured trauma patients have an increased risk for development of infectious complications. Severely injured patients with concomitant traumatic brain injury (PT+TBI) show the highest rate of post-traumatic infection. In this context, for the first time, we showed that broad application of single-shot antibiotics and prophylactic calculated antibiotic use need to be critically discussed in terms of their role in the development of post-traumatic infections and microbial selection for gram negative anaerobic bacteria. The leading entity of infection was pneumonia followed by infection of the urinary tract and soft tissue infections, mainly caused by gram-negative Enterobacteriaceae. Levels of IL-6 and CRP are significantly higher in trauma patients who develop an infectious complication. Both markers showed the efficacy of early biomarker tracking in the prediction of post-traumatic complication like infektons.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board, University Hospital Frankfurt, Goethe University (89/19). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IM, CS, and DH designed the study, obtained the ethical approval for human analyses, established the methods, and revised the manuscript. CS collected samples, carried out data analyses, performed the statistical analysis, and made the first draft of the manuscript. CR and MW carried out experiments. PS, DH, and IM critically reviewed the manuscript. MW, PS, and J-NF contributed intellectually to the completion of the study. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Bardenheuer M, Obertacke U, Waydhas C, Nast-Kolb D. Epidemiologie des Schwerverletzten. *Unfallchirurg.* (2000) 103:355–63. doi: 10.1007/s001130050550
2. Verboket R, Verboket C, Schöffski O, Tlatlik J, Marzi I, Nau C. Kosten und Erlöse von über den Schockraum eingelieferten Patienten mit leichtem Schädel-Hirn-Trauma. *Unfallchirurg.* (2019) 122:618–25. doi: 10.1007/s00113-018-0566-8
3. Störmann P, Wagner N, Köhler K, Auner B, Simon T-P, Pfeifer R, et al. Monotrauma is associated with enhanced remote inflammatory response and organ damage, while polytrauma intensifies both in porcine trauma model. *Eur J Trauma Emerg Surg.* (2020) 46:31–42. doi: 10.1007/s00068-019-01098-1
4. Balogh ZJ, Marzi I. Novel concepts related to inflammatory complications in polytrauma. *Eur J Trauma Emerg Surg.* (2018) 44:299–300. doi: 10.1007/s00068-018-0964-1
5. Frink M, van Griensven M, Kobbe P, Brin T, Zeckey C, Vaske B, et al. IL-6 predicts organ dysfunction and mortality in patients with multiple injuries. *Scand J Trauma Resusc Emerg Med.* (2009) 17:1–7. doi: 10.1186/1757-7241-17-49
6. Ma X-Y, Tian L-X, Liang H-P. Early prevention of trauma-related infection/sepsis. *Military Med Res.* (2016) 3:33. doi: 10.1186/s40779-016-0104-3
7. Meng M, Klingensmith NJ, Coopersmith CM. New insights into the gut as the driver of critical illness and organ failure. *Curr Opin Crit Care.* (2017) 23:143–8. doi: 10.1097/MCC.0000000000000386
8. Pape H -C, Dwenger A, Regel G, Auf'm'Kolck M, Gollub F, Wisner D, et al. Increased gut permeability after multiple trauma. *Br J Surg.* (1994) 81:850–2. doi: 10.1002/bjs.1800810619
9. Khodamoradi Y, Kessel J, Vehreschild JJ, Vehreschild MJGT. übersichtsarbeit Rolle der Mikrobiota bei der Prävention von Infektionen mit multiresistenten Bakterien. *Deutsches Arzteblatt Int.* (2019) 116:670–6. doi: 10.3238/arztebl.2019.0670
10. Relja B, Mörs K, Marzi I. Danger signals in trauma. *Eur J Trauma Emerg Surg.* (2018) 44:301–16. doi: 10.1007/s00068-018-0962-3
11. Woodcock T, Morganti-Kossmann MC. The role of markers of inflammation in traumatic brain injury. *Front Neurol.* (2013) 4:18. doi: 10.3389/fneur.2013.00018
12. Giannoudis PV, Hildebrand F, Pape HC. Inflammatory serum markers in patients with multiple trauma. *J Bone Joint Surg Br.* (2004) 86:313–23. doi: 10.1302/0301-620X.86B3.15035
13. Voth M, Duchene M, Auner B, Lustenberger T, Relja B, Marzi I. I-FABP is a novel marker for the detection of intestinal injury in severely injured trauma patients. *World J Surg.* (2017) 41:3120–7. doi: 10.1007/s00268-017-4124-2
14. Schindler CR, Lustenberger T, Woschek M, Störmann P, Henrich D, Radermacher P, et al. Severe Traumatic Brain Injury (TBI) modulates the kinetic profile of the inflammatory response of markers for neuronal damage. *J Clin Med.* (2020) 9:1667. doi: 10.3390/jcm9061667
15. Stensballe J, Christiansen M, TØnnesen E, Espersen K, Lippert FK, Rasmussen LS. The early IL-6 and IL-10 response in trauma is correlated with injury severity and mortality. *Acta Anaesthesiol Scand.* (2009) 53:515–21. doi: 10.1111/j.1399-6576.2008.01801.x
16. Hildebrand F, Pape H-C, Krettek C. Die Bedeutung der Zytokine in der posttraumatischen Entzündungsreaktion. *Unfallchirurg.* (2005) 108:793–803. doi: 10.1007/s00113-005-1005-1
17. Lustenberger T, Kern M, Relja B, Wutzler S, Störmann P, Marzi I. The effect of brain injury on the inflammatory response following severe trauma. *Immunobiology.* (2016) 221:427–31. doi: 10.1016/j.imbio.2015.11.011
18. von Elm E, Altmann DG, Egger M, Pocock SC, Gøtzsche PC, Vandenbroucke JP. Das strengthening the reporting of observational studies in epidemiology (STROBE-) statement. *Internist.* (2008) 49:688–93. doi: 10.1007/s00108-008-2138-4
19. Polytrauma Guideline Update Group. Level 3 guideline on the treatment of patients with severe/multiple injuries AWMF Register-Nr. 012/019. *Eur J Trauma Emerg Surg.* (2018) 44:3–271. doi: 10.1007/s00068-018-0922-y
20. Haasper C, Junge M, Ernstberger A, Brehme H, Hannawald L, Langer C, et al. Die Abbreviated Injury Scale (AIS). *Unfallchirurg.* (2010) 113:366–72. doi: 10.1007/s00113-010-1778-8

21. Baker SP, O'Neill B, Haddon W Jr, Long WB. The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care. *J Trauma.* (1974)14:187–96. doi: 10.1097/00005373-197403000-00001
22. Lefering R, Bouillon B, Neugebauer E. Traumascores: Ist der New ISS dem ISS überlegen? *Zurück in die Zukunft.* (2003) 2003:256–7. doi: 10.1007/978-3-642-55611-1_71
23. Lefering R, Huber-Wagner S, Nienaber U, Maegele M, Bouillon B. Update of the trauma risk adjustment model of the TraumaRegister DGU^{TM}: the revised injury severity classification, version II. *Critical Care.* (2014) 18:1–12. doi: 10.1186/s13054-014-0476-2
24. Morgan AS. Risk factors for infection in the trauma patient. *J Natl Med Assoc.* (1992) 84:1019–23.
25. Ponfick M, Bösl K, Lüdemann-Podubecka J, Neumann G, Pohl M, Nowak DA, et al. Erworbene muskelschwäche des kritisch kranken. Pathogenese, behandlung, rehabilitation, outcome. *Nervenarzt.* (2014) 85:195–204. doi: 10.1007/s00115-013-3958-6
26. Nicola R. Early total care versus damage control: current concepts in the orthopedic care of polytrauma patients. *ISRN Orthop.* (2013) 2013:329452. doi: 10.1155/2013/329452
27. Pfeifer R, Pape H-C. Diagnostik und Versorgungsstrategien beim polytraumatisierten Patienten. *Der Chirurg.* (2016) 87:165–73. doi: 10.1007/s00104-015-0139-0
28. Tschoeke SK, Hellmuth M, Hostmann A, Ertel W, Oberholzer A. The early second hit in trauma management augments the proinflammatory immune response to multiple injuries. *J Trauma.* (2007) 62:1396–403. doi: 10.1097/TA.0b013e318047b7f0
29. Keel M, Trentz O. Pathophysiology of polytrauma. *Injury.* (2005) 36:691–709. doi: 10.1016/j.injury.2004.12.037
30. Lasanianos NG, Kanakaris NK, Dimitriou R, Pape HC, Giannoudis PV. Second hit phenomenon: Existing evidence of clinical implications. *Injury.* (2011) 42:617–29. doi: 10.1016/j.injury.2011.02.011
31. Windolf J. Aktuelle Konzepte zur Prophylaxae posttraumatischer Infekte. *Trauma und Berufskrankheit.* (2005) 7:100–4. doi: 10.1007/s10039-004-0917-2
32. Wutzler S, Bläsius FM, Störmann P, Lustenberger T, Frink M, Maegele M, et al. Pneumonia in severely injured patients with thoracic trauma: results of a retrospective observational multi-centre study. *Scand J Trauma Resusc Emerg Med.* (2019) 27:1552. doi: 10.1186/s13049-019-0608-4
33. Schindler CR, Woschek M, Vollrath JT, Kontradowitz K, Lustenberger T, Störmann P, et al. MiR-142-3p expression is predictive for severe traumatic brain injury (TBI) in trauma patients. *Int J Mol Sci.* (2020) 21:1–16. doi: 10.3390/ijms21155381
34. Neumaier M, Vester H, Martetschläger F, Freude T, Scherer MA, Stöckle U. Optimaler Zeitpunkt zur prothetischen Versorgung von Schenkelhalsfrakturen: CRP als Marker der postoperativen immunologischen Reaktion. *Chirurg.* (2011) 82:921–6. doi: 10.1007/s00104-010-2043-y
35. Kany S, Vollrath JT, Relja B. Cytokines in inflammatory disease. *Int J Mol Sci.* (2019) 20:6008. doi: 10.3390/ijms20236008
36. Qiao Z, Wang W, Yin L, Luo P, Greven J, Horst K, et al. Using IL-6 concentrations in the first 24 h following trauma to predict immunological complications and mortality in trauma patients: a meta-analysis. *Eur J Trauma Emerg Surg.* (2018) 44:679–87. doi: 10.1007/s00068-017-0880-9
37. Gebhard F. Is Interleukin 6 an early marker of injury severity following major trauma in humans? *Arch Surg.* (2000) 135:291–5. doi: 10.1001/archsurg.135.3.291
38. Maier B, Lefering R, Lehnert M, Laurer HL, Steudel WI, Neugebauer EA, et al. Early versus late onset of multiple organ failure is associated with differing patterns of plasma cytokine biomarker expression and outcome after severe trauma. *Shock.* (2007) 28:668–74. doi: 10.1097/shk.0b013e318123e64e
39. Taniguchi T, Koido Y, Aiboshi J, Yamashita T, Suzaki S, Kurokawa A. The ratio of interleukin-6 to interleukin-10 correlates with severity in patients with chest and abdominal trauma. *Am J Emerg Med.* (1999) 17:548–51. doi: 10.1016/S0735-6757(99)90194-8
40. Giannoudis PV, Harwood PJ, Loughenbury P, van Griensven M, Krettek C, Pape HC. Correlation between IL-6 levels and the systemic inflammatory response score: Can an IL-6 cutoff predict a SIRS state? *J Trauma.* (2008) 65:646–52. doi: 10.1097/TA.0b013e3181820d48
41. Pape H-C, Griensven MV, Hildebrand FF, Tzioupis CT, Sommer KL, Krettek CC, et al. Systemic inflammatory response after extremity or truncal fracture operations. *J Trauma.* (2008) 65:1379–84. doi: 10.1097/TA.0b013e31818c8e8c
42. van der Poll T, Marchant A, van Deventer SJH. The role of interleukin-10 in the pathogenesis of bacterial infection. *Clin Microbiol Infect.* (1997) 3:605–7. doi: 10.1111/j.1469-0691.1997.tb00465.x
43. Schneider CP, Schwacha MG, Chaudry IH. The role of interleukin-10 in the regulation of the systemic inflammatory response following trauma-hemorrhage. *Biochim Biophys Acta.* (2004) 1689:22–32. doi: 10.1016/j.bbadis.2004.01.003
44. Santucci CA, Purcell TB, Mejia C. Leukocytosis as a predictor of severe injury in blunt trauma. *West J Emerg Med.* (2008) 9:81–5.
45. Paladino L, Subramanian RA, Bonilla E, Sinert RH. Leukocytosis as prognostic indicator of major injury. *West J Emerg Med.* (2010) 11:450–5.
46. Helling TS, Evans LL, Fowler DL, Hays LV, Kennedy FR. Infectious Complications in Patients with Severe Head Injury. *J Trauma.* (1988) 28:1575–7. doi: 10.1097/00005373-198811000-00009
47. Meybohm P, Schmitz-Rixen T, Steinbicker A, Schwenk W, Zacharowski K. Das patient-blood-management-konzept: gemeinsame empfehlung der deutschen gesellschaft für anästhesiologie und intensivmedizin und der deutschen gesellschaft für chirurgie. *Chirurg.* (2017) 88:867–70. doi: 10.1007/s00104-017-0506-0
48. Reutter F, Reuter DA, Hilgarth H, Heilek AM, Goepfert MS, Punke MA. Perioperative antibiotikaprophylaxe. *Anaesthesist.* (2014) 63:73–86. doi: 10.1007/s00101-013-2282-7
49. Schneider C, Grabein B. Antibiotikaprophylaxe und -therapie. In: in *Chirurgie Basisweiterbildung.* Berlin, Heidelberg: Springer Berlin Heidelberg (2013). p. 43–59. doi: 10.1007/978-3-642-23804-8_7
50. Zhang Y, Zhang J, Chen Y, Yu J, Cao G, Wu X, et al. Evaluation of meropenem penetration into cerebrospinal fluid in patients with meningitis after neurosurgery. *World Neurosurg.* (2017) 98:525–31. doi: 10.1016/j.wneu.2016.11.040
51. Sapan HB, Paturusi I, Islam AA, Yusuf I, Patellongi I, Massi MN, et al. Interleukin-6 and interleukin-10 plasma levels and mRNA expression in polytrauma patients. *Chinese J Traumatol.* (2017) 20:318–22. doi: 10.1016/j.cjtee.2017.05.003
52. Maier B, Schwerdtfeger K, Mautes A, Holanda M, Müller M, Steudel WI, et al. Differential release of interleukines 6, 8, and 10 in cerebrospinal fluid and plasma after traumatic brain injury. *Shock.* (2001) 15:421–6. doi: 10.1097/00024382-200115060-00002
53. Wutzler S, Lustenberger T, Relja B, Lehnert M, Marzi I. Pathophysiologie des Polytraumas: Intensivmedizin und "Timing" der Versorgung. *Chirurg.* (2013) 84:753–8. doi: 10.1007/s00104-013-2477-0
54. Gosling P, Dickson GR. Serum c-reactive protein in patients with serious trauma. *Injury.* (1992) 23:483–6. doi: 10.1016/0020-1383(92)90070-9
55. Neidhardt R, Keel M, Steckholzer U, Safret A, Ungethuem U, Trentz O, et al. Relationship of Interleukin-10 plasma levels to severity of injury and clinical outcome in injured patients. *J Trauma.* (1997) 42:863–70. doi: 10.1097/00005373-199705000-00017

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REVIEW
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Emerging Roles on Immunological Effect of Indoleamine 2,3-Dioxygenase in Liver Injuries

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Indoleamine 2,3-dioxygenase (IDO) is one of the initial rate-limiting enzymes of the kynurenine pathway (KP), which causes immune suppression and induction of T cell anergy. It is associated with the imbalance of immune homeostasis in numerous diseases including cancer, chronic viral infection, allergy, and autoimmune diseases. Recently, IDO has extended its role to liver field. In this review, we summarize the dysregulation and potentials of IDO in the emerging field of liver injuries, as well as current challenges for IDO targets. In particular, we discuss unexpected conclusions against previous work published. IDO is induced by pro-inflammatory cytokines in liver dysfunction and exerts an immunosuppressive effect, whereas the improvement of liver injury may require consideration of multiple factors besides IDO.

Keywords: IDO, liver injury, kynurenine pathway, immunoregulation, liver diseases

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INTRODUCTION

Tryptophan (Trp), as one of the nine essential amino acids, mediates energy metabolism, protein synthesis and significant bioactive molecular generation. In humans, Trp is only originated from food intake and mainly metabolized by the intestinal microbial pathway, serotonin pathway and kynurenine pathway (KP). The first two pathways consume a tiny fraction of free Trp. More than 95% of free Trp is metabolized through KP pathway to produce various key metabolites, which are extensively studied in immunology and neurology. Trp first generates N-formyl-L-kynurenine (NFK) by notable rate-limiting enzymes of Indoleamine 2,3-dioxygenase 1 (IDO1), IDO2 and tryptophan 2,3 -dioxygenase (TDO), further transforming into Kynurenine (Kyn). Kyn degradation includes three branches: (i) Kyn is metabolized into neuroactive and neurotoxic metabolites including 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic (3-HAA) and quinolinic acid (QA) by kynurenine monooxygenase (KMO) enzyme and other enzymes; (ii) Kyn directly catabolized to kynurenic acid (KA) by kynurenine aminotransferase (KAT) enzyme; (iii) Kyn further generated anthranilic (AA) by kynurenic (KYNA) enzyme (1, 2) (**Figure 1**).

IDO1, as the widely studied enzyme, is extensively located in various extra-hepatic cells and tissues under normal conditions but can be induced by pro-inflammatory factors such as interferon-γ (IFN-γ) during an immune response (3, 4). IDO2 shows 43% homology in amino acid sequence with IDO1 (5, 6) and located in brain, liver, thyroid and reproductive organs (6), but its mechanism is still uncertain. In this review, "IDO" hereafter refers to IDO1 or collective functional IDO enzyme activity unless otherwise specified. TDO is mainly present in the liver (3) and induced by corticosteroids, insulin and Trp (7). The most remarkable function of TDO

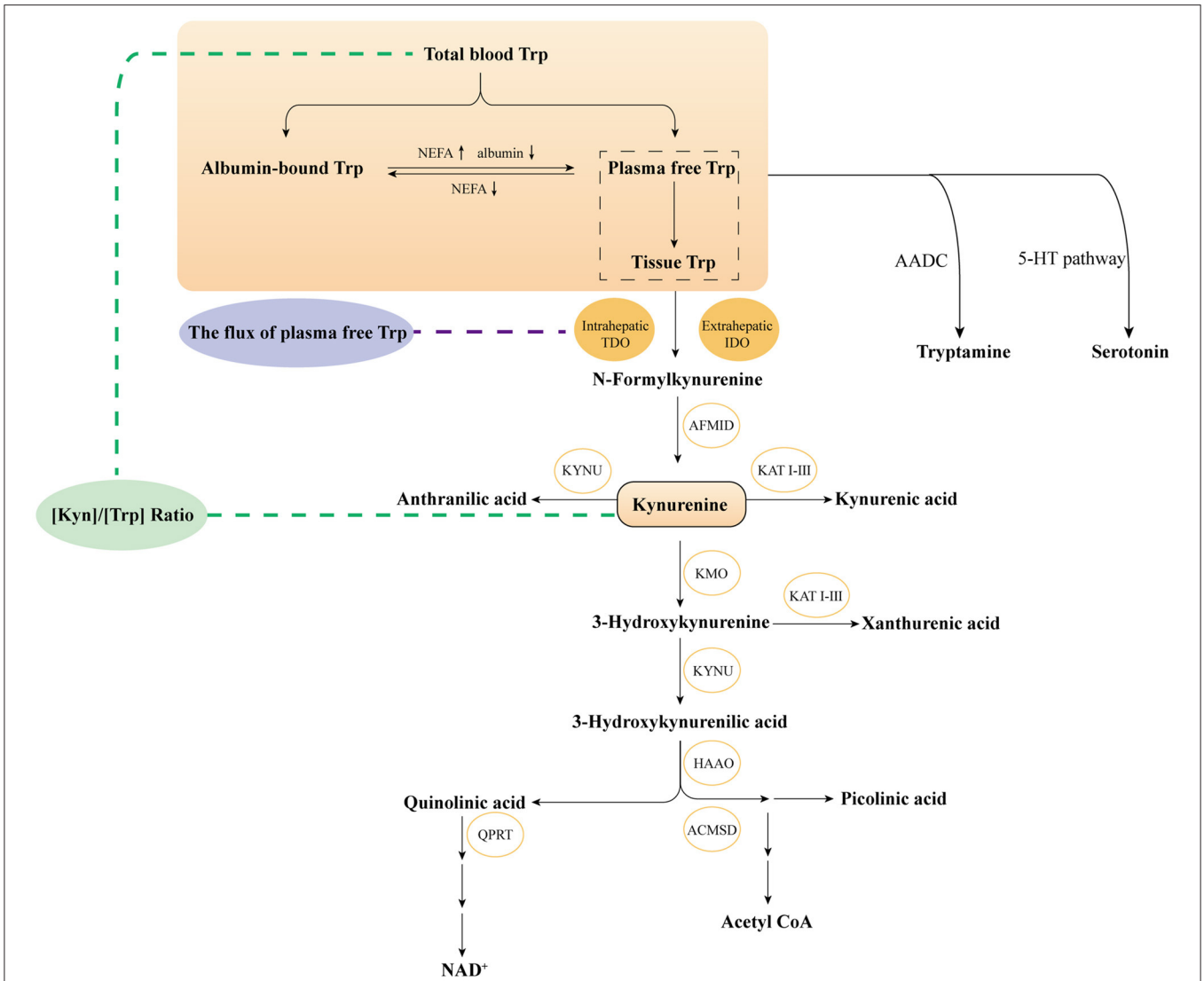

FIGURE 1 | Overviewthe KP of tryptophan metabolism. Trp, tryptophan; IDO, Indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; AFMID, kynurenine formamidase; KYNU, kynureninase; KAT I–III, kynurenine amino transferases I–III; KMO, kynurenine 3-monooxygenase; HAAO, 3-hydroxyanthranilate 3,4-dioxygenase; ACMSD, α-amino-β-carboxymuconate-ε- semialdehyde decarboxylase; QPRT, quinolinic acid phosphoribosyl transferase; 5-HT, 5-Hydroxytryptophan; AADC, aromatic-l-amino acid decarboxylase.

contributes to physiological system Trp level (8). It is noteworthy that TDO has a strict substrate specificity with only L-Trp-specific, while IDO can oxidize various substrates such as L-Trp and indoleamines (7, 9). In addition, the affinity for Trp is another crucial difference. IDO shows a much higher affinity with a Km of 3–50 μM in various sources. In contrast, TDO has very high Km values, whether in rat hepatocytes (100 μM), human liver (400 μM)or the purified human enzyme (190 μM) (10). The Trp metabolism contributes to immune regulation and has been well-covered in recent reviews (1, 11–13), whereas KP imbalance is related to numerous pathologies, including autoimmunity, viral infection, central nervous system (CNS) disorders, cardiovascular and cancer.

Intriguingly, although IDO1 with a physiological state is not expressed in the liver, recent studies on liver dysfunction have found that the liver's pathological state is significantly increased (1, 2, 7, 10, 14). Iwamoto and co-workers reported that IDO mRNA and protein expression of liver and hepatocytes were not detected in the control group, while those were significantly

Abbreviations: IDO, Indoleamine 2,3-dioxygenase; HCC, hepatocellular carcinoma; KP, kynurenine pathway; Trp, tryptophan; DCs, dendritic cells; TNF-α, tumor necrosis factor-α; IL-6, Interleukin-6; TGF-α, transforming growth factor-α; α-GalCer, α-galactosylceramide; 1-L-MT, Levo-1-methyl tryptophan; 1-D-MT, Dextro-1-methyl tryptophan; CCl_4, carbon tetrachloride; HFD, high-fat diet; Kyn, kynurenine; AHR, aryl hydrocarbon receptor; HCV, hepatitis C virus; HBV, hepatitis B virus; CTLs, cytotoxic T lymphocytes; mTOR, mammalian target of rapamycin; STAT3, signal transducer and activator of transcription 3.

enhanced in the acute hepatitis group, along with elevated IFN-γ and Kyn levels (15). Upregulation of IDO was consistent with previous results of HCV patients, and it was associated with IFN-γ induced by activated T cells in HCV-infected liver (16). Together, IDO dysregulation has been recorded in patients with viral hepatitis (15, 17), liver transplant (18, 19), autoimmune hepatitis (20) as well as hepatocellular carcinoma (HCC) (21, 22). Indeed, IDO expression and activity can be induced by numerous pro-inflammatory factors tumor necrosis factor-α (TNF-α), interleukin (IL)-6 (23–25), and IFN-γ (24, 26–31) and responsible for immune response. Moreover, the immune imbalance has been regarded as one of the prevailing mechanisms of liver injury (32). So, is the immunosuppressive effect of IDO related to liver dysfunction? A further investigation of IDO is urgent and available. Therefore, this review would like to discuss the functions of IDO and summarize current knowledge of IDO in liver dysfunction, along with the associated progress in therapeutically targeting IDO in each liver injury. Furthermore, the current challenges between KP and liver diseases will be especially emphasized, including the objective discussion of different conclusions to provide new strategies for future research and the development of clinical targets.

IMMUNOLOGICAL EFFECTS OF TRP METABOLISM

IDO was initially associated with establishing immune privilege and preventing T-cell-mediated allogeneic fetal rejection in mice (33). Since then, a growing line of evidence suggested IDO1 exerted crucial effects in orchestrating immune responses (34–39). It can be activated by numerous pro-inflammatory factors and T-helper cell-derived cytokines, including TNF-α, IL-6 (23–25), and IFN-γ (24, 26–31), and upregulated in different cell types including epithelial cells (23, 24, 40), macrophages (41), and dendritic cells (DCs) (42). Furthermore, enhanced IDO expression can also be modulated by inflammatory signals including transforming growth factor-α (TGF-α), nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) or transcription signal transducer and activator of transcription 3 (STAT3). These cytokines also drive liver-related inflammation and progress (32). The immunoregulatory effects mediated by IDO are mainly including as following ways: (i) Through Trp depletion, IDO inhibits the mammalian target of rapamycin (mTOR) kinase pathway and activates the General Control Non-depressible 2 (GCN2) kinase-dependent stress signaling pathway, thus inducing apoptosis and suppressing proliferation (43). However, more recent studies clarified GCN2 was activated only by extreme Trp shortage ($<1\,\mu M$) (43, 44). Thus the immunoregulatory roles of Trp metabolism are mainly caused by KP metabolites rather than depletion of Trp (45). (ii) Kyn and downstream catabolites induced by IDO can activate regulatory T (Treg) cells with stimulation of the aryl hydrocarbon receptor (AHR) pathway ultimately leading to T-cell function suppression (46). (iii) IDO mediates TGF-β-driven tolerance in pDCs, which has a non-enzymic function (47). By contrast, TDO mainly maintains blood Trp homeostasis, which is dramatically upregulated when present at supraphysiological blood Trp concentrations (48, 49). More recently, TDO expression was upregulated in human tumors and exhibited a similar immunosuppressive effect to benefit tumor cells (50). Overview of immune regulation pathways induced by IDO in humans is shown in **Figure 2**.

IDO AND CHEMICAL-INDUCED LIVER INJURY

Immunological Effects of IDO in Animal Models

The inhibition of IDO can aggravate or alleviate the severity of liver damage, which depends on the immunomodulatory effect of Trp metabolism and various experimental models. An experiment on the hepatitis mice model induced by α-galactosylceramide (α-GalCer) indicated that IDO could prevent excessive immune response to weaken liver injury in this model (51). This is the first research concentrated on the effects of IDO on acute liver injury. It is worth noting that α-GalCer-induced liver injury is thought to have the potential to partially mimic autoimmune hepatitis because it is caused by the activation and apoptosis of Vα14 NKT cells. These findings indicated that the expression of IDO can down-regulate the level of the proliferation of macrophages and natural killer (NK) cells and the TNF-αproducedby these immune cells inα-GalCer-induced liver injury. The authors inferred that IDO could prevent excessive immune response in α-GalCer-induced hepatitis model based on the above results.

The CCl_4-induced rodent models were intensively used to simulate liver fibrosis patients (52). The mechanism is primarily via trichloromethyl radicals metabolized, which destroys the biomembrane structure and triggers the inflammatory responses (53). It is reported that the liver injury in IDO-knockout (KO) mice treated with CCl_4 was exacerbated compared with WT mice (54). In the inflammatory condition caused by the injection of CCl_4, the authors observed IDO deficiency caused upregulation of pro-inflammatory cytokines and fibrogenic factors, contributing to the induction of activated hepatic stellate cells (HSCs) and the progression of liver fibrosis (54). Similarly, IDO inhibition with 1-D-MT after CCl_4 injection elevated the serum alanine aminotransferase (ALT) level and increased the severity of liver injury at 16 h after disease onset (55). 1-D-MT-treated rats produced higher TNF-α levels in the liver and IL-6 levels in the serum compared with those in mock and control groups, although no differences were measured in serum MIP-2 and keratinocyte chemoattractant (KC) levels (55). Both articles pointed to IDO activity upregulated after CCl_4 administration, while IDO inhibition exacerbated CCl_4-induced hepatitis with enhanced cytokine and chemokines. Interestingly, it is reported by Zhong et al. that IDO deficiency attenuated CCl4-induced cirrhosis (56). In addition, a more recent article (57) observed that $IDO2^{-/-}$ mice and administration of 1-D-MT or Kyn also prevented severe liver cell damage and liver fibrosis, which suggested that the mechanism is not attributable to the contribution of IDO1. The authors believed that Kyn produced

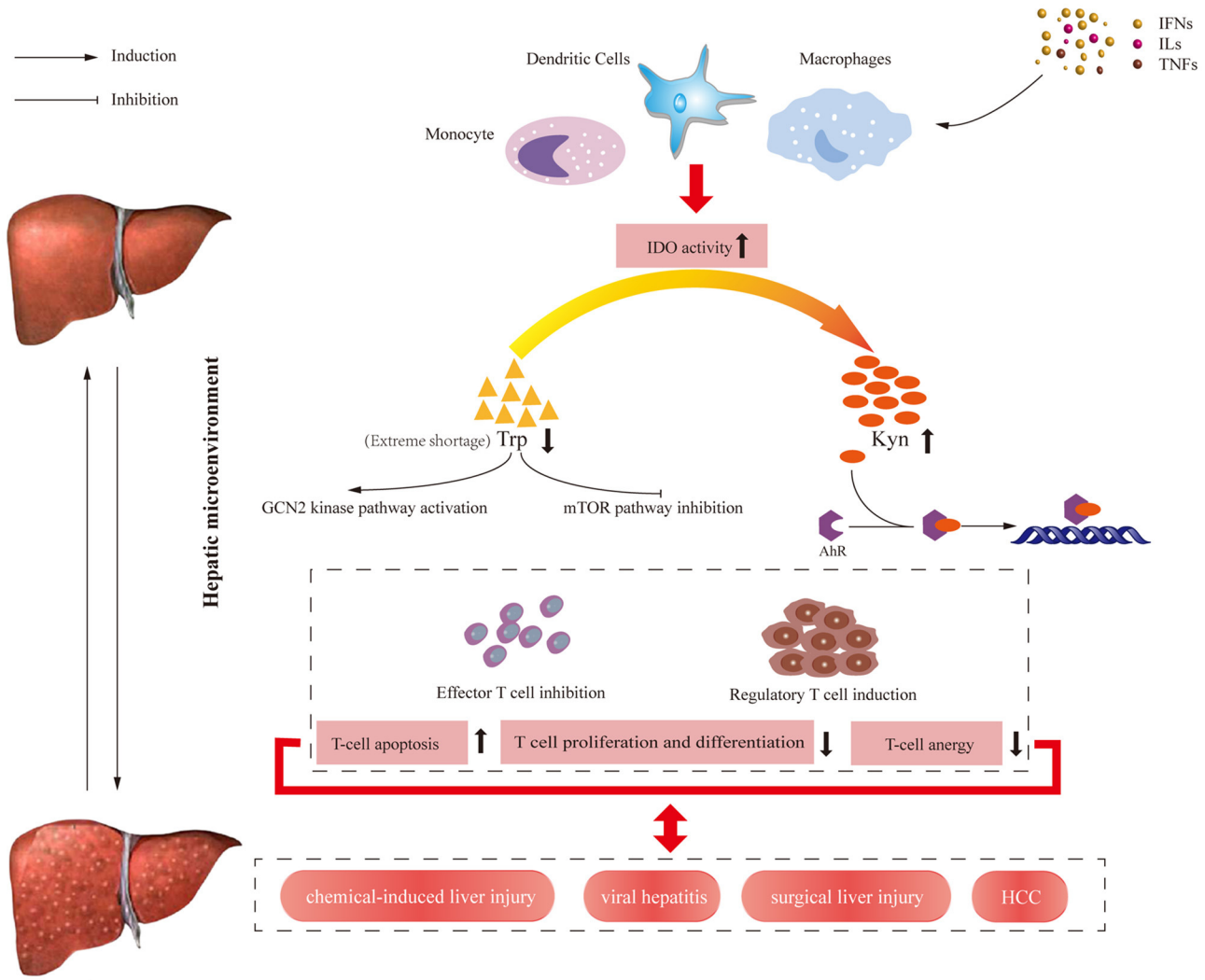

FIGURE 2 | Overview of immune regulation pathways induced by IDO enzyme in humans. At the transcriptional level, IDO enzyme is expressed by various cells of the immune system and activated by cytokines and other immunomodulatory molecules such as TNF-α, IL-6, and IFN-γ. On the one hand, IDO activity results in decreasing available Trp level, which activates the GCN2 kinase pathway and inhibits the mTOR pathway, leading to the reduced number of antigen-specific T cells. On the other hand, increasing Kyn level and KP metabolite concentrations induced by IDO activate AHR pathway, leading to the increased number of regulatory T cell. The results of these signal pathways contribute to the apoptosis of effector T cells and proliferation regulatory T cells. IFNs, interferons; ILs, interleukins; TNFs, tumor necrosis factors; IDO, Indoleamine 2,3-dioxygenase; HCC, hepatocellular carcinoma; Trp, tryptophan; Kyn, kynurenine; GCN2, general control non-depressible; AHR, aryl hydrocarbon receptor; mTOR, mammalian target of rapamycin.

by IDO2 in the liver might play a crucial role in CCl_4-induced acute liver injury through a mechanism involving AHR signal transduction (57). We found that the Ogiso's group (54) and the Li's group (55) only have taken IDO into account, whereas the Hoshi's group (57) and Zhong's group (56) considered IDO2 or TDO besides IDO1. Indeed, the different endings are possibly due to a compensatory mechanism among IDO1, IDO 2 and TDO. Previous evidence suggested TDO inhibition not only increased plasma and brain Trp but also the major Trp metabolite Kyn, which suggested a compensatory mechanism by extrahepatic IDO in the absence of intrahepatic TDO (58, 59). Moreover, a study reported TDO activity was also inhibited strongly by CCl_4 treatment (60), thus the incorporation of IDO1, IDO2 and TDO into liver dysfunction will be crucial revelation enlightenment.

Non-alcoholic steatohepatitis (NASH) is an inflamed fatty liver model subsequent to liver inflammation, fibrosis or even HCC. The animal model usually uses a high-fat diet (HFD)method. Nagano et al. reported that IDO gene silencing in the HFD-induced model aggravated hepatic inflammation and the progression of liver fibrosis. After being given pelleted HFD for 26 weeks, the IDO deficiency mice detected mixed

inflammatory cell infiltration, especially T lymphocytes and macrophages in the liver. The authors speculated that IDO deficiency increased the number of lymphocytes that migrated to the liver, thereby further exacerbating liver damage (61). However, the use of HFD has been demonstrated to regulate constant brain Trp by inhibiting TDO activity, as well as NEFA was increased caused by the HFD treatment (62). Therefore, only IDO-KO treated by HFD are not free from the interference of "external" modulating factors, such as the compensatory effect of the flux of free Trp and TDO activity caused by HFD. Moreover, this article calculated the L-Kyn/L-Trp ratio as the activity of IDO enzyme, which was controversial. What is more, liver TDO activity is another determinant of the ratio (63). We found the value of Kyn/Trp ratio in HFD-mice reported by Nagano et al. was ∼0.009, which was lower than a control ratio of 0.025 was reported by Ogawa et al. with the same procedure (64). It is likely contributed to the inhibition of TDO activity rather than the sole activity of IDO. Furthermore, the very low Kyn/Trp ratio in HFD mice further argues against IDO involvement. It is a pity that the authors did not report data on IDO in control mice.

Indeed, IDO deficiency does have beneficial effects in several other liver injury animal models, even though IDO expression was still increased in the model group. Using the concanavalin A (ConA)-induced liver injury mice model, Ting et al. observed that the inhibition or deficiency of IDO could alleviate murine liver damage by the reduction of inducible nitric oxide synthase (NOS) and 3-nitrotyrosine (65). As the T-cell mitotic plant lectin, ConA is recognized as a classic inducer for animal models of acute hepatitis, which can simulate the pattern of fulminant immunological liver injury (66, 67). ConA-induced hepatitis was recognized to be mainly related to ferroptosis, which was IDO-dependent. Noteworthy, *in vivo* and *in vitro* studies, IDO deficiency promoted the ferroptosis resistance by activating the expression of solute carrier family 7 member 11 (SLC7A11, also known as xCT), while reducing murine liver lesions and reactive nitrogen species (RNS) (65). In addition, another common bile duct ligation mice model also indicated IDO overexpression accelerated liver fibrosis and IDO-deficient fibrotic mice exhibited milder liver fibrosis than WT fibrotic mice by altered hepatic inflammatory cells (68). Researchers proved that IDO1 overexpression inhibited the maturation of CD11c + DCs in the liver and spleen, inhibited T cell proliferation mediated by mature DCs and worsened liver fibrosis, whereas in IDO1$^{-/-}$ mice the above pathological phenomena were reversed (69). Similarly, in diethylnitrosamine-induced HCC, IDO overexpression and higher Kyn levels were detected in IDO-wild-type mice compared to surrounding normal tissue. Furthermore, IDO-KO mice prevented the development of HCC, which was caused by the increasing the mRNA expression levels of CD8, perforin and granzyme B (70).

Based on these previous studies (**Table 1**), we found that a single discussion of the contribution of IDO on the liver is obviously over simplify the problem since Trp metabolism is a considerably complex pathway in liver injury. We speculated there might be existed a compensation mechanism besides IDO in KP, which brought to light a novel view in liver injuries (78).

Exploration of Therapeutic Targets for IDO

Insights Into Key Metabolites of KP

Studies on the metabolites of KP are widely used in various models of liver injury. By using wide-targeted metabolomics liquid chromatography-quadrupole time of flight mass spectrometry (LC-QTOF-MS) analysis, from the CCl_4-induced liver fibrosis rat model group, urinary and serum metabolomics L-Trp increased significantly from 2 to week 8, which can be regarded as effective biomarkers for the diagnosis of hepatic fibrosis and therapeutic targets (74). These results agreed with other liver injury models showing similar findings such as α-naphthyl isothiocyanate (ANIT) induced liver injury models or non-alcoholic fatty liver disease models, L-Trp was screened for potential biomarkers of the early detection of liver fibrosis (73, 75, 76). In patients, Clària et al. reported that KP activity showed a positive correlation with overall severity of cirrhosis. Furthermore, QA and KA were the most sensitive markers of KP activation (79). Thus, KP metabolites could provide a potential biomarker in the prognosis and diagnosis of liver dysfunction (80, 81).

IDO Inhibitors Applied in Animal Models of Chemical-Induced Liver Injury

Danshensu, which was identified as a novel IDO1 inhibitor by molecular docking and molecular dynamics analysis, was the main biologically active ingredient isolated from an edible traditional Chinese medicinal herb called *SalviaeMiltiorrhizae Radix et Rhizoma* (Danshen). Interestingly, fibrosis reduction and inhibition of IDO1 expression and STAT3 activity were observed *in vitro* TGF-β1-induced hepatic stellate cell model and *in vivo* CCl_4-induced rat hepatic fibrosis model after administration of Danshensu (82). Mechanistic studies indicated that Danshensu could inhibit JAK2-STAT3 signaling, which would further reduce the expression of IDO1 and downregulate the phosphorylation and nuclear localization of STAT3 (82). More importantly, overexpression of IDO1 diminished the anti-hepatic fibrosis effects of Danshensu. This study was critical preclinical data to suggest that reducing the IDO expression is beneficial to treating the liver injury. Meanwhile, another similar evidence was a characterized bioactive component isolated from the traditional Chinese medicinal herb *Panax ginseng C. A. Meyer* (Ginseng). Ginseng Rg1 also significantly reduced the aspartate transaminase (AST) and ALT expression levels in serum in CCl_4-induced liver fibrosis in mice (wild-type and those overexpressing IDO1 by *in vivo* AAV9 vector) and HSC-T6 cells (83). IDO1 can inhibit the maturation of DCs, and Ginseng Rg1 promoted the maturation of hepatic DCs by reducing the expression level of hepatic IDO1. In addition, oral administration of Ginseng Rg1 ameliorated the deterioration of liver fibrosis induced by IDO1 overexpression and the more pronounced inhibition of DCs maturation mediated by IDO1 overexpression (83). Notably, their therapeutic effect possibly was not achieved only by inhibiting IDO due to the complexity of traditional Chinese medicinal herbs, and the TDO activity and other factors have not been reported in both studies. Almost certainly, Trp metabolism including IDO exerts an important regulatory role in liver immunity.

TABLE 1 | Roles of IDO in animal models for liver injuries.

Year	Model	IDO status	Study design	Roles of IDO	Effect of IDO immune modulation	References
2009	HBV	+	Hepatitis B virus (HBV) transgenic (Tg) mice	Cytotoxic T lymphocytes transduction results in the upregulation of IDO, which might downregulate T-cell responsiveness	HBV infection facilitates the induction of IDO	(15)
2010	Liver transplantation	+	Rat orthotopic liver transplantations (OLT)	IDO may act as a local immunosuppressive molecule to protect transplanted cells, tissues and organs from immune attack	Protective effect against rat liver transplant rejection	(71)
2010	Attenuate liver injury	+	α-GalCer-inducedhepatitis	Decrease the number of TNF-α-producing immune cells in the liver	Protective effect against liver injury	(51)
2012	HBV	+	Hepatitis B virus (HBV)-transgenic (Tg)/IDO-knockout (KO) mice	IDO deficiency attenuated liver injury in HBV-Tg mice injected with HBV-specific CTL	Aggravated HBV	(72)
2012	Liver transplantation	+	Rat liver transplantation	The IDO level of KCs was closely associated with immune tolerance induction	Protective effect against rat liver transplant rejection	(18)
2012	Acute hepatic injury	+	CCl_4-induced hepatitis model	IDO deficiency exacerbated liver injury in CCl_4-induced hepatitis by inducing TNF-α and IL-6	Protective effect against liver injury	(55)
2013	Liver injury	NA	High-fat diet-induced hepatic inflammation and fibrosis	The deficiency of IDO may increase T cell activation, either directly or indirectly, by suppressing Tregs and thus contributed to a worsening of hepatic inflammation	Protective effect against hepatic fibrosis	(61)
2014	Liver injury	NA	The CCl_4 liver-injured rats	The level of Trp increased	Biomarker	(73)
2016	Hepatic fibrosis	+	The CCl_4-induced liver fibrosis in Mice	The deficiency of IDO aggravated the CCl_4-Inducedliver fibrosis in Mice by suppressing the inflammatory response induced by TNF-α	Protective effect against liver injury	(54)
2016	Hepatocarcinogenesis	-	DEN-induced hepatocarcinogenesis	IDO up-regulation may contribute to the development and progression of liver carcinogenesisbyinduction of both inflammation and an immunosuppressive microenvironment	Aggravated hepatocarcinogenesis	(70)
2017	Hepatic fibrosis	UK	CCl_4-induced liver fibrosis rat model	Trp level is changed at all time points and could be regarded as effective biomarkers for the early detection of liver fibrosis	Biomarkers	(74)
2017	Liver fibrosis	Humans,-; mice,+	CCl_4-induced liver fibrosis mice	IDO1 deficiency attenuated CCl_4-induced fibrosisthrough Th17 cells down-regulation andTDO compensation	Aggravated liver injury	(56)
2017	Liver fibrosis	NA	CCl_4-induced liver fibrosis rat model	Trplevel was increased	Biomarker	(74)
2018	Liver injury	NA	ANIT-induced liver injury in rats	Trp was identified as potential biomarkers of cholestasis	Biomarkers	(75)
2020	Liver injury	UK	CCl_4-induced acute liver injury	IDO2 deficiency attenuated CCl_4-induced acute liver injury by AHR pathway	Aggravated liver injury	(76)
2020	Acute immune hepatitis (AIH)	+	ConA-induced AIH mice	1-MT alleviated murine liver damage with the reduction of inducible nitric oxide synthase and 3-nitrotyrosine expression alleviated murine liver damage	Aggravated liver injury	(65)
2021	Idiosyncratic drug-induced liver injury (IDILI)	UK	PD-$1^{-/-}$mouse model of IDILI	1-D-MT decreased amodiaquine-induced liver injury in femalePD-$1^{-/-}$mice.	The immuneresponse has many redundant feedback mechanisms that canlead to paradoxical effects	(77)
2021	BDL	+	BDL mice model	IDO1 affects the process of immune cells recruitment via inhibiting DCs maturation and subsequent T cells proliferation, resulting in the promotion of hepatic fibrosis	Aggravated liver injury	(68)

In contrast to control group, the increase of IDO expression in model group is expressed as "+," the decrease of that as "-." UK, unknown; IDO, indoleamine 2,3 dioxygenase; Kyn, kynurenine; Trp, tryptophan.

ANTI-VIRAL INFECTION EFFECT OF IDO IN VIRAL HEPATITIS

IDO Against HBV and HCV Infection by Regulating Immune Tolerance Microenvironment

Bacterial and viral infections are critical risk factors for liver injury. Epidemiological data demonstrated that chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) are the most common forms of infectious liver disease. It is estimated that the total number of deaths due to liver disease caused by HBV is about 800,000, while about 500,000 deaths are caused by HCV (84). The HCV infection progression is possibly mediated by innate and adaptive immune responses in infected patients (85). Impairment of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses (86–88) and abnormal DCs function has been observed in HCV infection (89) as well as the upregulation of IDO expression in HCV infection (25). Persistent IDO expression within the liver microenvironment may play a crucial role in reducing HCV-specific T-cell responses (6). Yang et al. (90) revealed that plasma level of IDO was associated with TGF-β expression and severity of chronic HCV infection. In the acute infection stage, the expression of IDO1 is upregulated to promote the process of anti-viral defense, as IFN-γ did so in HCV-infected liver cells to inhibit anti-HCV T cells in the chronic infection phase.

Similar to HCV, IDO overexpression was associated with HBV. The expression and activity of IDO in patients with chronic HBV infection were significantly higher than those in healthy controls (91). Further studies explicated that IDO expression was correlated with HBV viral load and responsible for immunotolerance against HBV (91). HBV infection facilitated IDO induction, mainly through response to the hepatocyte pro-inflammatory cytokine IFN-γ in mice model studies. The up-regulation of IDO was accountable for transduction of cytotoxic T lymphocytes and ultimately down-regulated responsiveness of T cells (15). Similarly, the increase of IDO expression in the livers of murine fulminant hepatitis model induced by HBV-specific cytotoxic T lymphocytes (CTLs) was also examined in another study. Moreover, IDO inhibition could reduce liver injury, with Kyn and IFN-γ cooperatively involved in the progression of liver injury (72). IDO was demonstrated as a potential and novel favorable therapeutic target for chronic HBV infection (37, 91).

Possible Benefits of the Target IDO in Viral Hepatitis

IDO exhibited an essential role in HBV and HCV infections by constructing an immunotolerogenic microenvironment. Moreover, the prevailing theory is that the molecular mechanisms of the tolerogenic state are accompanied by chronic HBV and HCV infection, with only a weak response of CTLs against the HBV surface antigen (HBsAg). Nevertheless, another mechanism of IDO activity was discovered in a murine hepatitis model. Ito et al. immunized with a combination of α-GalCer, a specific agonist for NK inducing IDO and HBsAg on wild-type and IDO-KO mice. Only IDO-KO mice showed an increased expression of the cytokines IL-2 and IL-12b leading to the induction of HBsAg-specific CTLs (92). Besides, α-GalCer induced IDO production in $CD11b^+$ cells, which inhibited the proliferation of HBsAg-specific CTLs (92). These data suggested that inhibition of IDO activity enhanced the induction of HBsAg-specific CTLs after immunization with HBsAg and α-GalCer. Thus, IDO played the role of the central mediator in the IFN-γ-induced anti-viral response since it mediated Trp depletion followed by suppressing HBV or HCV replication. Additionally, published literature has shown that the inhibitory effect of IDO1 can be used to treat chronic HCV patients, and its mechanism may be attributed to the reduced inducible nitric oxide synthase and 3-nitrotyrosine expression (65, 93).

As described above, IDO suppressed the degree of the immune response in viral hepatitis. In this case, IDO inhibition could be expected to enhance the immune response and shield mice from viral infections. Nevertheless, Duhalde Vega et al. got contrary conclusions from a hepatitis virus A59 (MHV-A59) infected mice model that 1-L-MT aggravated liver injury, and the survival rate of MHV-infected animals treated with 1-L-MT was severely decreased compared to the control group without treatment of 1-L-MT (94). Excitingly, they continued their follow-up exploration and demonstrated TDO inhibition by LM10 resulted in decrease levels of the Ab to MHV induced by the same virus infection (78, 95). The two contrary responses might be clarified by the compensation mechanism controlling Trp metabolism (96). It is confirmed by Too et al. that deletion of the IDO1 or IDO2 gene does not alter brain Trp, Kyn or the Kyn/Trp ratio, whereas TDO2 deletion increases brain Trp without altering brain Kyn (78). The above evidence indicated that there might exist compensatory effects of TDO when IDO is inhibited. Therefore, inhibition of IDO alone cannot alter the progression of liver injury.

It is known that pro-inflammatory cytokines elevated caused by viral infections can induce IDO instead of TDO. Further, another study about the contribution between IDO and TDO explained that the Trp metabolism that occurred during infection was related to several KP metabolites involved in immune response mediated by IDO rather than the amount of Trp depletion (45, 96). Thus the pro-inflammatory Kyn metabolites mediated by IDO may be the real pathogenic factor in viral infections.

Notably, it was reported in this article by Duhalde Vega et al. that liver (Trp) also increased in MHV-infected mice, instead of the expected decrease compared with that of control (95). Badawy et al. supported that the flux of plasma free (non-albumin-bound) Trp was another vital factor (63). In MHV-induced hepatitis, the plasma free Trp may be upregulated, which is supported by evidence of decreasing albumin and increasing non-esterified fatty acids (NEFA) in hepatitis patients (81, 95, 97, 98). And the increase of the plasma free Trp promotes the flux of Trp through TDO.

Based on these, once TDO activity is inhibited by LM10 treatment on MHV-infected mice, the degradation of intrahepatic Trp mediated by TDO failed and the flux of Trp through TDO is prevented, leading to the decrease of pro-inflammatory Kyn metabolites.Consequently, the flux of Trp through TDO together with IDO induction enhanced

pro-inflammatory Kyn metabolites by two mechanisms in the MHV-infected mice.

Obviously, the immune response is very complicated, and the various effects on viral infection should not be simply interpreted as the sole IDO inhibition or Kyn decrease. Overall, the expression of IDO might have a significant effect on the prognosis of viral-induced patients. Trp metabolism may provide new strategies as an adjuvant therapy intervention for viral hepatitis.

IMMUNOLOGICAL EFFECT OF IDO IN SURGICAL LIVER INJURY

IDO Suppressing Liver Regeneration and Protecting Against Immune Rejection in Liver Transplantation

Compared with immune responses after a chemical- or viral-induced liver injury, post-hepatectomy and partial liver transplantation are also closely related to IDO activity by suppressing liver regeneration and protecting against immune rejection. This vital role of IDO in transplantation originated from a landmark study published in science magazine in 1998. Mellor et al. found that IDO suppressed the maternal T-cell response against fetus and inhibition of IDO with 1-MT caused abortion of allogeneic concepti but not syngeneic concepti (33). This work showed the most inspiring results of late years, which has completely changed the statement of IDO, from what was initially thought to be an innate defense mechanism to a potent immunomodulatory enzyme of which can down-regulate immune activation and inhibit T cell response and promote tolerance induction eventually. Since then, several studies on the effect of IDO in transplantation have been published. To determine whether IDO is the cause of liver tolerogenicity, Madeleine et al. studied IDO expression in liver-induced acceptance of kidney grafts in sensitized patients undergoing combined auxiliary liver-kidney transplantation. Combined auxiliary graft transplantation showed an increase of tryptophan degradation in peripheral blood and expression of IDO mRNA compared to regular renal transplantations (99). These results were proved by Xing et al. in a comparable rat experimental model where they demonstrated that IDO could be significantly overexpressed in rat livers after syngeneic and allograft liver transplantation compared to the sham-operated group (100). In addition, the results of immunohistochemistry showed that the number of IDO-positive cells was positively correlated with the exacerbation of rejection, and the level of inflammatory cell infiltration in the portal area was different *in vitro*, the expression of IDO gene and enzyme activity was increased in the IFN-γ-treated DC group within 7 days after transplantation compared to the untreated DC group and their survival rates were also significantly improved (100). Therefore, these results suggested that IDO might be involved in the spontaneous tolerance of liver allograft. IDO-positive DC induced by IFN-γ might relieve acute rejection and accelerate local tryptophan metabolism through IDO enzyme expression, resulting in post-liver transplantation immune tolerance (101, 102).

Besides living donor's liver transplantation, liver regeneration is another highly orchestrated process influenced by various factors. Liver regeneration is initiated immediately after the loss of hepatocytes for partial hepatectomy or partial liver transplantation, while its failure leads to hepatic injury and death. Hepatocyte proliferation is regulated by several growth factors such as TGF-α. Then hepatocytes need priming by TNF-α and IL-6, which are secreted by Kupffer cells, leading to the transition of growth factors from resting phase (G0) to interstitial phase (G1) (103, 104). Hideyuki et al. demonstrated that the liver-body weight ratio and the number of Ki-67-positive cells per field were significantly higher in IDO1-KO mice than WT mice. Likewise, the hepatic mRNA expression of cell cycle genes (cyclin D1, cyclin E) and pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) were significantly higher in IDO1-KO mice than in WT mice (105). Moreover, the administration of the 1-DL-MT at the time of transplantation resulted in promoting liver regeneration (105). Therefore, these results indicated that IDO1 suppressed the production of inflammatory cytokines and subsequently inhibited hepatocyte proliferation during liver regeneration.

Therapeutic Implications for IDO in Surgical Liver Injury

As previous studies described above, tryptophan catabolism via the IDO pathway is required for immune tolerance in allogeneic concepti rather than syngeneic concepti (33). Increasing evidence suggested that IDO might be a potential target to prevent acute rejection in spontaneously tolerant mouse liver allografts (106) and delayed rejection (107). Jerome et al. applied recombinant adeno-associated virus 2/8 (rAAV2/8) to deliver the transgene to allograft prior to transplantation (108). However, the median survival of recipients of allografts pretreated with recombinant adeno-associated virus 2/8–liver-specific promoter 1–IDO (rAAV2/8-LSP1-rIDO) vectors was 11–15 days, which was not significantly greater than that of the control group pretreated with recombinant adeno-associated virus 2/8–liver-specific promoter 1–enhanced green fluorescent protein (rAAV2/8-LSP1-eGFP) or untreated Piebald Virol Glaxo-to-Lewis liver allografts (108). These results indicated that transfecting single adenovirus-mediated IDO in rat liver allografts could not prevent acute rejection. Interestingly, Yakun et al. challenged to explore the effects of adenovirus-mediated combined genes of CTLA4Ig and IDO in the immune tolerance after orthotopic liver transplantation in rat models. Combined transfection of CTLA4Ig-IDO exhibited milder acute rejection and a higher survival rate in inducing immune tolerance after liver transplantation than the groups that using single adenovirus-mediated genes (CTLA4Ig or IDO) alone. These results were in line with previous reports indicating the therapeutic value of using DCs with IFN-γ-induced IDO expression to treat acute rejection after rat liver transplantation (100). The data exhibited a positive correlation between increased IDO-positive cells in the portal area and the severity of rejection. Meanwhile, experimental results from other *in vivo* models also pointed out that although IDO expression is upregulated in rejected grafts, merely IDO does not play a crucial role in the

process of acute rejection (108–112). This result may be caused by a strong rejection process that IDO cannot reverse (113). Therefore, the combined application of IDO and other targets may become a feasible and valid method for immunological tolerance in liver transplantation and may become a promising clinical method for treating immune liver disease.

THE IMMUNOSUPPRESSIVE ROLE OF IDO IN HCC

The Mechanisms of IDO Regulating Immune Responses in HCC

HCC is the third most common cause of cancer-associated deaths worldwide and confers a poor prognosis (114). Tumor-immune escape mechanisms have been currently proposed as emerging topics that were involved in HCC progression. Therefore, it has begun to focus on tumor immunology to reveal the immunosuppressive effect of IDO recently.

The prevailing theory suggested IDO has a tumor-promoting outcome in the occurrence and development of some solid tumors through immunosuppressive effects (115–117). IDO expression in various histologic cancer types seemed to build an immune-suppressive microenvironment (118, 119) by regulating immune cells including T effector cells (120), regulatory T cells (121), and Myeloid-derived suppressors cells (MDSC) (122, 123). In HCC pathogenesis, IDO was expressed in HCC cells after IFN-γ stimulation, which was a prognostic factor for poor survival of HCC patients (115, 124). One new subset of human $CD14^+$ $CTLA\text{-}4^+$ regulatory dendritic cells ($CD14^+$DCs) was identified in HCC patients, which significantly suppressed T cell response *in vitro* via IDO (125). This finding added new insights into HCC induced immunosuppression mechanism related to IDO. Another study indicated that expression of PGE2 and IDO by HCC-associated fibroblasts might represent a novel mechanism by inducing NK cell dysfunction and creating favorable conditions for tumor progression (126).

Interestingly, some contradictory data demonstrated that induction of the IDO enzyme by IFN-γ exhibited opposite anticancer immune reactions in tumor-infiltrating cells of HCC. The recurrence-free survival rate of IDO-positive HCC patients was significantly higher than that of IDO-negative HCC patients, which was the first report to suggest that IDO expression at the molecular level might be essential for TIL to suppress tumor proliferation in HCC (127). Notably, IFN-γ is known to antitumor, namely a low, rather than a high IFN-γ level predicts HCC recurrence after therapy (128, 129). Indeed, the elevation of IFN-γ in HCC may reflect liver dysfunction rather than inflammation (130). Thus, besides IDO, the role of IFN-γ is not exclusive to the present experiment by Ishio et al. In addition, IDO was strongly induced in HCC cells following IFN-γ does not simply mean that IDO is expressed (strongly or otherwise) in HCC. Likewise, mRNA expression of IDO in HCC patients was performed in this article, which is also not always synonymous with IDO enzyme functional activity (129, 130). The mechanisms of primary and secondary resistant tumors were complex, including factors related to both the tumor and the host itself. Among them, immune metabolism dysregulation played an eventful role in the disease development of HCC patients.

Therapeutic Implications of IDO for HCC

IDO Inhibitors

Using both subcutaneous and hepatic orthotopic models, Zachary et al. (131) found that adding IDO inhibitor1-D-MT can increase the therapeutic efficacy in resistant HCC tumors induced by high IDO. The negative results of current clinical trials for other tumors like ECHO-301 trial (132) made some researchers doubt about IDO inhibition strategy. The above discussion mentioned in this review, for different conclusions of IDO in various liver injuries, just provides some enlightenment in IDO/TDO target drug development. Indeed, new IDO inhibitors are under clinical trials, such as navoximod (NLG-919), an oral inhibitor that can inhibit IDO1 and TDO, and BMS-986205, an irreversible IDO1 inhibitor (133). Of course, we also got some excellent inspiration from off-target data of IDO inhibitors. The iper-activation of the mTOR pathway, the gut microbiota alteration, and the prolonged activation of AHR might be the main reasons for these compounds' lack of efficacy (134). In addition, these off-target effects could be expected to find new combinations and predictive biomarkers to select the most suitable crowd (135). Immune checkpoint blockade anti-CTLA-4 treatment increased IDO induction in RIL-175 tumor cells via IFN-γ (131), and similar findings were observed with anti-PD-1 therapy (131). These shreds of evidence indicated that IDO was still encouraging as an immune checkpoint inhibitor in HCC.

Based on the tumor-promoting effects of IDO and TDO, studies seeking small-molecule inhibitors for cancer treatment have been ongoing for recent years, including KHK2455, Epacadostat (INCB 024360), Indoximod, Linrodostat (BMS-986205) and Celecoxib (http://www.clinicaltrials.gov/). Ongoing results are shown in **Table 2**. These IDO inhibitors showed promising results in the treatment of patients with advanced malignancies. Furthermore, BMS-986205, a highly potent and highly selective IDO1 inhibitor, is currently evaluated in Phase I/II clinical trials combined with the PD-1 inhibitor nivolumab as first or second-line therapy to HCC patients (**Table 2**). The current research phase is recruiting, and the estimated study completion date is June 1, 2022, which is the current clinical trial of IDO inhibitors in liver cancer and has essential incentive significance for the future treatment of IDO inhibitors in the field of liver disease. In addition, another immune checkpoint blockade with anti-CTLA-4 and anti-PD-1 bifunctional antibodies has been approved for various advanced malignancies, including HCC (114, 136). The evidence above indicates that IDO inhibitors could provide better synergistic effects with other targeted immunotherapies and should prioritize clinical evaluation in HCC.

Prognostic Factors

Increasing pieces of evidence indicate that IDO inhibitors have great potential for the treatment of HCC patients. However, in fact, the immune responses among patients are widely different. This phenomenon may be closely related to the difference in IDO expression level of HCC-tumors, explaining why several

TABLE 2 | List of current investigated IDO1 inhibitors. Information about the above trials can be accessed at https://www.clinicaltrials.gov/.

IDO	Notes	Indications	Phase	Status	Mechanism	Reference
IDO peptides	Nivolumab and PD-L1/IDO peptide vaccine	Metastatic melanoma	Phase 1 Phase 2	Recruiting	Combination therapy with nivolumab and PD-L1/IDO peptide vaccine to patients with metastatic melanoma	NCT03047928
KHK2455	Combined with avelumab	Urothelial carcinoma	Phase 1	Recruiting	KHK2455 (IDO inhibitor) plus avelumab in adult subjects with advanced bladder cancer	NCT03915405
Epacadostat	Combined with pembrolizumab	Sarcoma	Phase 2	Active, not recruiting	A study of Epacadostat, an IDO1 inhibitor, in combination with pembrolizumab in patients with metastatic and/or locally advanced sarcoma	NCT03414229
Indoximod	Combined with docetaxel	Non-small cell lung cancer Progression of non-small cell lung cancer Non-small cell lung cancer recurrent	Phase 1	Active, not recruiting	Immunotherapy combination study in advanced previously treated non-small cell lung cancer	NCT02460367
Epacadostat (INCB 024360)	Combined with CDX-1401 and poly ICLC	Fallopian tube carcinoma Ovarian carcinoma Primary peritoneal carcinoma	Phase 1 Phase 2	Active, not recruiting	DEC-205/NY-ESO-1 fusion protein CDX-1401, Poly ICLC, and IDO1 inhibitor INCB024360 in treating patients with ovarian, fallopian tube, or primary peritoneal cancer in remission	NCT02166905
Linrodostat (BMS-986205)	Combined with nivolumab	Endometrial adenocarcinoma Endometrial carcinosarcoma	Phase 2	Recruiting	Study of BMS-986205 and nivolumab in endometrial cancer or endometrial carcinosarcoma that has not responded to treatment	NCT04106414
Celecoxib 200 mg capsule	As single agent	Endometrium cancer	Phase 2	Recruiting	Neoadjuvant Celecoxib in newly diagnosed patients with endometrial carcinoma	NCT03896113
Linrodostat (BMS-986205)	Combined with relatlimab and nivolumab	Advanced cancer	Phase 1 Phase 2	Recruiting	An investigational study of immunotherapy Combinations in participants with solid cancers that are advanced or have spread	NCT03459222
Linrodostat (BMS-986205)	Combined with nivolumab and temozolomide	Glioblastoma	Phase 1	Recruiting	Nivolumab, BMS-986205, and radiation therapy with or without temozolomide in treating patients with newly diagnosed glioblastoma	NCT04047706
Epacadostat (INCB 024360)	Combined with pembrolizumab	Head and neck cancer	Phase 3	Active, not recruiting	Pembrolizumab plus Epacadostat, pembrolizumab monotherapy, and the extremeregimen in recurrent or metastatic head and neck squamous cell carcinoma (KEYNOTE-669/ ECHO-304)	NCT03358472
Epacadostat (INCB 024360)	Combined with pembrolizumab	Renal cell carcinoma (RCC)	Phase 3	Active, not recruiting	Pembrolizumab (MK-3475) plus Epacadostat vs. standard of care in mRCC (KEYNOTE-679/ ECHO-302)	NCT Number
Epacadostat (INCB 024360)	Combined with pembrolizumab	Metastatic pancreatic adenocarcinoma	Phase 2	Recruiting	Epacadostat, pembrolizumab, and CRS-207, with or without CY/GVAX pancreas in patients with metastatic pancreas cancer	NCT03260894
Indoximod	Combined with chemotherapy and radiation	Glioblastoma Medulloblastoma Ependymoma Diffuse intrinsic pontine glioma	Phase 2	Recruiting	Pediatric trial of Indoximod with chemotherapy and radiation for relapsed brain tumors or newly diagnosed diffuse intrinsic pontine glioma	NCT03006302

immune IDO inhibitor monotherapy studies show disillusionary results such as ECHO-301 (NCT02752074). Subsequently, the high level of IDO expression was established as an important prognostic factor for the overall survival of HCC patients (115). Moreover, IDO overexpression on tumors was significantly correlated with high metastasis rates (124). Thus, IDO may be a novel favorable prognostic indicator for HCC. However, elevated levels of IDO1 are associated with poor patient prognosis in some circumstances (124, 137), but this is not always the case (127, 138). Thus, the disease-free survival was associated with

the level of IDO1 expression in HCC patients. Moreover, there is a direct proportion between IDO1 expression levels and the ability of peripheral blood mononuclear cells of HCC patients to lyse HCC cell lines *in vitro* (127). These combined observations indicate that IDO1 may have great potential as a marker of prognosis in HCC patients.

Interestingly enough, IDO overexpression on tumors may have essential implications for immune-checkpoint therapy (139). It would be ideal for the prediction of patients for the degree of the immune response. The univariate analysis for overall survival (OS) showed that patients of early non-small cell lung cancer with higher levels of both genes (PD-L1/IDO-2) or (PD-L2/IDO-1) were associated with a worse OS. High levels of PD-L1/IDO-2 and PD-L2/IDO-1 co-expression have been independent negative prognostic factors (139). There are crucial implications of the features of IDO mentioned above for future immune checkpoint therapy (140).

An effective combination of immunomodulators and other treatments is another challenge for identifying predictive biomarkers (136). Recent studies indicated that combined multiple therapeutic options, such as combinations of anti-CTLA-4 with anti-PD-1/PD-L1 agents or combinations of anti-PD-1/PD-L1 agents and conventional treatments, have the most significant potential for successful treatments (141). The latest research directions are investigation in other biomarkers such as microsatellite instability, tumor mutational burden, BRAF and polybromo 1 (116, 142). These immune checkpoint inhibitors have shown unprecedented potentials based on positive results of biomarkers in multiple tumors.

CONCLUSION

To date, the increasing interest in liver dysfunction has been associated with IDO enzyme. We summarize the immunomodulatory role of IDO, current treatment advances and challenges in various liver injury models including chemical-induced liver injury, viral liver injury, surgical liver injury and HCC. However, emerging contrary outcomes occurred, which attracts some arguments against the involvement of IDO enzyme in liver dysfunction even in Trp metabolism. Surprisingly, IDO is an integral part of KP in regulating liver dysfunction, which might be compensated with other factors such as TDO. The essential part of KP in the liver which is related to various KP metabolites, result in immune response and immunoregulation mediated by the activity of IDO rather than the amount of metabolic tryptophan. TDO might also be responsible for the degradation of intrahepatic Trp (45, 96). However, the relationship between "quantity" and "effect" is equally important, thus we need to consider other crucial factors, including the activity of IDO, TDO, Kyn monooxygenase and kynureninase, the flux of plasma free (non-albumin-bound), Trp through TDO, the Kyn/Trp ratio, the level of KP metabolites and any other factor that alters plasma Kyn disposition (63). In addition, we gave a more rational appraisal against some aspects of present IDO research, such as the somewhat indiscriminate use of the ratio in plasma or serum of concentration of Kyn to that of Trp, i.e., the (Kyn)/(Trp) ratio, to express IDO activity. Thus, it may be more informative to assess the IDO or TDO status by other means, i.e., mRNA expression and enzymatic activity. It is good enlightenment for future studies on the mechanism of KP in liver dysfunction, even other diseases. For instance, this review may provide some new inspired movements of the expected promising IDO1 inhibitors in initial studies but terminated in recent phase III trial. Dual IDO and TDO inhibitors or effective combination of other immunomodulators may also have great prospects.

AUTHOR CONTRIBUTIONS

LX and JL reviewed the literature and wrote the manuscript. CS, YX, and Y-WS reviewed and revised the manuscript. ZJ reviewed the literature and contributed to the conceptualization of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. arszalek-Grabska M, Walczak K, Gawel K, Wicha-Komsta K, Turski WA. Kynurenine emerges from the shadows – current knowledge on its fate and function. *Pharmacol Ther.* (2021) 225:107845. doi: 10.1016/j.pharmthera.2021.107845
2. Knyihár-Csillik E, Chadaide Z, Mihály A, Krisztin-Péva B, Fenyo R, Vécsei L. Effect of 6-hydroxydopamine treatment on kynurenine aminotransferase-I (KAT-I) immunoreactivity of neurons and glial cells in the rat substantia nigra. *Acta Neuropathol.* (2006) 112:127–37. doi: 10.1007/s00401-006-0086-4
3. Capece L, Arrar M, Roitberg AE, Yeh SR, Estrin DA. Substrate stereo-specificity in tryptophan dioxygenase and indoleamine 2,3- dioxygenase. *Proteins.* (2010) 78:2961–72. doi: 10.1002/prot.22819
4. Stefan Löb AK, Derek Zieker, Björn LDMB, Hans-Georg R, Gerhard O, Peter T. IDO1 and IDO2 are expressed in human tumors: levo- but not dextro-1-methyl tryptophan inhibits tryptophan catabolism. *Cancer Immunol Immunother.* (2009) 58:153–7. doi: 10.1007/s00262-008-0513-6
5. Merlo L, Duhadaway JB, Montgomery JD, Peng WD, Mandik-Nayak L. Differential roles of IDO1 and IDO2 in T and B Cell inflammatory immune responses. *Front Immunol.* (2020) 11:1861. doi: 10.3389/fimmu.2020.01861
6. Helen JB, Angeles S-P, Silvia W, Christopher JDA, Florian A, Jenny M, et al. Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. *Gene.* (2007) 396:203–13. doi: 10.1016/j.gene.2007.04.010
7. Badawy AAB. Kynurenine pathway of tryptophan metabolism: regulatory and functional aspects. *Int J Tryptophan Res.* (2017) 10:117864691769193. doi: 10.1177/1178646917691938

8. Platten M, Nollen EAA, Röhrig UF, Fallarino F, Opitz CA. Tryptophan metabolism as a common therapeutic target in cancer, neurodegeneration and beyond. *Nat Rev Drug Discov.* (2019) 18:379–401. doi: 10.1038/s41573-019-0016-5
9. Pallotta MT, Rossini S, Suvieri C, Coletti A, Orabona C, Macchiarulo A, et al. Indoleamine 2,3-dioxygenase 1 (IDO1): an up-to-date overview of an eclectic immunoregulatory enzyme. *FEBS J.* (2021). doi: 10.1111/febs.16086
10. Badawy AB. Tryptophan availability for kynurenine pathway metabolism across the life span: control mechanisms and focus on aging, exercise, diet and nutritional supplements. *Neuropharmacology.* (2017) 112:248–63. doi: 10.1016/j.neuropharm.2015.11.015
11. Jones SP, Franco NF, Bianca V, Gayathri S, Brown DA, Josien DB, et al. Expression of the kynurenine pathway in human peripheral blood mononuclear cells: implications for inflammatory and neurodegenerative disease. *PLoS ONE.* (2015) 10:e0131389. doi: 10.1371/journal.pone.0131389
12. Zou MH. Tryptophan-kynurenine pathway is dysregulated in inflammation, and immune activation. *Front Biosci.* (2015) 20:1116–43. doi: 10.2741/4363
13. Sorgdrager FJH, Naudé PJW, Kema IP, Nollen EA, Deyn PPD. Tryptophan metabolism in inflammaging: from biomarker to therapeutic target. *Front Immunol.* (2019) 10:2565. doi: 10.3389/fimmu.2019.02565
14. Prendergast GC, Malachowski WJ, Mondal A, Scherle P, Muller AJ. Indoleamine 2,3-Dioxygenase and its therapeutic inhibition in cancer. *Int Rev Cell Mol Biol.* (2017) 336:175–203. doi: 10.1016/bs.ircmb.2017.07.004
15. Iwamoto N, Ito H, Ando K, Ishikawa T, Hara A, Taguchi A, et al. Upregulation of indoleamine 2,3-dioxygenase in hepatocyte during acute hepatitis caused by hepatitis B virus-specific cytotoxic T lymphocytes in vivo. *Liver Int.* (2009) 29:277–83. doi: 10.1111/j.1478-3231.2008.01748.x
16. Larrea E, Riezu-Boj JI, Gil-Guerrero L, Casares N, Aldabe R, Sarobe P, et al. Upregulation of indoleamine 2,3-dioxygenase in hepatitis C virus infection. *J Virol.* (2007) 81:3662–6. doi: 10.1128/JVI.02248-06
17. Yoshio S, Kanto T. Host–virus interactions in hepatitis B and hepatitis C infection. *J Gastroenterol.* (2016) 51:409–20. doi: 10.1007/s00535-016-1183-3
18. Luan X, Liao W, Lai X, He Y, Liu Y, Gong J, et al. Dynamic changes of indoleamine 2,3-dioxygenase of kupffer cells in rat liver transplant rejection and tolerance. *Transplant Proc.* (2012) 44:1045–7. doi: 10.1016/j.transproceed.2012.01.033
19. Gerald Brandacher, Raimund Margreiter, Fuchs D. Implications of IFN-gamma-mediated tryptophan catabolism on solid organ transplantation. *Curr Drug Metab.* (2007) 8:273–82. doi: 10.2174/138920007780362536
20. Lytton SD, Osiecki M, Cukrowska B, Wierzbicka A, Goliszek M, Socha P, et al. Tryptophan-kynurenine profile in pediatric autoimmune hepatitis. *Immunol Res.* (2019) 67:39–47. doi: 10.1007/s12026-019-9068-1
21. Asghar K, Farooq A, Zulfiqar B, Rashid MU. Indoleamine 2,3-dioxygenase: as a potential prognostic marker and immunotherapeutic target for hepatocellular carcinoma. *World J Gastroenterol.* (2017) 23:2286. doi: 10.3748/wjg.v23.i13.2286
22. De Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer.* (2006) 6:24–37. doi: 10.1038/nrc1782
23. Xiao Y, Christou H, Liu L, Visner G, Mitsialis SA, Kourembanas S, et al. Endothelial indoleamine 2,3-dioxygenase protects against development of pulmonary hypertension. *Am J Respir Crit Care Med.* (2013) 188:482–91. doi: 10.1164/rccm.201304-0700OC
24. Sakash JB, Byrne GI, Lichtman A, Libby P. Cytokines induce indoleamine 2,3-dioxygenase expression in human atheroma-associated cells: implications for persistent chlamydophila pneumoniae infection. *Infect Immun.* (2002) 70:3959–61. doi: 10.1128/IAI.70.7.3959-3961.2002
25. Koyo H, Tatsuya K, Shoko K, Sachiyo Y, Tokuhiro M, Naruyasu K, et al. Association of enhanced activity of indoleamine 2,3-dioxygenase in dendritic cells with the induction of regulatory T cells in chronic hepatitis C infection. *J Gastroenterol.* (2013) 48:660–70. doi: 10.1007/s00535-012-0667-z
26. Mailankot M, Nagaraj RH. Induction of indoleamine 2,3-dioxygenase by interferon-gamma in human lens epithelial cells: apoptosis through the formation of 3-hydroxykynurenine. *Int J Biochem Cell Biol.* (2010) 42:1446–54. doi: 10.1016/j.biocel.2010.04.014
27. Alberati-Giani D, Ricciardi-Castagnoli P, Khler C, Cesura AM. Regulation of the kynurenine pathway by IFN-γ in murine cloned macrophages and microglial cells. *J Neurochem.* (2002) 66:996–1004. doi: 10.1046/j.1471-4159.1996.66030996.x
28. Mellor A. IDO expression by dendritic cells : tolerance and tryptophan catabolism. *Nat Rev Immunol.* (2004) 4:762–74. doi: 10.1038/nri1457
29. Guillonneau C, Hill M, Hubert FX, Chiffoleau E, Anegon I. CD40Ig treatment results in allograft acceptance mediated by CD8CD45RC T cells, IFN-gamma, and indoleamine 2,3-dioxygenase. *J Clin Invest.* (2007) 117:1096–106. doi: 10.1172/JCI28801
30. Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J Immunol.* (2000) 164:3596–9. doi: 10.4049/jimmunol.164.7.3596
31. Reena Rani MBJ, Senad D, De'Broski RH. IFN-γ-Driven IDO production from macrophages protects IL-4Rα-deficient mice against lethality during schistosoma mansoni infection. *Am J Pathol.* (2012) 180:2001–8. doi: 10.1016/j.ajpath.2012.01.013
32. Kubes P, Jenne C. Immune responses in the liver. *Annu Rev Immunol.* (2018) 36:247–77. doi: 10.1146/annurev-immunol-051116-052415
33. Munn D, Zhou M, Attwood J, Bondarev I, Conway S, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science.* (1998) 281:1191–3. doi: 10.1126/science.281.5380.1191
34. Barth H, Raghuraman S. Persistent infectious diseases say – IDO. Role of indoleamine-2,3-dioxygenase in disease pathogenesis and implications for therapy. *Crit Rev Microbiol.* (2012) 40:360. doi: 10.3109/1040841X.2012.742037
35. Mangge H, Stelzer I, Reininghaus EZ, Weghuber D, Postolache TT, Fuchs D. Disturbed tryptophan metabolism in cardiovascular disease. *Curr Med Chem.* (2014) 21:1931–7. doi: 10.2174/09298673216661403041 05526
36. Filippini P, Del Papa N, Sambataro D, Del Bufalo A, Locatelli F, Rutella S. Emerging concepts on inhibitors of indoleamine 2,3-dioxygenase in rheumatic diseases. *Curr Med Chem.* (2012) 19:5381–93. doi: 10.2174/092986712803833353
37. Modoux M, Rolhion N, Mani S, Sokol H. Tryptophan metabolism as a pharmacological target. *Trends Pharmacol Sci.* (2021) 42:60–73. doi: 10.1016/j.tips.2020.11.006
38. Le Naour J, Galluzzi L, Zitvogel L, Kroemer G, Vacchelli E. Trial watch: IDO inhibitors in cancer therapy. *OncoImmunology.* (2020) 9:1777625. doi: 10.1080/2162402X.2020.1777625
39. Liu XH, Zhai XY. Role of tryptophan metabolism in cancers and therapeutic implications. *Biochimie.* (2021) 182:131–9. doi: 10.1016/j.biochi.2021.01.005
40. Wang Q, Zhang M, Ding Y, Wang Q, Zhang W, Song P, et al. Activation of NAD(P)H oxidase by tryptophan-derived 3-hydroxykynurenine accelerates endothelial apoptosis and dysfunction in vivo. *Circ Res.* (2014) 114:480–92. doi: 10.1161/CIRCRESAHA.114.302113
41. Bogdan C. Macrophages as host, effector and immunoregulatory cells in leishmaniasis: impact of tissue micro-environment and metabolism. *Cytokine X.* (2020) 2:100041. doi: 10.1016/j.cytox.2020.100041
42. Yun TJ, Lee JS, Machmach K, Shim D, Choi J, Wi YJ, et al. Indoleamine 2,3-dioxygenase-expressing aortic plasmacytoid dendritic cells protect against atherosclerosis by induction of regulatory T cells. *Cell Metab.* (2016) 23:852–66. doi: 10.1016/j.cmet.2016.04.010
43. Munn DH, Sharma M, Baban B, Harding HP, Zhang Y, Ron D, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity.* (2005) 22:633–42. doi: 10.1016/j.immuni.2005.03.013
44. Sonner JK, Deumelandt K, Ott M, Thomé C, Platten M. The stress kinase GCN2 does not mediate suppression of antitumor T cell responses by tryptophan catabolism in experimental melanomas. *Oncoimmunology.* (2016) 5:e1240858. doi: 10.1080/2162402X.2016.1240858
45. Badawy AB, Namboodiri A, Moffett JR. The end of the road for the tryptophan depletion concept in pregnancy and infection. *Clin Sci.* (2016) 130:1327–33. doi: 10.1042/CS20160153
46. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J Exp Med.* (1999) 189:1363–72. doi: 10.1084/jem.189.9.1363

47. Pallotta MT, Orabona C, Volpi C, Vacca C, Grohmann U. Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nat Immunol.* (2011) 12:870–8. doi: 10.1038/ni.2077
48. van Baren N, Van den Eynde BtJ. Tryptophan-degrading enzymes in tumoral immune resistance. *Front Immunol.* (2015) 6:34. doi: 10.3389/fimmu.2015.00034
49. Knox WE. Two mechanisms which increase in vivo the liver tryptophan peroxidase activity: specific enzyme adaptation and stimulation of the pituitary-adrenal system. *Br J Exp Pathol.* (1951) 32:462–9.
50. Chen LB, Zhu SP, Liu TP, Zhao H, Hu R. Cancer associated fibroblasts promote renal cancer progression through a TDO/Kyn/AhR dependent signaling pathway. *Front Oncol.* (2021) 11:628821. doi: 10.3389/fonc.2021.628821
51. Ito H, Hoshi M, Ohtaki H, Taguchi A, Ando K, Ishikawa T, et al. Ability of IDO to attenuate liver injury in α-galactosylceramide–induced hepatitis model. *J Immunol.* (2010) 185:4554–60. doi: 10.4049/jimmunol.0904173
52. Chatamra K, Proctor E. Phenobarbitone-induced enlargement of the liver in the rat: its relationship to carbon tetrachloride-induced cirrhosis. *Br J Exp Pathol.* (1981) 62:283–8.
53. Tomasi A, Albano E, Banni S, Botti B, Corongiu F, Dessi MA, et al. Free-radical metabolism of carbon tetrachloride in rat liver mitochondria. A study of the mechanism of activation. *Biochem J.* (1987) 246:313–7. doi: 10.1042/bj2460313
54. Ogiso H, Ito H, Ando T, Arioka Y, Kanbe A, Ando K, et al. The deficiency of indoleamine 2,3-dioxygenase aggravates the CCl4-induced liver fibrosis in mice. *PLoS ONE.* (2016) 11:e0162183. doi: 10.1371/journal.pone.0162183
55. Li D, Cai H, Hou M, Fu D, Ma Y, Luo Q, et al. Effects of indoleamine 2,3-dioxygenases in carbon tetrachloride-induced hepatitis model of rats. *Cell Biochem Funct.* (2012) 30:309–14. doi: 10.1002/cbf.2803
56. Weichao Zhong LG, Zhenting Z, Haiyan L, Chun C, Peng H, Weiliang H, et al. Indoleamine 2,3-dioxygenase 1 deficiency attenuates CCl4-induced fibrosis through Th17 cells down-regulation and tryptophan 2,3-dioxygenase compensation. *Oncotarget.* (2017) 8:40486–500. doi: 10.18632/oncotarget.17119
57. Hoshi M, Osawa Y, Nakamoto K, Morita N, Yamamoto Y, Ando T, et al. Kynurenine produced by indoleamine 2,3-dioxygenase 2 exacerbates acute liver injury by carbon tetrachloride in mice. *Toxicology.* (2020) 438:152458. doi: 10.1016/j.tox.2020.152458
58. Kanai M, Funakoshi H, Takahashi H, Hayakawa T, Mizuno S, Matsumoto K, et al. Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. *Mol Brain.* (2009) 2:8. doi: 10.1186/1756-6606-2-8
59. Yuasa HJ, Ball HJ, Ho YF, Austin CJD, Whittington CM, Belov K, et al. Characterization and evolution of vertebrate indoleamine 2, 3-dioxygenases. *Comp Biochem Physiol B Biochem Mol Biol.* (2009) 153:137–44. doi: 10.1016/j.cbpb.2009.02.002
60. Snyder IS, Agarwal MK, Berry LJ. Influence of carbon tetrachloride on inducible liver enzymes and response to endotoxin in mice. *J Bacteriol.* (1967) 94:1817. doi: 10.1128/jb.94.6.1817-1823.1967
61. Nagano J, Shimizu M, Hara T, Shirakami Y, Kochi T, Nakamura N, et al. Effects of indoleamine 2,3-dioxygenase deficiency on high-fat diet-induced hepatic inflammation. *PLoS ONE.* (2013) 8:e73404. doi: 10.1371/journal.pone.0073404
62. Badawy A, Morgan CJ, Davis NR, Dacey A. High-fat diets increase tryptophan availability to the brain: importance of choice of the control diet. *Biochem J.* (1984) 217:863. doi: 10.1042/bj2170863
63. Badawy AAB, Guillemin G. The Plasma [Kynurenine]/[Tryptophan] Ratio and Indoleamine 2,3-Dioxygenase: Time for Appraisal. *Int J Tryptophan Res.* (2019) 12:117864691986897. doi: 10.1177/1178646919868978
64. Ogawa K HT, Shimizu M, Ninomiya S, J N. Suppression of azoxymethane-induced colonic preneoplastic lesions in rats by 1-methyltryptophan, an inhibitor of indoleamine 2,3dioxygenase. *Cancer Sci.* (2012) 103:951–8. doi: 10.1111/j.1349-7006.2012.02237.x
65. Zeng T, Deng G, Zhong W, Gao Z, Ma S, Mo C, et al. Indoleamine 2, 3-dioxygenase 1enhanceshepatocytes ferroptosis in acute immune hepatitis associated with excess nitrative stress - ScienceDirect. *Free Radic Biol Med.* (2020) 152:668–79. doi: 10.1016/j.freeradbiomed.2020.01.009
66. Tiegs G, Hentschel J, Wendel AA. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J Clin Invest.* (1992) 90:196–203. doi: 10.1172/JCI115836
67. Heymann F, Hamesch K, Weiskirchen R, Tacke F. The concanavalin a model of acute hepatitis in mice. *Lab Anim.* (2015) 49(Suppl. 1):12–20. doi: 10.1177/0023677215572841
68. Mo C, Xie S, Liu B, Zhong W, Zeng T, Huang S, et al. Indoleamine 2,3-dioxygenase 1 limits hepatic inflammatory cells recruitment and promotes bile duct ligation-induced liver fibrosis. *Cell Death Dis.* (2021) 12:16. doi: 10.1038/s41419-020-03277-0
69. Zhou Q, Shi Y, Chen C, Wu F, Chen Z. A narrative review of the roles of indoleamine 2,3-dioxygenase and tryptophan-2,3-dioxygenase in liver diseases. *Ann Transl Med.* (2021) 9:174. doi: 10.21037/atm-20-3594
70. Avila MA, Shibata Y, Hara T, Nagano J, Nakamura N, Ohno T, et al. The role of indoleamine 2,3-dioxygenase in diethylnitrosamine-induced liver carcinogenesis. *PLoS ONE.* (2016) 11:e0146279. doi: 10.1371/journal.pone.0146279
71. Yu-Chun L, Chao-Long C, Toshiaki N, Shigeru G, Ying-Hsien K, Li-Wen H, et al. Immunological role of indoleamine 2,3-dioxygenase in rat liver allograft rejection and tolerance. *J Gastroenterol Hepatol.* (2010) 23:e243–50. doi: 10.1111/j.1440-1746.2007.05036.x
72. Hirofumi O, Hiroyasu I, Kazuki A, Tetsuya I, Masato H, Tatsuya A, et al. Kynurenine production mediated by indoleamine 2,3-dioxygenase aggravates liver injury in HBV-specific CTL-induced fulminant hepatitis. *Biochim Biophys Acta.* (2014) 1842:1464–71. doi: 10.1016/j.bbadis.2014.04.015
73. Yao W, Gu H, Zhu J, Barding G, Cheng H, Bao B, et al. Integrated plasma and urine metabolomics coupled with HPLC/QTOF-MS and chemometric analysis on potential biomarkers in liver injury and hepatoprotective effects of Er-Zhi-Wan. *Anal Bioanal Chem.* (2014) 406:7367–78. doi: 10.1007/s00216-014-8169-x
74. Chang H, Meng HY, Liu SM, Wang Y, Yang XX, Lu F, et al. Identification of key metabolic changes during liver fibrosis progression in rats using a urine and serum metabolomics approach. *Sci Rep.* (2017) 7:11433. doi: 10.1038/s41598-017-11759-z
75. Han Y, Yunzhou L, Zongying X, Dingnan W, Shaohua S, Huifang D, et al. Identification of potential biomarkers in cholestasis and the therapeutic effect of melatonin by metabolomics, multivariate data and pathway analyses. *Int J Mol Med.* (2018) 42:2515–26. doi: 10.3892/ijmm.2018.3859
76. Lin H, Teng H, Wu W, Li Y, Lv G, Huang X, et al. Pharmacokinetic and metabolomic analyses of mangiferin calcium salt in rat models of type 2 diabetes and non-alcoholic fatty liver disease. *BMC Pharmacol Toxicol.* (2020) 21:59. doi: 10.1186/s40360-020-00438-x
77. Cho T, Kok LY, Uetrecht J. Testing possible risk factors for idiosyncratic drug-induced liver injury using an amodiaquine mouse model and co-treatment with 1-methyl-d-tryptophan or acetaminophen. *ACS Omega.* (2021) 6:4656–62. doi: 10.1021/acsomega.0c05352
78. Too LK, Li KM, Suarna C, Maghzal GJ, Stocker R, Mcgregor IS, et al. Deletion of TDO2, IDO-1 and IDO-2 differentially affects mouse behavior and cognitive function. *Behav Brain Res.* (2016) 312:102–17. doi: 10.1016/j.bbr.2016.06.018
79. Clària J, Moreau R, Fenaille F, Amorós A, Junot C, Gronbaek H, et al. Orchestration of tryptophan-kynurenine pathway, acute decompensation, and acute-on-chronic liver failure in cirrhosis. *Hepatology.* (2019) 69:1686–701. doi: 10.1002/hep.30363
80. Badawy A. Letter to the editor: the kynurenine pathway in hepatic cirrhosis. *Hepatology.* (2019) 70:1077–8. doi: 10.1002/hep.30680
81. Hijikata Y, Hara K, Shiozaki Y, Murata K, Sameshima Y. Determination of free tryptophan in plasma and its clinical applications. *J Clin Chem Clin Biochem.* (1984) 22:291–300. doi: 10.1515/cclm.1984.22.4.291
82. Cao G, Zhu R, Jiang T, Tang D, Su T. Danshensu, a novel indoleamine 2,3-dioxygenase1 inhibitor, exerts anti-hepatic fibrosis effects via inhibition of JAK2-STAT3 signaling. *Phytomedicine.* (2019) 63:153055. doi: 10.1016/j.phymed.2019.153055
83. Mo C, Xie S, Zeng T, Lai Y, Lv Z. Ginsenoside-Rg1 acts as an IDO1 inhibitor, protects against liver fibrosis via alleviating IDO1-mediated the inhibition of DCs maturation. *Phytomedicine.* (2021) 84:153524. doi: 10.1016/j.phymed.2021.153524

84. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the global burden of disease study 2010. *Lancet.* (2012) 380:2095–128. doi: 10.1016/S0140-6736(12)61728-0
85. Koziel MJ. Cellular immune responses against hepatitis C virus. *Clin Infect Dis.* (2005) 41(Suppl. 1):S25–31. doi: 10.1086/429492
86. Xiao-Song H RB, Lopez-Labrador FX. Quantitative analysis of hepatitis C virus-specific CDS8+ T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci USA.* (1999) 96:5692–7. doi: 10.1073/pnas.96.10.5692
87. Schirren CA, Jung MC, Gerlach JT, Worzfeld T, Baretton G, Mamin M, et al. Liver-derived hepatitis C virus (HCV)-specific CD4(+) T cells recognize multiple HCV epitopes and produce interferon gamma. *Hepatology.* (2000) 32:597–603. doi: 10.1053/jhep.2000.9635
88. Grabowska AM, Lechner F, Klenerman P, Tighe PJ, Robins RA. Direct ex vivo comparison of the breadth and specificity of the Tcells in the liver and peripheral blood of patients with chronic HCV infection. *Eur J Immunol.* (2001) 31:2388–94. doi: 10.1002/1521-4141(200108)31:8<2388::AID-IMMU2388>3.0.CO;2-L
89. Barnes E, Salio M, Cerundolo V, Francesco L, Pardoll D, Klenerman P, et al. Monocyte derived dendritic cells retain their functional capacity in patients following infection with hepatitis C virus. *J Viral Hepat.* (2010) 15:219–28. doi: 10.1111/j.1365-2893.2007.00934.x
90. Yang R, Gao N, Chang Q, Meng X, Wang W. The role of IDO, IL-10, and TGF-β in the HCV-associated chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. *J Med Virol.* (2018) 91:265–71. doi: 10.1002/jmv.25083
91. Chen YB, Li SD, He YP, Shi XJ, Chen Y, Gong JP. Immunosuppressive effect of IDO on T cells in patients with chronic hepatitis B. *Hepatol Res.* (2009) 39:463–8. doi: 10.1111/j.1872-034X.2008.00476.x
92. Ito H, Ando T, Ando K, Ishikawa T, Saito K, Moriwaki H, et al. Induction of hepatitis B virus surface antigen-specific cytotoxic T lymphocytes can be up-regulated by the inhibition of indoleamine 2, 3-dioxygenase activity. *Immunology.* (2014) 142:614–23. doi: 10.1111/imm.12274
93. Schulz S, Landi A, Garg R, Wilson JA, van Drunen Littel-van den Hurk S. Indolamine 2,3-dioxygenase expression by monocytes and dendritic cell populations in hepatitis C patients. *Clin Exp Immunol.* (2015) 180:484–98. doi: 10.1111/cei.12586
94. Vega MD, Aparício J, Retegui LA. Levo-1-methyl tryptophan aggravates the effects of mouse hepatitis virus (MHV-A59) infection. *Int Immunopharmacol.* (2015) 24:377–82. doi: 10.1016/j.intimp.2014.12.031
95. Mdv A, Jla A, Mfmb C, Lar A. The autoimmune response elicited by mouse hepatitis virus (MHV-A59) infection is modulated by liver tryptophan-2,3-dioxygenase (TDO). *Immunol Lett.* (2020) 217:25–30. doi: 10.1016/j.imlet.2019.11.004
96. Moffett JR, Ma AN. Tryptophan and the immune response. *Immunol Cell Biol.* (2003) 81:247–65. doi: 10.1046/j.1440-1711.2003.t01-1-01177.x
97. Badawy AAB. Liver tryptophan 2,3-dioxygenase in the mouse hepatitis virus (MHV-A59) model. *Immunol Lett.* (2020) 225:23–4. doi: 10.1016/j.imlet.2020.05.007
98. Sullivan DH, Roberson PK, Johnson LE. Association between inflammation-as-sociated cytokines, serum albumins, and mortality in the elderly. *J Am Med Dir Assoc.* (2007) 8:458–63. doi: 10.1016/j.jamda.2007.04.004
99. Ingelsten M, Gustafsson K, Oltean M, Karlsson-Parra A, Olausson M, Haraldsson B, et al. Is indoleamine 2,3-dioxygenase important for graft acceptance in highly sensitized patients after combined auxiliary liver-kidney transplantation? *Transplantation.* (2009) 88:911–9. doi: 10.1097/TP.0b013e3181b72e49
100. Sun X, Gong Z-J, Wang Z-W, Li T, Zhang J-Y, Sun H-C, et al. IDO-competent-DCs induced by IFN-γ attenuate acute rejection in rat liver transplantation. *J Clin Immunol.* (2012) 32:837–47. doi: 10.1007/s10875-012-9681-4
101. Weng MZ, Xu YG, Zhang Y, Zhang JY, Quan ZW, Xu JM, et al. Indoleamine 2,3-dioxygenase as a predictor of acute rejection after orthotopic liver transplantation in rat model. *Transplant Proc.* (2011) 43:3969–72. doi: 10.1016/j.transproceed.2011.09.061
102. Lin YC, Goto S, Tateno C, Nakano T, Cheng YF, Jawan B, et al. Induction of indoleamine 2,3-dioxygenase in livers following hepatectomy prolongs survival of allogeneic hepatocytes after transplantation. *Transplant Proc.* (2008) 40:2706–8. doi: 10.1016/j.transproceed.2008.08.001
103. Hepatol J. Liver regeneration. *J Hepatol.* (2012) 57(Suppl. 1):692–4. doi: 10.1016/j.jhep.2012.04.016
104. Tacke F, Luedde T, Trautwein C. Inflammatory pathways in liver homeostasis and liver injury. *Clin Rev Allergy Immunol.* (2009) 36:4–12. doi: 10.1007/s12016-008-8091-0
105. Ogiso H, Ito H, Kanbe A, Ando T, Hara A, Shimizu M, et al. The inhibition of indoleamine 2,3-dioxygenase accelerates early liver regeneration in mice after partial hepatectomy. *Dig Dis Sci.* (2017) 62:2386–96. doi: 10.1007/s10620-017-4651-6
106. Miki T, Sun H, Lee YH, Tandin A, Valdivia LA. Blockade of tryptophan catabolism prevents spontaneous tolerogenicity of liver allografts. *Transplant Proc.* (2001) 33:129–30. doi: 10.1016/S0041-1345(00)02792-5
107. Laurence JM, Wang C, Park ET, Buchanan A, Clouston A, Allen R, et al. Blocking indoleamine dioxygenase activity early after rat liver transplantation prevents long-term survival but does not cause acute rejection. *Transplantation.* (2008) 85:1357–61. doi: 10.1097/TP.0b013e31816fc27f
108. Laurence JM, Wang C, Zheng M, Cunningham S, Earl J, Tay SS, et al. Overexpression of indoleamine dioxygenase in rat liver allografts using a high-efficiency adeno-associated virus vector does not prevent acute rejection. *Liver Transpl.* (2009) 15:233–41. doi: 10.1002/lt.21662
109. Pree I, Bigenzahn S, Fuchs D, Koporc Z, Nierlich P, Winkler C, et al. CTLA4Ig promotes the induction of hematopoietic chimerism and tolerance independently of indoleamine-2,3-dioxygenase. *Transplantation.* (2007) 83:663–7. doi: 10.1097/01.tp.0000255594.23445.29
110. Wu Y, Wang Y, Li M, Yang X, Gong J, Zhang W. Gadolinium chloride suppresses acute rejection and induces tolerance following rat liver transplantation by inhibiting kupffer-cell activation. *Exp Ther Med.* (2014) 8:1777–82. doi: 10.3892/etm.2014.2015
111. Anthonyj D, Kennethp B, Amarp D, Lindaferrell J, Stephena G, Johnhart P, et al. Banff schema for grading liver allograft rejection: An international consensus document. *Hepatology.* (1997) 25:658–63. doi: 10.1002/hep.510250328
112. Mulley WR, Nikolic-Paterson DJ. Indoleamine 2,3-dioxygenase in transplantation. *Nephrology.* (2010) 13:204–11. doi: 10.1111/j.1440-1797.2007.00921.x
113. Yakun Wu ZY, Junhua G, Min L, Yiming L, Jianping G. Effects of combined genes of CTLA4Ig and IDO in post-liver transplantation immune tolerance of rats. *Ann Hepatol.* (2016) 15:729-37. doi: 10.5604/16652681.1212524
114. Keenan BP, Fong L, Kelley RK. Immunotherapy in hepatocellular carcinoma: the complex interface between inflammation, fibrosis, and the immune response. *J Immunother Cancer.* (2019) 7:267. doi: 10.1186/s40425-019-0749-z
115. Wang Y, Yao R, Zhang L, Xie X, Chen R, Ren Z. IDO and intra-tumoral neutrophils were independent prognostic factors for overall survival for hepatocellular carcinoma. *J Clin Lab Anal.* (2019) 33:e22872. doi: 10.1002/jcla.22872
116. Davar D, Bahary N. Modulating tumor immunology by inhibiting indoleamine 2,3-dioxygenase (IDO): recent developments and first clinical experiences. *Target Oncol.* (2018) 13:125–40. doi: 10.1007/s11523-017-0547-9
117. Ala M. The footprint of kynurenine pathway in every cancer: a new target for chemotherapy. *Eur J Pharmacol.* (2021) 896:173921. doi: 10.1016/j.ejphar.2021.173921
118. Prendergast GC, Mondal A, Dey S, Laury-Kleintop LD, Muller AJ. Inflammatory reprogramming with IDO1 inhibitors: turning immunologically unresponsive 'cold' tumors 'hot'. *Trends Cancer.* (2018) 4:38–58. doi: 10.1016/j.trecan.2017.11.005
119. Lilla H, Nikoletta D, Gábor K, Zsolt K, Dénes P, Zoltán S, et al. The role of indoleamine-2,3-dioxygenase in cancer development, diagnostics, and therapy. *Front Immunol.* (2018) 9:151. doi: 10.3389/fimmu.2018.00151
120. Francesca F, Ursula G, Sylvaine Y, Barbara CM, Douglas RC, Carmine V, et al. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor ζ-chain and induce a regulatory phenotype in naive T cells. *J Immunol.* (2006) 176:6752–61. doi: 10.4049/jimmunol.176.11.6752

121. Munn DH, Sharma MD, Johnson TS, Rodriguez P. IDO, PTEN-expressing Tregs and control of antigen-presentation in the murine tumor microenvironment. *Cancer Immunol Immunother.* (2017) 66:1–10. doi: 10.1007/s00262-017-2010-2
122. Holmgaard RB, Zamarin D, Li Y, Gasmi B, Wolchok JD. Tumor-expressed IDO recruits and activates MDSCs in a Treg-dependent manner. *Cell Rep.* (2015) 13:412–24. doi: 10.1016/j.celrep.2015.08.077
123. Ornstein MC, Diaz-Montero CM, Rayman P, Elson P, Haywood S, Finke JH, et al. Myeloid-derived suppressors cells (MDSC) correlate with clinicopathologic factors and pathologic complete response (pCR) in patients with urothelial carcinoma (UC) undergoing cystectomy. *Urol Oncol.* (2018) 36:405–12. doi: 10.1016/j.urolonc.2018.02.018
124. Pan K, Wang H, Chen M-S, Zhang H-K, Weng D-S, Zhou J, et al. Expression and prognosis role of indoleamine 2,3-dioxygenase in hepatocellular carcinoma. *J Cancer Res Clin Oncol.* (2008) 134:1247–53. doi: 10.1007/s00432-008-0395-1
125. Han Y, Chen Z, Yang Y, Jiang Z, Gu Y, Liu Y, et al. Human CD14+CTLA-4+regulatory dendritic cells suppress T-cell response by cytotoxic T-lymphocyte antigen-4-dependent IL-10 and indoleamine-2,3-dioxygenase production in hepatocellular carcinoma. *Hepatology.* (2014) 59:567–79. doi: 10.1002/hep.26694
126. Tuanjie Li YY, Xuefeng H, Guoying W, Wei L, Changchang J, Yan T, et al. Hepatocellular carcinoma-associated fibroblasts trigger NK cell dysfunction via PGE2 IDO. *Cancer Let.* (2012) 318:154–61. doi: 10.1016/j.canlet.2011.12.020
127. Ishio T, Goto S, Tahara K, Tone S, Kitano S. Immunoactivative role of indoleamine 2,3-dioxygenase in human hepatocellular carcinoma. *J Gastroenterol Hepatol.* (2010) 19:319–26. doi: 10.1111/j.1440-1746.2003.03259.x
128. Hiroaki I, Lloyd JO, Schreiber RD. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev.* (2002) 13:95–109. doi: 10.1016/S1359-6101(01)00038-7
129. Lee IC, Huang YH, Chau GY, Huo TI, Su CW, Wu JC, et al. Serum interferon gamma level predicts recurrence in hepatocellular carcinoma patients after curative treatments. *Int J Cancer.* (2013) 133:2895–902. doi: 10.1002/ijc.28311
130. Attallah AM, El-Far M, Zahran F, Shiha GE, Farid K, Omran MM, et al. Interferon-gamma is associated with hepatic dysfunction in fibrosis, cirrhosis, and hepatocellular carcinoma. *J Immunoassay Immunochem.* (2016) 37:597–610. doi: 10.1080/15321819.2016.1179646
131. Brown ZJ, Yu SJ, Heinrich B, Ma C, Fu Q, Sandhu M, et al. Indoleamine 2,3-dioxygenase provides adaptive resistance to immune checkpoint inhibitors in hepatocellular carcinoma. *Cancer Immunol Immunother.* (2018) 67:1305–15. doi: 10.1007/s00262-018-2190-4
132. Long GV, Dummer R, Hamid O, Gajewski TF, Mitchell TC. Epacadostat plus pembrolizumab versus placebo plus pembrolizumab in patients with unresectable or metastatic melanoma (ECHO-301/KEYNOTE-252): a phase 3, randomised, double-blind study. *Lancet Oncol.* (2019) 20:1083–97. doi: 10.1016/S1470-2045(19)30274-8
133. Ming L, Wang X, Wang L, Ma X, Gong Z, Zhang S, et al. Targeting the IDO1 pathway in cancer: from bench to bedside. *J Hematol Oncol.* (2018) 11:100. doi: 10.1186/s13045-018-0644-y
134. Muller AJ, Manfredi MG, Zakharia Y, Prendergast GC. Inhibiting IDO pathways to treat cancer: lessons from the ECHO-301 trial and beyond. *Semin Immunopathol.* (2018) 41:41–8. doi: 10.1007/s00281-018-0702-0
135. Günther J, Dbritz J, Wirthgen E. Limitations and off-target effects of tryptophan-related IDO inhibitors in cancer treatment. *Front Immunol.* (2019) 10:1801. doi: 10.3389/fimmu.2019.01801
136. van Doorn DJ, Takkenberg RB, Klümpen H-J. Immune checkpoint inhibitors in hepatocellular carcinoma: an overview. *Pharmaceuticals.* (2020) 14:3. doi: 10.3390/ph14010003
137. Ferdinande L, Decaestecker C, Verset L, Mathieu A, Lopez XM, Negulescu AM, et al. Clinicopathological significance of indoleamine 2,3-dioxygenase 1 expression in colorectal cancer. *Br J Cancer.* (2012) 106:141–7. doi: 10.1038/bjc.2011.513
138. Riesenberg R, Weiler C, Spring O, Eder M, Buchner A, Popp T, et al. Expression of indoleamine 2,3-dioxygenase in tumor endothelial cells correlates with long-term survival of patients with renal cell carcinoma. *Clin Cancer Res.* (2007) 13:6993–7002. doi: 10.1158/1078-0432.CCR-07-0942
139. Ludovini V, Bianconi F, Siggillino A, Vannucci J, Baglivo S, Berti V, et al. High PD-L1/IDO-2 and PD-L2/IDO-1 co-expression levels are associated with worse overall survival in resected non-small cell lung cancer patients. *Genes.* (2021) 12:273. doi: 10.3390/genes12020273
140. Melaiu O, Lucarini V, Giovannoni R, Fruci D, Gemignani F. News on immune checkpoint inhibitors as immunotherapy strategies in adult and pediatric solid tumors. *Semin Cancer Biol.* (2020) S1044-579X:30156–5. doi: 10.1016/j.semcancer.2020.07.001
141. Lee JB, Ha SJ, Kim HR. Clinical insights into novel immune checkpoint inhibitors. *Front Pharmacol.* (2021) 12:681320. doi: 10.3389/fphar.2021.681320
142. Wang Z, Zhu J, Liu Y, Liu C, Ma L. Development and validation of a novel immune-related prognostic model in hepatocellular carcinoma. *J Transl Med.* (2020) 18:67. doi: 10.1186/s12967-020-02255-6

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The Influence of Sevelamer Hydrochloride and Calcium Carbonate on Markers of Inflammation and Oxidative Stress in Hemodialysis at Six Months of Follow-Up

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Patients with end-stage renal disease (ESRD) present alterations in mineral and bone metabolism. Hyperphosphatemia in ESRD is considered an independent risk factor for cardiovascular disease (CVD), increasing morbidity, and mortality. Sevelamer hydrochloride is a calcium-free, non-absorbable phosphate-chelating polymer. Calcium carbonate chelator is helpful in controlling serum phosphate levels. There is insufficient information on the influence of sevelamer hydrochloride and calcium carbonate on the behavior of oxidative stress (OS) markers and inflammation in patients on hemodialysis (HD). A randomized open clinical trial was carried out on patients to evaluate sevelamer hydrochloride and calcium carbonate influence at 6 months of study follow-up. Levels of oxidants (LPO, NO, and 8-isoprostanes), antioxidants (SOD and TAC), oxidative DNA damage (8-OHdG and hOGG1), pro-inflammatory cytokines (IL-6 and TNF-α), and inflammation markers (ferritin and C-reactive protein) were measured with colorimetric and ELISA methods. We found a significant increase in oxidants LPO and NO, and antioxidants SOD and TAC, and downregulation of IL-6 and TNF-α. Ferritin decrease at 6 months follow-up in the sevelamer hydrochloride group. Increase in C-reactive protein was found in the group of patients treated with calcium carbonate. In conclusion, we found an oxidative state imbalance with increase in LPO and NO oxidants. The activity of the antioxidant enzymes (SOD and TAC) was also found to increase, suggesting a compensatory effect in the face of increase in oxidants. The same phenomenon was observed with increase in the oxidative damage marker to DNA and the increase in the DNA repair enzyme, suggesting a compensatory effect. Pro-inflammatory cytokines were predominantly downregulated by TNF-α in the group that ingested sevelamer hydrochloride in the final determination at 6 months of follow-up.

Serum ferritin levels decreased significantly at the end of follow-up in patients on HD in the sevelamer hydrochloride group. The management of hyperphosphatemia with sevelamer hydrochloride appears to have obvious anti-inflammatory and antioxidant benefits.

Keywords: oxidative stress markers, ESRD, calcium carbonate, antioxidants, sevelamer hydrochloride

INTRODUCTION

Disorders of mineral and bone metabolism are frequent complications in patients with end-stage renal disease (ESRD). Hyperphosphatemia is an inevitable consequence of ESRD, the presence of which is associated with increased morbidity and mortality (1). Oxidative stress (OS) is characterized by an imbalance between decrease in antioxidant defense mechanisms and increase in oxidant products. OS appears in early stages of chronic kidney disease (CKD), progresses along with worsening kidney failure, and is aggravated by the hemodialysis (HD) process. Observational studies have reported that hyperphosphatemia is an independent risk factor for cardiovascular disease (CVD) capable of increased mortality in patients on dialysis (2). Control of serum phosphorus (Pi) is key to improving clinical outcomes for patients on dialysis. The predominant drug treatment for hyperphosphatemia has been calcium-based phosphate binders (calcium carbonate or calcium acetate). However, calcium salts are related to arterial calcification (3). New phosphate binders have made it possible to dispense with calcium in the formulation (4). Sevelamer hydrochloride is a non-absorbable, calcium-free phosphate-chelating polymer that has been available since 1998 (5). Sevelamer hydrochloride is an anion exchange resin, allowing it to exchange negatively charged ions. Sevelamer hydrochloride has a high affinity for phosphate ions and exerts its therapeutic action in the intestine, exchanging chloride for phosphate and forming an insoluble compound that is excreted in feces (6).

Figure 1 briefly shows the binding mechanism of sevelamer hydrochloride and calcium carbonate with ingested Pi. Some studies have reported potential benefits of the use of sevelamer hydrochloride. However, there are insufficient data to establish the superiority of phosphate binders without calcium content over those containing calcium with CVD development (7). Sevelamer hydrochloride helps prevent calcium overload, reduces hyperphosphatemia, and translates into better clinical results. Sevelamer hydrochloride is associated with better survival in patients on HD (8). Experimental, observational,

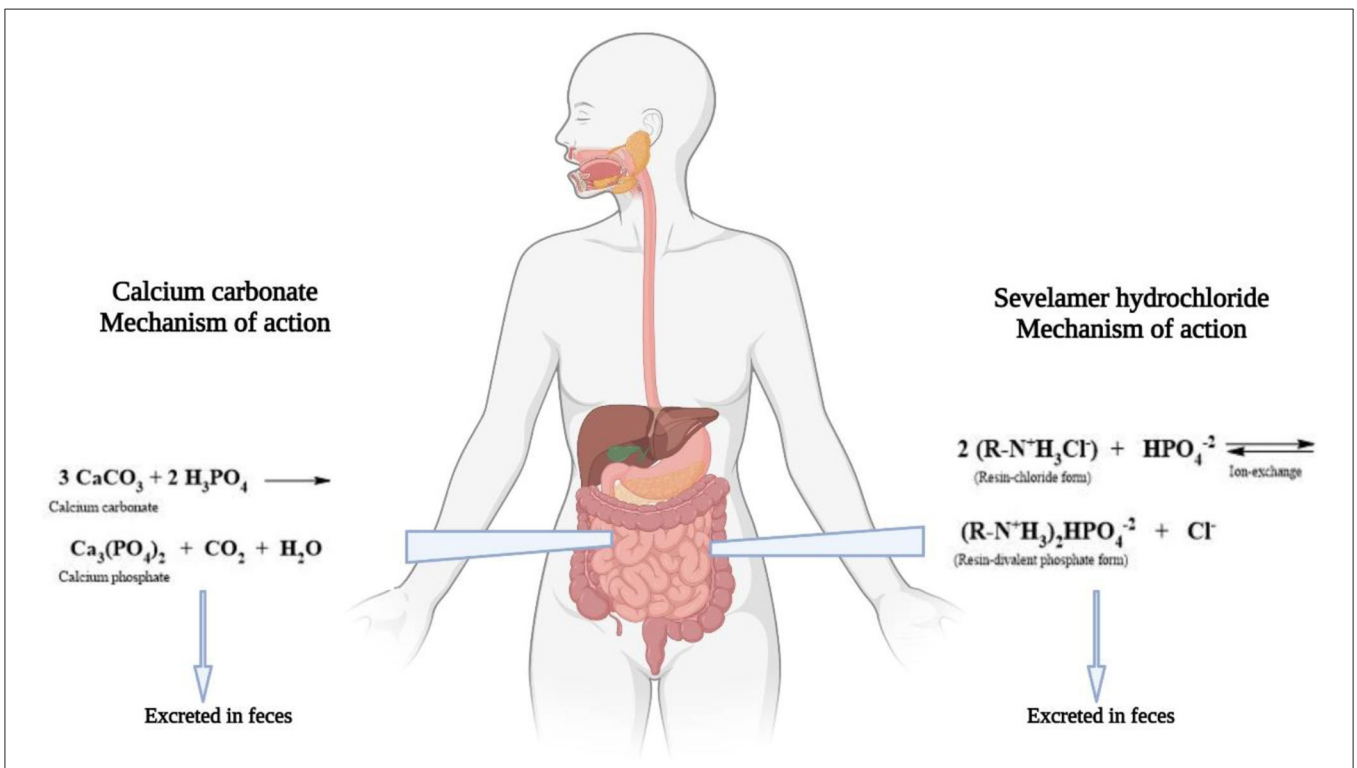

FIGURE 1 | Sevelamer hdyrochloride and calcium carbonate mechanism. Binding of sevelamer hydrochloride and calcium carbonate with phosphate. Right side shows the ion exchange between the resin and the phosphate. Left side shows the reaction between calcium carbonate and phosphate.

and clinical trials have shown sevelamer hydrochloride to have pleiotropic effects beyond the control of hyperphosphatemia. Sevelamer hydrochloride acts on inflammation, OS, lipid profile, atherogenesis, vascular calcification, and endothelial dysfunction, and reduces uremic toxins (9, 10). Patients on HD have a high risk of mortality from all causes and cardiovascular events. Excessive OS and chronic inflammation emerge as new contributors of accelerated atherosclerosis and elevated mortality in ESRD. Patients on HD show an increase in free radicals due to retention of uremic toxins, reduction in antioxidants, activation of leukocytes, infectious processes, and presence of comorbidities (11). The potential benefits of sevelamer hydrochloride to decrease inflammation and OS markers need to be studied (12).

The purpose of the study was to evaluate the influence of sevelamer hydrochloride and calcium carbonate on OS markers and inflammation in patients on HD at 6 months of follow-up.

PATIENTS AND METHODS

Patients

A randomized open clinical trial was carried out on patients to evaluate sevelamer hydrochloride and calcium carbonate influence at 6 months of study follow-up. An open randomized clinical trial was designated, because it was not possible to mask the drugs. The medications were obtained through a prescription that was exchanged at the pharmacy by the patient himself. The initial doses of sevelamer hydrochloride were two tablets of 800 mg every 12 h. It was necessary to adjust the dose (±1 tablet) each month to achieve a target serum Pi of 5 mg/dl and considering drug tolerance. Serum Pi was monitored each month to determine the need for dose adjustment using a colorimetric method for its determination. The doses administered at the end of the study were 1,600, 2,400, and 3,200 mg. The titration of the drug was gradual and according to each patient requirement (13).

Randomization and treatment allocation were based on simple randomization using the Microsoft Excel software that generates random numbers. Male and female patients aged 18–60 years were included. Medications were administered for comorbidities, for arterial hypertension (nifedipine, metoprolol, losartan), for DM (insulin), for anemia (folic acid, erythropoietin). The diet of ESRD patients includes limiting fluids, consuming a low-protein diet, and reducing their intake of salt, potassium, Pi, and other electrolytes. In ESRD patients it is important to consume enough calories to avoid losing weight. No dietary supplements were administered. All patients who have ESRD, with GFR <10 ml/min/1.73 m^2, were subjected to three HD sessions per week with the same type of filter without the possibility of determining albuminuria. The patients were attended in the HD Unit of the Department of Nephrology of the High Specialty Medical Unit of the Mexican Institute of Social Security (IMSS) in Guadalajara, Jalisco, Mexico. Patients who ingested antioxidants (vitamins E and C) 3 months before the study and patients with clinical or biochemical data of an infectious process were not included. Patients who withdrew informed consent, with <80% adherence to treatment and patients with adverse drug events were excluded from the study.

Data Collection

Sixty-four patients were enrolled, and two intervention groups with 32 patients were formed. One group was composed of patients treated with sevelamer hydrochloride, and the other was made up of patients treated with calcium carbonate. Treatment adherence was verified by the investigator in charge of prescribing the drug to each patient. Adherence was determined by recording the drug supply of a pharmacy according to the stipulated time and loaded into the file of each patient. Body mass index (BMI) and treatment group were recorded. Biochemical data were obtained the first day of treatment immediately before starting HD and at 6 months of follow-up: hemoglobin, urea, uric acid, cholesterol, triglycerides, albumin, urea, serum creatinine, and uric acid, serum electrolytes (sodium, potassium, chlorine, Pi, calcium, and magnesium), ferritin, iron saturation, bicarbonate, C-reactive protein (CRP), vitamin D, pH, and parathyroid hormone (PTH). Venous blood samples, 5 ml, were collected in a dry tube and 5 ml in a tube with 7.2 mg of dipotassium ethylene-diamine-tetra-acetic acid (K2 EDTA). The samples were centrifuged at 3,500 rpm for 10 min to obtain serum and plasma. Six months after starting treatment (sevelamer hydrochloride or calcium carbonate), blood samples were collected to compare the results. The aliquots obtained were stored at −80°C to analyze later inflammation markers, oxidants, antioxidants, and oxidative damage to DNA.

OS Markers

Lipoperoxidation (LPO) Products

The levels of LPO in plasma were measured using an FR22 assay kit (Oxford Biomedical Research Inc., Oxford, MI, United States) and according to the instructions of the manufacturer.

8-iso Prostaglandin $F_{2\alpha}$ 8-Isoprostanes (8-IP)

An immunoassay reagent kit from Abcam Company® (Cambridge, England) was used according to the instructions of the manufacturer.

Nitric Oxide (NO)

Before determining NO levels, the serum samples were deproteinized by adding 6 mg of zinc sulfate to 400 μl of the sample vortex for 1 min, and the samples were centrifuged at 10,000 × g for 10 min at 4°C. The determination of NO was carried out per the kit (NB98; Oxford Biochemical Research Inc., Oxford, MI, United States).

Antioxidants

Superoxide Dismutase (SOD)

The instructions of the kit manufacturer (SOD No. 706002; Cayman Chemical Company®, Ann Arbor, Michigan, USA) were followed to measure SOD activity. The levels are reported in U/ml.

Total Antioxidant Capacity (TAC)

Evaluations of total antioxidant capacity (TAC) were performed according to the instructions of the kit manufacturer (Total Antioxidant Power Kit, No. TA02.090130; Oxford Biomedical Research®, Oxford, MI, United States).

Marker of Oxidative DNA Damage

The serum levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were measured using an 8-hydroxy-2-deoxyguanosine ELISA kit ab201734 (Abcam®, Cambridge, MA, United States).

DNA Repair Enzyme—Oxoguanine Glycosylase

It was performed by the manufacturer of human 8-oxoguanine DNA glycosidase (hOGG1) ELISA kit no. MBS702793, MyBioSource® (San Diego, USA).

Pro-inflammatory Cytokines

TNF-α and IL-6

The sandwich ELISA method of the commercial 900-K25 and 900-K16 kit suggested by the manufacturer Peprotech (New Jersey, USA) was followed. The absorbances of the wells were recorded at 405 nm with a correction of 650 nm.

Ferritin and C-Reactive Protein (CRP)

They were determined by immuno-turbidimetry. Antibody-coated latex particles were agglutinated by ferritin or CRP present in the patient sample. The agglutination process caused an absorbance change proportional to the concentration of the inflammation marker in the sample, and the concentration in the sample was determined by comparison with a standard.

Statistical Analysis

Sample size calculation was performed by comparing two means formula (14). In the available literature, we did not find longitudinal studies on the effect of sevelamer on OS markers. Therefore, we used as a reference the variation of TNF-α in patients on HD treated with sevelamer reported by Peres et al. (13). There were no losses from the included population. The patients underwent HD three times a week with the same type of filter; no intention-to-treat analysis was required. The results are presented in measures of central tendency and dispersion for quantitative variables. Qualitative variables are shown in frequencies and percentages. The Kolmogorov–Smirnov test was performed to determine the normality of the data. For intra-group differences, the Wilcoxon rank test was performed according to the distribution obtained. Mann–Whitney *U*-test or Student's *t*-test was performed on independent samples for treatment inter-group differences. ANOVA or Kruskal-Wallis test was performed for dose inter-group differences. All results with a value of $p \leq 0.05$ were documented as statistically significant.

Ethical Considerations

The study was followed under the ethical principles for medical research in human beings stipulated in the declaration of Helsinki 64th General Assembly, Fortaleza Brazil, October 2013, and the Standards of Good Clinical Practice according to the guidelines of the International Conference on Harmonization. Under the General Health Law's provisions following the Regulations of the General Health Law on Research for Health Article 17 of Mexico, the study corresponds to category III. All the patients signed the Low Information Consent in the presence of witnesses. The study was evaluated and approved by the Local Ethics and Research Committee at the *Centro Médico Nacional de Occidente*, Instituto Mexicano del Seguro Social (R-2021-1301-063).

TABLE 1 | Baseline anthropometric data.

	Sevelamer hydrochloride	Calcium carbonate	*P*
Gender (%)			
Male	41.90	33.3	0.61
Female	58.1	66.6	
Weight (kg)	64.79 ± 19.10	63.55 ± 22.46	0.8
BMI (Kg/m^2)	24.95 ± 5.93	24.02 ± 5.28	0.72
BMI (%)			
Normal	64.5	71.4	0.44
Overweight	12.9	14	
Obesity	22.6	14.3	
Time in HD (years)	8.06 ± 4.78	7.05 ± 5.06	0.038¥
DM (%)			
Yes	74.2	71.4	0.11
No	25.8	28.6	
AH (%)			
Yes	32.3	19	0.31
No	67.7	81	

Values are expressed as mean ± standard deviation or percentages. ¥ Chi^2 test. BMI, Body mass index; DM, Diabetes Mellitus; AH, Arterial Hypertension.

RESULTS

Sixty-four patients were included, 32 in the sevelamer hydrochloride group and 32 patients in the calcium carbonate group. Thirty-eight female and 26 male patients were included. Body weight was similar in both groups, 64.79 ± 19.1 kg of patients treated with sevelamer hydrochloride and 63.55 ± 22.46 kg of those treated with calcium carbonate. The time in the HD program was also similar, 8.06 ± 4.78 years in the sevelamer hydrochloride group and 7.05 ± 5.06 years in the group treated with calcium carbonate. In the sevelamer hydrochloride group, a normal BMI was found in 64.5% of the patients, 12.9% were overweight, and 22.6% were obese. In the calcium carbonate group, the BMI was normal in 71.4%, 14% of the patients were overweight, and 14.3% were obese. 74.2% of the patients in the sevelamer hydrochloride group and 71.4% of the patients treated with calcium carbonate had diabetes mellitus (DM). 32.3% of the patients treated with sevelamer hydrochloride and 19% of those treated with calcium carbonate had arterial hypertension (AH) (**Table 1**).

Table 2 shows the causes of kidney failure in both treatment groups. The unknown cause is highlighted.

Biochemical Data

The baseline hemoglobin levels in both groups were similar, 10.5 ± 1.63 mg/dl in the sevelamer hydrochloride group and 10.14 ± 2.23 mg/dl in the calcium carbonate group. In the determination at six months of follow-up, the hemoglobin levels in the sevelamer hydrochloride group increased to 12.15 ±

1.93 mg/dl ($p = 0.001$) without observing changes in those who received calcium carbonate. 10.85 ± 2.57 mg/dl ($p = 0.23$). Ferritin levels decreased significantly in the sevelamer hydrochloride group, 201.67 ± 60.39 ng/ml with respect to the 396.24 ± 709.77 ng/ml in the calcium carbonate group ($p = 0.004$). CRP levels showed an increase in both groups (**Table 3**).

TABLE 2 | ESRD causes.

	Sevelamer hydrochloride n- 32	Calcium carbonate n-32
Unknown	23	22
DM2	5	4
Polycystic kidney disease	1	1
Systemic lupus erythematous	1	2
Nephrolithiasis	1	–
Other	1	3

OS Markers

LPO, 8-IP, NO

The LPO levels increased significantly between the baseline levels 15.7 ± 3.79 mM and the final determination 35.85 ± 6.32 mM ($p = 0.022$) in the sevelamer hydrochloride group. The same behavior was observed in basal NO levels 1,196.01 ± 112.08 μg/ml compared with the increase found at the end of follow-up, 1,387.61 ± 175.97 μg/ml ($p = 0.001$). The group treated with calcium carbonate had similar results between the baseline and 6 months of treatment. The 8-IP levels in the sevelamer hydrochloride group were 50.46 ± 10.2 vs. 73.11 ± 13.34 pg/ml ($p = 0.07$) (**Table 3**).

Antioxidants

The activity of the SOD enzyme was found to be significantly increased in the final determination of those treated with sevelamer hydrochloride, 7.69 ± 3.19 U/L ($p < 0.001$) and with calcium carbonate, 8.78 ± 5.43 U/L ($p = 0.016$). The levels of TAC were significant increased at the end of the follow-up for

TABLE 3 | Sevelamer hydrochloride or Calcium carbonate dose.

	Sevelamer hydrochloride n-32		WCX	Calcium carbonate n-32		WCX	U-MW
	Baseline	Six-months	*p*	Baseline	Six-months	*p*	*p*
Oxidants							
LPO (mM)	15.70 ± 3.78	35.85 ± 6.31	**0.022****	12.92 ± 2.03	27.53 ± 8.31	0.11	0.68
NO (μg/mL)	1196.01 ± 112.08	1387.61 ± 175.97	**<0.001****	1215.70 ± 163.65	1322.02 ± 200.54	**<0.001****	0.8
8-IP (pg/mL)	50.46 ± 10.20	73.11 ± 13.34	0.07	112.82 ± 14.62	90.32 ± 9.08	0.12	**<0.001***
Markers of oxidative damage to DNA							
8-OHdG (ng/mL)	6.29 ± 2.22	9.52 ± 7.21	**0.033****	8.60 ± 4.77	8.88 ± 5.43	0.84	0.69
hOGG1 (pg/mL)	3.67 ± 0.53	21.84 ± 6.54	**<0.001****	7.89 ± 1.52	14.68 ± 4.86	0.22	0.10
Antioxidants							
SOD (U/L)	4.75 ± 3.07	7.69 ± 3.19	**<0.001****	6.03 ± 4.39	8.78 ± 5.43	**0.016****	0.51
TAC (μM)	0.62 ± 0.19	0.82 ± 0.22	**<0.001****	0.69 ± 0.21	0.87 ± 0.34	**<0.001****	0.86
Pro-inflammatory cytokines							
IL-6 (pg/mL)	3.00 ± 1.86	2.41 ± 0.92	0.37	3.49 ± 1.66	2.70 ± 1.19	**0.037****	0.38
TNF- α (pg/mL)	2.44 ± 1.92	2.01 ± 0.47	**0.003****	2.12 ± 0.73	1.75 ± 0.53	**0.009****	0.97
Ferritin (ng/mL)	234.63 ± 404.33	201.67 ± 60.39	**0.004****	158.44 ± 153.60	396.24 ± 709.77	0.59	0.10
CRP (mg/L)	7.04 ± 7.34	11.65 ± 10.55	0.08	7.08 ± 9.39	9.53 ± 4.10	**0.043****	0.65
Biochemical data							
Hb (mg/dL)	10.50 ± 1.63	12.15 ± 1.93	**0.001****	10.14 ± 2.23	10.85 ± 2.57	0.26	0.23
Urea (mg/dL)	108.43 ± 37.93	102.80 ± 32.17	0.42	130.27 ± 43.11	128.91 ± 30.14	0.26	0.69
Uric acid (μmol/L)	5.16 ± 1.48	5.18 ± 1.19	0.79	5.27 ± 1.94	5.15 ± 1.67	0.6	0.76
SCr (mg/dL)	10.56 ± 2.34	10.41 ± 3.1	0.6	10.01 ± 3.2	9.51 ± 3.2	0.6	0.1
Na (mmol/L)	138.79 ± 2.29	136.95 ± 0.96	**0.008 ****	138.65 ± 2.55	138.09 ± 2.63	0.49	0.75
K (mmol/L)	5.17 ± 0.52	5.15 ± 0.59	0.38	5.19 ± 1.13	5.51 ± 0.77	0.60	**0.048**
Pi (mmol/L)	6.29 ± 1.61	5.71 ± 52	**0.040****	5.81 ± 2.39	6.15 ± 1.60	0.59	0.79
Ca (mg/mL)	8.86 ± 1.14	8.93 ± 1.02	0.63	8.71 ± 1.43	8.05 ± 2.34	0.44	0.22
HCO_3 (mEq/L)	23.62 ± 3.16	22.14 ± 4.90	0.79	21.62 ± 4.49	23.41 ± 2.74	**0.047****	**<0.001****

*Values are expressed as mean ± standard deviation or standard error of the mean. *Mann-Whitney U-test. **Wilcoxon rank test. LPO, Lipoperoxides; NO, Nitric Oxide; 8-IP, 8-isoprostanes; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; hOGG1, Oxoguanine glycosylase; SOD, Superoxide dismutase; TAC, Total Antioxidant Capacity; IL-6, Interleukin 6; TNF-α, Tumor necrosis factor alpha; Hb, hemoglobin; Na, Sodium; K, Potassium; Pi, Phosphate; CRP, C-reactive protein; Vit. D, vitamin D; HCO3, Bicarbonate. Data in bold are statistically significant p-values.*

the sevelamer hydrochloride (0.82 ± 0.22 μM, $p < 0.001$) and calcium carbonate, (0.87 ± 0.34 μM, $p < 0.001$) groups (**Table 3**).

Pro-inflammatory Cytokines

We found a significant decrease in the expression of the pro-inflammatory cytokine TNF-α between baseline levels, 2.44 ± 1.92 pg/ml, and the final result, 2.01 ± 1.47 pg/ml ($p = 0.003$), in patients treated with sevelamer hydrochloride. The same behavior was observed in those who received calcium carbonate between the expression of basal TNF-α, 2.12 ± 0.73 pg/ml, and final result, 1.75 ± 0.53 pg/ml ($p = 0.009$). Ferritin levels were decreased at the end of follow-up 201.67 ± 60.39 ng/ml ($p = 0.04$), in the patients who ingested sevelamer hydrochloride. CRP levels were increased in the patients treated with calcium carbonate, 10.66 ± 6.07 mg/L ($p = 0.008$) (**Table 3**).

Oxidative Damage to DNA

The expression of the oxidative DNA damage marker (8-OHdG) in the final determination increased, 9.52 ± 7.21 ng/ml ($p = 0.033$), compared with the baseline determination of 6.29 ± 2.22 ng/ml in the hydrochloride sevelamer group. The same behavior was observed with the DNA repair enzyme in the final determination, 21.84 ± 6.54 pg/ml ($p < 0.001$), of the patients treated with sevelamer hydrochloride compared with the baseline value of 3.67 ± 0.53 pg/ml. The group that ingested calcium carbonate did not change the levels between the baseline and final determination (**Table 3**).

Hemodialysis and Sevelamer Hydrochloride Dose

Three daily doses (1,600, 2,400, and 3,200 mg) of sevelamer hydrochloride were administered to the patients in the HD program. Patients who ingested 3,200 mg had a significant increase in LPO in the final determination of the study, 48.85 ± 42.79 mM ($p = 0.011$), compared with the baseline level of 9.18 ± 4.52 mM. NO levels were also increased in the final determination in patients who received 1,600 mg, 1,372.14 ± 177.5 μg/ml ($p = 0.031$), and 3,200 mg, 1,319.25 ± 158.35 μg/ml ($p = 0.028$). The DNA repair enzyme was found to be significantly increased in the final determination in patients who ingested 1,600 mg of sevelamer hydrochloride 17.74 ± 9.02 ($p = 0.028$).

The activity of the antioxidant enzyme SOD was significantly increased at 6 months of follow-up only in patients who ingested 1,600 mg of sevelamer hydrochloride 0.91 ± 0.25 μM ($p = 0.047$). TAC levels were found to be increased in the final determination in patients who ingested 1,600 mg, 0.91 ± 0.25 μM, ($p = 0.004$), and 3,200 mg, 0.85 ± 0.28 μM ($p = 0.011$) of sevelamer hydrochloride.

The expression of IL-6 decreased significantly in the final determination in those who ingested 1,600 mg, 2.3 ± 1.01 pg/ml ($p = 0.03$). The expression of TNF-α decreased at the end of the study in those who ingested 3,200 mg, 1.8 ± 0.54 pg/ml ($p = 0.038$). Hemoglobin significantly improved in the final determination in patients who ingested 1,600 mg, 12.13 ± 2.81 mg/day ($p = 0.012$), and 3,200 mg, 11.2 ± 1.63 mg/dl ($p = 0.03$) of sevelamer hydrochloride. Ferritin levels decreased significantly at the end of the study in those who ingested 1,600 mg, 190.99 ± 282.23 ng/ml, ($p = 0.028$), and 3,200 mg, 231.47 ± 154.03 ng/ml ($p = 0.05$) of sevelamer hydrochloride. The levels of CRP, 9.63 ± 7.36 mg/L ($p = 0.043$), and vitamin D, 28.7 ± 12.65 nmol/L ($p = 0.018$), increased at the end of the study in patients who ingested 1,600 mg of sevelamer hydrochloride (**Table 4**).

Hemodialysis and Calcium Carbonate Dose

Patients who ingested calcium carbonate in daily doses of 2–4 g showed an increase in NO, 1,313.13 ± 42.38 μg/ml ($p = 0.043$), and even those who ingested ≥ 5 g per day had increased NO levels at 6 months of follow-up, 1,410.69 ± 172.87 μg/ml ($p = 0.008$). The DNA repair enzyme was significantly increased at the final determination in patients who ingested 2–4 g of calcium carbonate, 16.23 ± 5.78 pg/ml ($p = 0.009$).

The activity of the antioxidant enzyme SOD was significantly increased in those who ingested ≥ 5 g in the final determination of the study, 8.13 ± 4.96 U/L ($p = 0.017$). TAC was determined to be significantly increased at 6 months of follow-up in those who ingested 1 (0.84 ± 0.29 μM, $p = 0.028$), 2–4, (0.89 ± 0.07 μM, $p = 0.007$), and ≥ 5 g (0.7 8 ± 0.13 μM ($p = 0.011$).

The expression of TNF-α decreased significantly in the final determination of those who ingested 2–4 g, 1.99 ± 0.33 pg/ml ($p = 0.001$). Hemoglobin improved with the doses of 2–4 (11.51 ± 2.5 mg/dl) and ≥5 g (11.93 ± 2.26 mg/dl, $p = 0.032$). CRP was significantly increased in patients who ingested 2–4 g at 6-month follow-up 10.66 ± 6.07 mg/L ($p = 0.008$). PTH was significantly increased in patients who ingested 1 g of calcium carbonate, 652.86 ± 217.31 pg/ml ($p = 0.043$) (**Table 4**).

Correlation Between Clinical Data and Markers of Inflammation and OS

Table 5 shows the moderate negative correlations found between SOD and PTH (rho = −0.052, $p = 0.035$), TAC and CRP (rho = −0.58, $p = 0.017$), for sevelamer hydrochloride. For calcium carbonate, moderate negative correlations were found for hOGG1 and HCO3 (rho = −0.68, $p = 0.05$), and LPO and pH (rho = −0.67, $p = 0.035$). Those with high correlations included 8-OHdG and urea (rho = −0.7, $p = 0.024$), and hOGG1 and K (rho = 0.7, $p = 0.038$) (**Table 5**).

DISCUSSION

Most patients on HD are treated with phosphate binders to reduce serum levels and intestinal absorption of this mineral (15). It is known that dietary Pi restriction is not sufficient to maintain serum concentration within recommended levels. The management of hyperphosphatemia includes various chelators, such as sevelamer hydrochloride and calcium carbonate (16). Sevelamer has been observed in previously published research to reduce the absorption of advanced glycation products (AGE), bacterial toxins, and bile acids, suggesting that this mechanism reduces inflammatory, oxidative, and atherogenic stimuli in addition to its direct action of reducing serum Pi (17). Recently, some antioxidant and anti-inflammatory pleiotropic effects of sevelamer hydrochloride not associated with Pi depletion

TABLE 4 | Doses of sevelamer hydrochloride intervention Calcium carbonate dose.

	1,600 mg n-16		WCX	2,400 mg n-5		WCX	3,200 mg n-11		WCX	K-W
	Baseline	**Six-months**	***p***	**Baseline**	**Six-months**	***p***	**Baseline**	**Six-months**	***p***	***p***
Oxidants										
LPO (mM)	14.48 ± 9.25	37.36 ± 36.51	0.12	24.20 ± 17.55	11.43 ± 4.02	0.65	9.18 ± 4.52	48.85 ± 42.79	**0.011**	0.54
NO (μg/mL)	1,209.88 ± 92.41	1,372.14 ± 177.50	**0.031**	1,301.14 ± 134.41	1,383.12 ± 272.53	0.65	1,120.51 ± 150.09	1,319.25 ± 158.35	**0.028**	0.89
8-IP (pg/mL)	51.96 ± 31.15	89.33 ± 84.95	0.16	18.41	55.78	—	53.28 ± 35.85	65.53 ± 34.06	0.51	0.90
Markers of oxidative damage to DNA										
8-OHdG (ng/mL)	7.32 ± 4.90	8.06 ± 3.64	0.57	7.65	7.33	—	6.23 ± 1.74	7.01 ± 2.71	0.37	0.48
hOGG1 (ng/mL)	3.33 ± 0.73	19.61 ± 8.80	**0.017**	7.95	14.5	—	3.25 ± 0.32	14.31 ± 10.24	0.051	0.29
Antioxidants										
SOD (U/L)	5.00 ± 3.04	7.93 ± 4.35	**0.047**	6.24 ± 5.53	3.70 ± 2.43	0.65	5.42 ± 4.40	8.18 ± 4.06	0.16	0.89
TAC (μM)	0.71 ± 0.17	0.91 ± 0.25	**0.004**	0.73 ± 0.00	0.69 ± 0.12	0.65	0.53 ± 0.20	0.85 ± 0.28	**0.011**	0.49
Pro-inflammatory cytokines										
IL-6 (pg/mL)	3.24 ± 1.79	2.30 ± 1.01	**0.030**	1.91	2.47	—	3.05 ± 1.97	2.63 ± 0.91	0.95	0.42
TNF-α (pg/mL)	3.25 ± 2.83	2.70 ± 2.14	0.09	1.12	1.41	—	2.28 ± 0.91	1.80 ± 0.54	**0.038**	0.95
Ferritin (ng/mL)	201.58 ± 242.00	190.99 ± 282.23	**0.028**	21.15 ± 0.49	11.45 ± 2.05	0.18	378.36 ± 220.96	231.47 ± 154.03	**0.050****	0.24
CRP (mg/L)	6.47 ± 6.87	9.63 ± 7.36	**0.043****	5.40 ± 3.23	10.80 ± 5.23	0.18	9.92 ± 8.69	9.15 ± 4.60	0.77	0.87
Biochemical data										
Hb (mg/dL)	10.12 ± 1.82	12.13 ± 2.81	**0.012****	13.00 ± 1.27	12.05 ± 1.48	0.65	9.87 ± 1.11	11.20 ± 1.63	**0.030****	0.79
Urea (mg/dL)	113.92 ± 34.45	99.20 ± 32.75	0.23	125.00 ± 16.97	134.00 ± 12.73	0.18	110.89 ± 46.75	115.67 ± 32.60	0.72	0.42
Uric acid (μmol/L)	5.05 ± 1.60	4.85 ± 1.34	0.65	6.15 ± 1.06	4.80 ±	—	5.80 ± 1.68	5.84 ± 1.38	0.46	0.70
SCr (mg/dL)	10.24 ± 2	10.03 ± 2.9	0.72	11.5 ± 0.91	9.4 ± 4.1	0.65	11.73 ± 2.9	10.40 ± 4.22	0.24	0.17
Pi (mg/dL)	6.57 ± 1.61	5.45 ± 1.12	0.08	6.50 ± 0.00	4.50 ± 2.40	0.18	6.88 ± 1.78	6.58 ± 1.64	0.29	0.49
Cl (mmol/L)	106.43 ± 27.89	98.91 ± 2.95	0.14	98.50 ± 2.12	97.00 ± 1.41	0.65	99.56 ± 2.83	102.00 ± 3.24	**0.041****	0.42
Vitamin D (nmol/L)	19.79 ± 10.18	28.70 ± 12.65	**0.018****	35.05 ± 18.74	35.20 ± 12.59	0.65	28.69 ± 13.65	27.18 ± 9.65	0.46	0.82
Calcium carbonate dose										
	1 g n–6			**2-4 g n-11**			**≥5 g n-15**			
	Baseline	**Six-months**	**Baseline**	**Six-months**	**Baseline**	**Six-months**	**Baseline**	**Six-months**		
Oxidants										
LPO (mM)	16.21 ± 10.81	42.90 ± 31.01	0.11	14.27 ± 2.10	33.33 ± 8.76	0.12	9.71 ± 3.62	27.27 ± 9.04	0.06	0.16
LPO (mM)	16.21 ± 10.81	42.90 ± 31.01	0.11	14.27 ± 2.10	33.33 ± 8.76	0.12	9.71 ± 3.62	27.27 ± 9.04	0.06	0.16
NO (μg/mL)	1,257.37 ± 96.35	1,269.13 ± 165.38	0.75	1,169.39 ± 37.29	1,313.13 ± 42.38	**0.022****	1,222.37 ± 103.72	1,410.69 ± 172.87	**0.008****	0.39
8-IP (pg/mL)	53.95 ± 34.28	98.36 ± 65.91	0.10	87.62 ± 15.62	84.42 ± 12.37	0.91	70.70 ± 60.51	75.12 ± 42.07	0.77	0.90

(Continued)

TABLE 4 | Continued

	1 g n-6			2-4 g n-11			≥5 g n-15			
	Baseline	**Six-months**	**Baseline**	**Six-months**	**Baseline**	**Six-months**	**Baseline**	**Six-months**		
Markers of oxidative damage to DNA										
8-OHdG (ng/mL)	7.35 ± 2.37	12.71 ± 2.88	0.10	7.30 ± 1.06	8.47 ± 1.26	0.17	8.49 ± 3.37	8.90 ± 7.03	0.44	0.08
hOGG1 (ng/mL)	5.91 ± 2.96	46.40 ± 26.45	0.28	6.00 ± 1.05	17.71 ± 5.72	**0.014****	4.66 ± 1.19	15.31 ± 7.5	0.11	0.16
Antioxidants										
SOD (U/L)	4.78 ± 3.06	7.08 ± 4.23	0.24	6.31 ± 1.00	8.34 ± 0.96	0.10	4.58 ± 3.04	8.13 ± 4.96	**0.017***	0.57
TAC (μM)	0.57 ± 0.17	0.84 ± 0.29	**0.028**	0.65 ± 0.04	0.89 ± 0.07	**0.007****	0.65 ± 0.22	0.78 ± 0.13	**0.011***	0.21
Pro-inflammatory cytokines										
IL-6 (pg/mL)	3.82 ± 2.47	3.87 ± 1.36	0.59	3.42 ± 0.42	2.58 ± 0.18	0.08	2.50 ± 0.62	2.60 ± 1.53	0.72	0.41
TNF-α (pg/mL)	2.14 ± 0.55	1.79 ± 0.08	0.28	2.56 ± 0.43	1.99 ± 0.33	**0.001****	1.92 ± 0.42	1.75 ± 0.38	0.59	0.33
Ferritin (ng/mL)	184.28 ± 146.37	144.16 ± 74.63	0.06	247.69 ± 106.41	320.87 ± 162.70	0.16	176.83 ± 86.49	199.26 ± 104.42	0.11	0.66
CRP (mg/L)	10.30 ± 11.26	20.53 ± 22.33	1.00	5.34 ± 4.82	10.66 ± 6.07	**0.008****	8.13 ± 9.71	7.87 ± 4.11	0.24	0.57
Biochemical data										
Hb (mg/dL)	10.00 ± 0.98	12 ± 0.84	0.08	10.23 ± 2.28	11.51 ± 2.50	**0.026****	10.74 ± 1.46	11.93 ± 2.26	**0.032****	0.71
Urea (mg/dL)	79.20 ± 42.32	107 ± 34.73	0.42	126.85 ± 40.02	115.39 ± 31.78	0.08	120.29 ± 36.62	107.75 ± 39.86	0.610	0.19
Uric acid (μmol/L)	4.44 ± 1.41	5.93 ± 0.40	0.11	5.29 ± 1.58	5.13 ± 1.51	0.79	5.22 ± 1.75	5.12 ± 1.56	1.000	0.28
SCr (mg/dL)	8.95 ± 2.51	9.58 ± 3.8	0.75	10.9 ± 3.45	10.31 ± 3.43	0.3	10.42 ± 1.76	9.95 ± 3.16	0.86	0.88
Cholesterol (mg/dL)	182.67 ± 40.30	174.25 ± 28.55	0.46	143.62 ± 30.41	159.63 ± 33.22	0.25	1058.93 ± 901.35	151.11 ± 22.03	**0.035****	0.36
Pi (mg/dL)	5.48 ± 1.98	5.76 ± 0.82	0.89	6.36 ± 2.36	6.14 ± 1.79	0.22	6.24 ± 1.24	5.40 ± 1.28	0.092	0.90
K (mmol/L)	4.95 ± 0.61	5.06 ± 0.43	1.00	5.37 ± 0.97	5.34 ± 0.61	0.50	5.07 ± 0.61	5.26 ± 0.87	0.47	**0.011*****
HCO_3(mEq/L)	24.18 ± 2.43	23.68 ± 1.89	0.68	21.70 ± 4.40	22.01 ± 5.12	0.36	24.32 ± 2.88	23.58 ± 1.84	0.20	**0.020****

*Values are expressed as mean ± standard deviation or standard error of the mean. ***Kruskal-Wallis test. **Wilcoxon rank test. LPO, Lipoperoxides; NO, Nitric Oxide; 8-IP, 8-isoprostanes; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; hOGG1, Oxoguanine glycosylase; SOD, Superoxide dismutase; TAC, Total Antioxidant Capacity; IL-6, Interleukin 6; TNF- α, Tumor necrosis factor alpha; Hb, hemoglobin; SCr, Serum creatinine; K, Potassium; Pi, Phosphate; Cl, Chlorine; CRP, C Reactive Protein; HCO3, Bicarbonate. It was not possible to measure the glomerular filtration rate since its value is <10 mL/min/1.73 m^2. Data in bold are statistically significant p-values.*

TABLE 5 | Correlation between oxidative stress markers and clinical parameters.

Redox biomarker	Clinical parameter	Sevelamer hydrochloride		Calcium carbonate	
		rho	*p*	rho	*p*
LPO	pH	0.53	**0.025**	−0.67	**0.035**
8-IP	Cl	0.56	**0.046**	−0.04	0.91
8-IP	HCO_3	0.58	**0.049**	−0.21	**0.056**
8-OHdG	Urea	−0.14	0.67	−0.7	**0.024**
hOGG	K	−0.01	0.98	0.7	**0.038**
hOGG1	Urea	0.65	**0.016**	0.49	**0.09**
hOGG	Cholesterol	−0.35	0.15	−0.72	**0.043**
hOGG	HCO_3	−0.38	0.25	−0.68	**0.05**
SOD	PTH	−0.52	**0.035**	0.61	**0.15**
TAC	CRP	−0.58	**0.017**	−0.1	0.87
TAC	Cl	0.43	**0.046**	0.2	0.58
IL−6	TSAT	−0.67	**0.0.13**	−0.14	0.76
IL−6	Vit D	0.2	0.65	1	**<0.01**
IL−6	HCO_3	−0.65	**0.022**	0.12	0.75

Data in bold are statistically significant p-values.

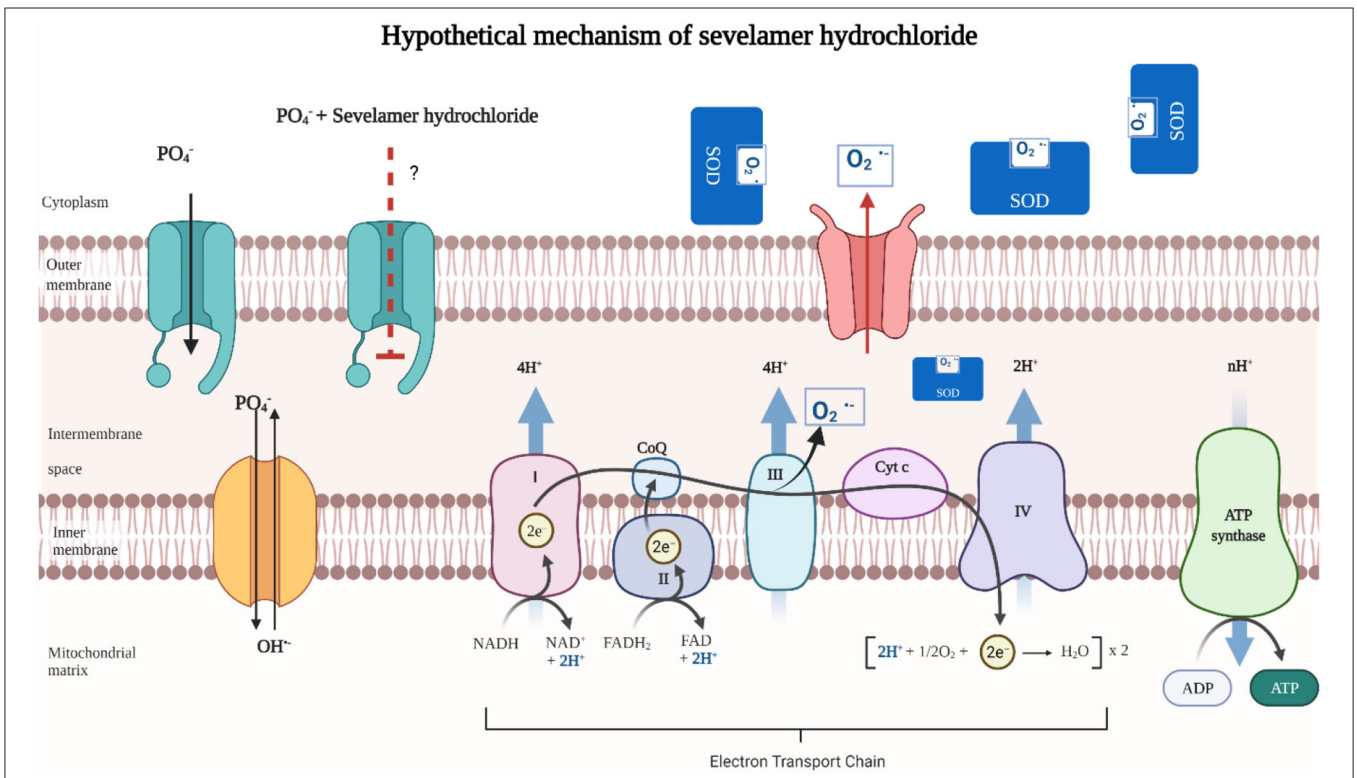

FIGURE 2 | Hypothetical mechanism of sevelamer hydrochloride in oxidative stress (OS). Increase in extracellular Pi causes mitochondrial OS conditioned by mitochondrial hyperpolarization produced by a phosphate-hydroxyl exchange. Hyperpolarization stimulates the electron transport chain and increases the concentration of superoxide radicals by complex III. The binding of Pi to sevelamer hydrochloride could explain the antioxidant mechanism by reducing mitochondrial OS (20).

have been described (18). Elevated extracellular Pi causes mitochondrial OS related to mitochondrial hyperpolarization (19). The binding of Pi to sevelamer hydrochloride could explain the antioxidant mechanism by reducing mitochondrial OS. In this clinical study, the influence of sevelamer hydrochloride and calcium carbonate on markers of inflammation, oxidants,

antioxidants, and oxidative DNA damage in patients on HD with 6 months of follow-up was evaluated (**Figure 2**).

Calcium carbonate is the first-line treatment for lowering Pi in ESRD. However, its main effect is to avoid significant hyperphosphatemia without normalizing Pi serum levels. Adequate adherence to treatment with calcium carbonate avoids the uncontrolled rise of hyperphosphatemia (21).

Lipoperoxidation produces increased OS, leading to the formation of molecules such as aldehydes (malondialdehyde), and whose measurement is used to determine OS (22). HD can remove water-soluble, low molecular-weight LPO products. However, the HD process can also favor the increase in LPO depending on the time spent in the HD program (23). The prolonged stay of a patient in renal replacement therapy is considered a contributing factor to increasing inflammation and OS (24). In this study, the results at 6-months follow-up showed an increase in LPO in patients treated with the 3,200 mg dose of sevelamer hydrochloride.

NO is a molecule with a wide variety of fundamental physiological functions, such as maintenance of muscle tone. It is an intracellular messenger, a cytotoxic agent, and has a primordial effect on the vascular endothelium. NO has specific functions in the kidney, regulating hemodynamics, salt and water reabsorption, renin secretion, and tubule-glomerular feedback. Its bioavailability abnormalities are causally related to various cardiovascular and renal disorders (20). In patients with ESRD, there could be decreased NO synthesis due to kidney damage (25). The disturbed balance of NO is related to the imbalance of endothelin-1 (ET-1) (26). NO is involved in the pathogenesis of hypertension and rebound hypotension during the HD process (27). The female gender tends to have better kidney function and higher NO concentration (as usual) due to the action of estrogens (28). According to previous studies, the bioavailability of NO in patients with DM is low (29, 30). Endothelial dysfunction is a key step in the development of atherosclerosis in HD. Endothelial dysfunction has been attributed to altered NO bioactivity and increased formation of oxygen-derived free radicals. The underlying mechanisms of the altered bioavailability of NO in patients on HD are not fully understood. However, the activation of cytokines during HD can increase NO production (31, 32). The increase in NO levels in the patients who ingested sevelamer hydrochloride could suggest that they were under nitrosative stress in the final follow-up. This phenomenon occurred in patients who ingested doses of 1,600 and 3,200 mg. In the group that ingested calcium carbonate, NO levels also increased in the determination at 6 months of follow-up and when the patients ingested amounts >2 g, suggesting that they were also under nitrosative stress.

8-Hydroxy-2-deoxyguanosine (8-OHdG) is a marker that determines oxidative damage to DNA (33, 34). OS determined by the 8-OHdG marker is an independent predictor of all-cause mortality and morbidities in patients on HD (35, 36). Previously, it has been found that the oxidative damage to DNA evaluated with the marker 8-OHdG in peripheral blood lymphocytes increased more in patients on chronic HD than in non-dialyzed patients (37). On the other hand, sevelamer hydrochloride has previously been reported to have the ability to reduce oxidative damage to genetic material (38). The authors of a recent study reported the potential effect of sevelamer treatment on inflammation and possible oxidative RNA modifications (39). The results obtained at 6 months of follow-up of the patients on HD included in these studies who ingested sevelamer hydrochloride showed a significant increase in the oxidative DNA damage marker different from previously reported.

Repair of oxidative DNA damage is essential to maintain the integrity of genetic material, prevent mutagenesis, and decrease damage caused by reactive oxygen species. The hOGG1 enzyme is responsible for identifying and repairing oxidative damage to DNA through base cleavage mechanisms (40). High levels of pro-inflammatory cytokines and certain oxidants can decrease the activity of the DNA repair enzyme, especially in patients with ESRD (41, 42). The authors of a recent publication reported the downregulation of DNA repair enzymes in patients dependent on the type of peritoneal transport (43). Significant increase in oxidative DNA damage repair enzyme levels in patients treated with sevelamer hydrochloride, at 6 months of follow-up, is notable, especially in those who ingested 1,600 mg of the phosphate binder. Given this finding, we could hypothesize that the significant increase in DNA repair enzyme in the sevelamer hydrochloride group of patients on HD could be compensating for the increased oxidative damage marker in DNA. In contrast, no modification of the oxidative DNA damage marker or DNA repair enzyme was observed in those who ingested calcium carbonate.

The SOD enzyme is the first antioxidant defense of the body. The dismutation of the superoxide anion characterizes the SOD enzyme into oxygen and hydrogen peroxide (44). Increased activity of the antioxidant enzyme SOD in patients on HD with or without hyperglycemic status is well-documented. Increased SOD activity is considered an indicator of future vascular complications in patients on HD (45, 46). Elevated extracellular Pi causes mitochondrial OS related to mitochondrial hyperpolarization (20). The binding of Pi to sevelamer hydrochloride could explain the antioxidant mechanism by reducing mitochondrial OS. In this study, a significant increase in the activity of the SOD enzyme was found in the groups treated with sevelamer hydrochloride and with calcium carbonate in the determination at 6 months of follow-up. The increase in the activity of the SOD enzyme was notorious in those who ingested 3,200 mg of sevelamer hydrochloride (**Figure 2**).

Total antioxidant capacity determines the sum of endogenous and exogenous antioxidants (47). Advanced glycation end (AGE) products are excreted in the urine of subjects with normal renal function. However, in patients with ESRD who require HD, AGE products accumulate in the body from insufficient urinary excretion and limited clearance during dialysis (48). Treatment with sevelamer hydrochloride has previously been reported to reduce systemic and cellular AGE levels by restoring innate antioxidant defenses, improving inflammatory status, and reducing chronic OS (49, 50). At the end of the follow-up period of this study, SOD enzyme activity and TAC levels were found to be increased in the groups treated with sevelamer hydrochloride and calcium carbonate. These findings could suggest an attempt

to compensate for the increase in oxidant molecules LPO and NO.

Mediators that orchestrate inflammatory response are cytokines. Among the cytokines, IL-6, and TNF-α have profound effects on the pro-inflammatory process in patients undergoing HD (51). According to our results, sevelamer hydrochloride showed an anti-inflammatory effect (52). Sevelamer hydrochloride can bind the endotoxins present in the intestinal lumen. In this way, the negatively charged lipid. Endotoxins are a powerful stimulus with the ability to activate the innate immune system that favors the transcription and production of pro-inflammatory cytokines. A part of endotoxins binds to sevelamer. In this way, sevelamer hydrochloride could exert its anti-inflammatory effect (53, 54). IL-6 decreased significantly in the determination at 6 months of follow-up in patients who ingested calcium carbonate and those who ingested 1,600 mg of sevelamer hydrochloride. TNF-α showed downregulation at 6 months of follow-up in the sevelamer hydrochloride and calcium carbonate groups. TNF-α decreased its levels primarily in those who ingested 3,200 mg of sevelamer hydrochloride and in those who ingested 2–4 g of calcium carbonate.

Sevelamer hydrochloride (more significant effect) and calcium carbonate showed anti-inflammatory and antioxidant effects independent of the phosphate inhibitory effect as previously reported (55, 56). Inflammatory markers, such as CRP, progressively increase with deterioration in renal function (57). Available data, such as CRP, TNF-α, IL-6, and arteriovenous fistula dysfunction, demonstrated that these systemic inflammatory markers were elevated in patients on HD (58). CRP was found to be increased in the final determination at the follow-up in those who ingested calcium carbonate, 2–4 g of calcium carbonate, and 1,600 mg of sevelamer hydrochloride. Alteration of serum ferritin may be a determining factor in mortality in adults on HD, and high ferritin levels lead to

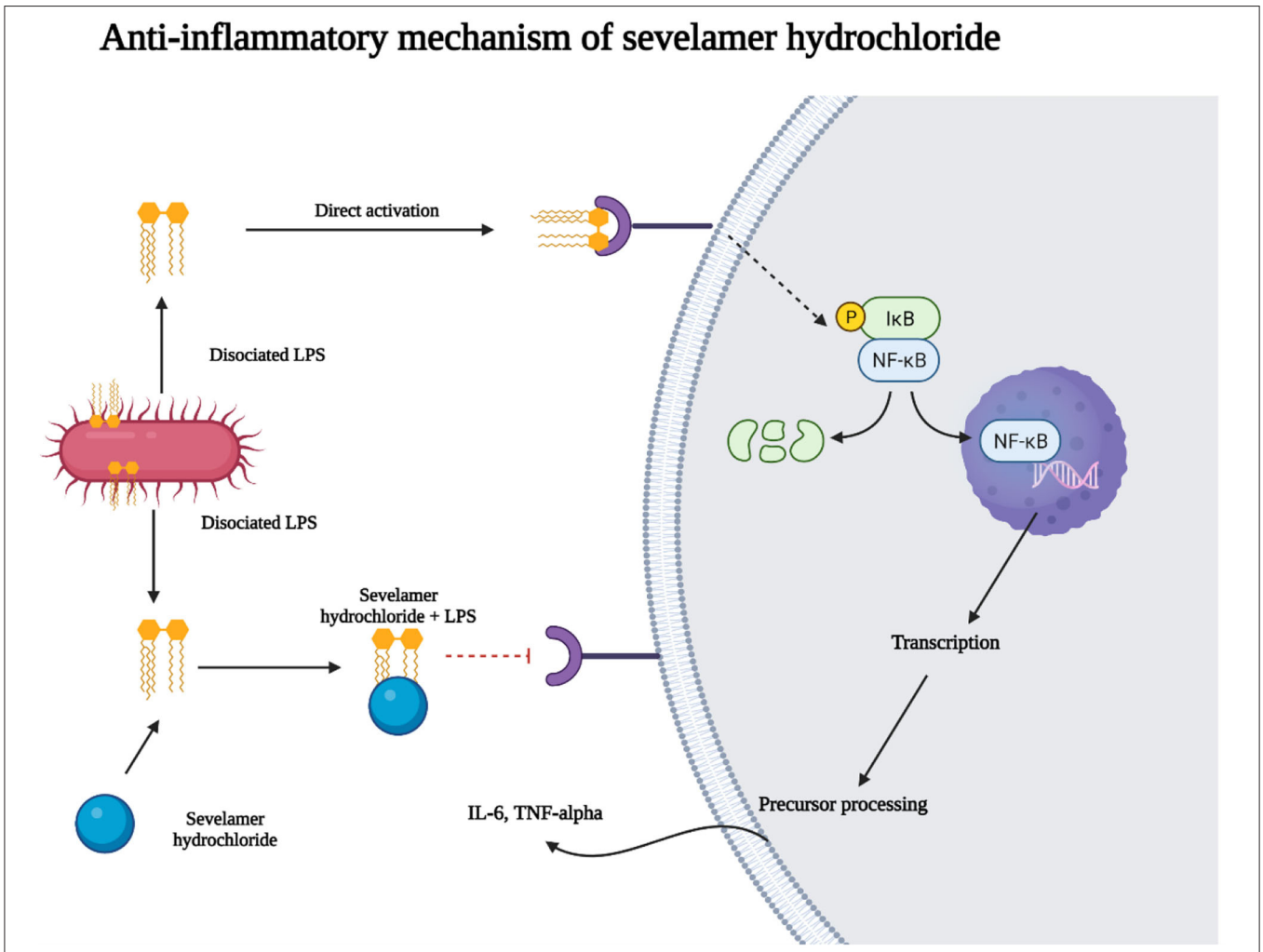

FIGURE 3 | Anti-inflammatory of sevelamer hydrochloride. Sevelamer hydrochloride can bind to endotoxins present in the intestinal lumen. In this way, the negatively charged lipid A portion of endotoxins binds to sevelamer. Endotoxins are a component of the cell wall of gram-negative bacteria and are a potent stimulus for activating the innate immune system that leads to the transcription of pro-inflammatory cytokines. In this way, sevelamer hydrochloride could exert its anti-inflammatory effect.

high mortality (59). The alteration of serum ferritin can be a determining factor in mortality in adults on HD. At the end of the follow-up of the patients who ingested sevelamer hydrochloride, ferritin levels decreased. In patients who ingested 1,600 and 3,200 mg of sevelamer hydrochloride, ferritin levels were decreased (**Figure 3**).

CONCLUSIONS

The pleiotropic antioxidant and anti-inflammatory effects of sevelamer hydrochloride were fundamental in the results of this research study. OS was manifested by a significant increase in LPO and NO levels in the final determination at 6 months of follow-up in the sevelamer hydrochloride group and with the intake of 1,600 and 3,200 mg. The increase in NO at the end of the follow-up favored OS in the calcium carbonate group when they ingested doses of 2–4 and >5 g. OS is not only explained by the intake of Pi binders. ESRD and HD could play an essentially active role in their presence. As a probable compensatory mechanism for the increase in OS markers, levels of antioxidant enzymes were found to be increased at 6 months of follow-up. Increase in the SOD enzyme and TAC activity was observed in the sevelamer hydrochloride group and calcium carbonate groups in the final determination at the follow-up. A more significant increase in SOD activity was observed when the patients ingested 1,600 and 3,200 mg of sevelamer hydrochloride. In the calcium carbonate group, the increase in TAC was observed when they ingested from 1, 2–4, and more than 5 g. The behavior of pro-inflammatory cytokines was downregulated; primarily, TNF-α decreased its levels in the final determination at six months of follow-up in the groups that ingested sevelamer hydrochloride and calcium carbonate. Decrease in TNF-α is predominant in those who ingested 2–4 g of calcium carbonate or 3,200 mg of sevelamer hydrochloride. IL-6 decreased in those who ingested 1,600 mg of sevelamer hydrochloride and in the final determination of the calcium carbonate group, suggesting that both phosphate inhibitors have anti-inflammatory effects. Ferritin levels decreased significantly at 6 months of follow-up in the sevelamer hydrochloride group. This phenomenon was predominantly observed when they ingested the 1,600 and 3,200 mg doses, suggesting an anti-inflammatory improvement.

Contrary to the sevelamer hydrochloride, those who ingested calcium carbonate did not show changes in their levels. CRP levels increased in the final determination of the calcium carbonate group. The beneficial influence of sevelamer hydrochloride on pro-inflammatory cytokines and OS markers could be essential in managing phosphate binders in patients on HD by offering advantages in morbidity. However, the mechanism of action of sevelamer hydrochloride in OS is not fully understood. Long-term studies with a larger number of patients are required to know all the benefits that they can provide to patients with ESRD.

Study Limitations

The limitations of this study are based on the small number of patients in the HD program and the short follow-up time. We consider it worthwhile to design another stratified analysis that considers several phosphorus inhibitors for different follow-up times.

Study Strengths

The study offers a broader view on the simple use of two phosphate binders, the widely known calcium carbonate, and sevelamer hydrochloride, with probable anti-inflammatory pleiotropic effects. The information reported on the impact of sevelamer hydrochloride on the repair of oxidative DNA damage is scarce. In this study, we found the overexpression of the DNA repair enzyme possibly compensating for the significant increase in the oxidative DNA damage marker in patients on HD. This study provides new information on the possible regulatory effects of sevelamer hydrochloride on DNA repair.

DATA AVAILABILITY STATEMENT

The data raw that support the conclusions of this article will be made available by the authors, with prior authorization from the Research and Ethics Committee.

ETHICS STATEMENT

The study was followed under the ethical principles for medical research in human beings stipulated in the declaration of Helsinki 64th General Assembly, Fortaleza Brazil, October 2013, and the Standards of Good Clinical Practice according to the guidelines of the International Conference on Harmonization. Under the General Health Law's provisions following the Regulations of the General Health Law on Research for Health Article 17 of Mexico, the study corresponds to category III. All the patients signed the Low Information Consent in the presence of witnesses. The study was evaluated and approved by the Local Ethics and Research Committee at the CentroMédico Nacional de Occidente, Instituto Mexicano del Seguro Social (R-2021-1301-063).

AUTHOR CONTRIBUTIONS

ED-D, AG-S, AGM-D, and JC-G: conception and design. CP-N, JA-S, BJ-M, AB-L, and ER-C: acquisition, analysis, and interpretation of data. AG-S, ED-D, and AGM-D: drafting the article and revising it critically for important intellectual content. ED-D, AG-S, AGM-D, JC-G, CP-N, JA-S, BJ-M, AB-L, and ER-C: final approval of the version to be published. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Cozzolino M, Ureña-Torres P, Vervloet MG, Brandenburg V, Bover J, Goldsmith D, et al. Is chronic kidney disease-mineral bone disorder (CKD-MBD) really a syndrome? *Nephrol Dial Transplant.* (2014) 29:1815–20. doi: 10.1093/ndt/gft514
2. Taniguchi M, Fukagawa M, Fujii N, Hamano T, Shoji T, Yokoyama K, et al. Serum phosphate and calcium should be primarily and consistently

controlled in prevalent hemodialysis patients. committee of renal data registry of the japanese society for dialysis therapy. *Ther Apher Dial.* (2013) 17:221–8. doi: 10.1111/1744-9987.12030
3. Young EW, Albert JM, Satayathum S, Goodkin DA, Pisoni RL, Akiba T, et al. Predictors and consequences of altered mineral metabolism: the dialysis outcomes and practice patterns study. *Kidney Int.* (2005) 67:1179–87. doi: 10.1111/j.1523-1755.2005.00185.x
4. Scialla JJ, Kendrick J, Uribarri J, Kovesdy CP, Gutiérrez OM, Jimenez EY, et al. State-of-the-art management of hyperphosphatemia in patients with CKD: An NKF-KDOQI controversies perspective. *Am J Kidney Dis.* (2021) 77:132–41. doi: 10.1053/j.ajkd.2020.05.025
5. Suki WN, Zabaneh R, Cangiano JL, Reed J, Fischer D, Garrett L, et al. Effects of sevelamer and calcium-based phosphate binders on mortality in hemodialysis patients. *Kidney Int.* (2007) 72:1130–37. doi: 10.1038/sj.ki.5002466
6. Tzanno-Martins C, Biavo BM, Ferreira-Filho O, et al. Clinical efficacy, safety and anti-inflammatory activity of two sevelamer tablet forms in patients on low-flux hemodialysis. *Int J Immunopathol Pharmacol.* (2014) 27:25–35. doi: 10.1177/039463201402700105
7. Patel L, Bernard LM, Elder GJ. Sevelamer versus calcium-based binders for treatment of hyperphosphatemia in CKD: a meta-analysis of randomized controlled trials. *Clin J Am Soc Nephrol.* (2016) 11:232–44. doi: 10.2215/CJN.06800615
8. Komaba H, Wang M, Taniguchi M, Yamamoto S, Nomura T, Schaube DE, et al. Initiation of sevelamer and mortality among hemodialysis patients treated with calcium-based phosphate binders. *Clin J Am Soc Nephrol.* (2017) 12:1489–97. doi: 10.2215/CJN.13091216
9. Rodríguez-Osorio L, Zambrano DP, Gracia-Iguacel C, Rojas-Rivera J, Ortiz A, Egido J, et al. Use of sevelamer in chronic kidney disease: beyond phosphorus control. *Nefrologia.* (2015) 35:207–17. doi: 10.1016/j.nefroe.2015.05.011
10. Peres AT, Dalboni MA, Canziani ME, Manfredi SR, Carvalho JTG, Batista MC, et al. Effect of phosphate binders on oxidative stress and inflammation markers in hemodialysis patients. *Hemodial Int.* (2009) 13:271–77. doi: 10.1111/j.1542-4758.2009.00369.x
11. Liakopoulos V, Roumeliotis S, Gorny X, Dounousi E, Mertens PR. Oxidative stress in hemodialysis patients: a review of the literature. *Oxid Med Cell Longev.* (2017) 2017:3081856. doi: 10.1155/2017/3081856
12. Ikee R, Tsunoda M, Sasaki N, Sato N, Hashimoto N. Emerging effects of sevelamer in chronic kidney disease. *Kidney Blood Press Res.* (2013) 37:24–32. doi: 10.1159/000343397
13. Evenepoel P, Selgas R, Caputo F, Foggensteiner L, Heaf JG, Ortiz A, et al. Efficacy and safety of sevelamer hydrochloride and calcium acetate in patients on peritoneal dialysis. *Nephrol Dial Transplant.* (2009) 24:278–85. doi: 10.1093/ndt/gfn488
14. Sakpal TV. Sample size estimation in clinical trial. *Perspect Clin Res.* (2010) 1:67–9.
15. Gonzalez-Parra E, Tuñón J, Egido J, Ortiz A. Phosphate: a stealthier killer than previously thought? *Cardiovasc Pathol.* (2012) 21:372–81. doi: 10.1016/j.carpath.2012.02.008
16. Spaia S. Phosphate binders: sevelamer in the prevention and treatment of hyperphosphataemia in chronic renal failure. *Hippokratia.* (2011) 15:22–6.
17. Rastogi A. Sevelamer revisited: pleiotropic effects on endothelial and cardiovascular risk factors in chronic kidney disease and end-stage renal disease. *Ther Adv Cardiovasc Dis.* (2013) 7:322–42. doi: 10.1177/1753944713513061
18. Yu-Feng L, Chiang-Ting C, Wei-Chih K. Pleiotropic effects of sevelamer beyond phosphate binding in end-etage renal disease patients A randomized, open-label, parallel-group study. *Clin Drug Investig.* (2011) 31:257–67. doi: 10.2165/11539120-000000000-00000
19. Nguyen TT, Quan X, Hwang KH, Xu S, et al. Mitochondrial oxidative stress mediates high-phosphate-induced secretory defects and apoptosis in insulin-secreting cells. *Am J Physiol Endocrinol Metab.* (2015) 308: E933–41. doi: 10.1152/ajpendo.00009.2015
20. Lee J, Bae EH, Ma SK, Kim SW. Altered nitric oxide system in cardiovascular and renal diseases. *Chonnam Med J.* (2016) 52:81–90. doi: 10.4068/cmj.2016.52.2.81
21. Di Iorio B, Molony D, Bell C, Cucciniello E, Bellizzi V, Russo D, et al. Sevelamer versus calcium carbonate in incident hemodialysis patients: results of an open-label 24-month randomized clinical trial. *Am J Kidney Dis.* (2013) 62:771–8. doi: 10.1053/j.ajkd.2013.03.023
22. Boaz M, Matas Z, Biro A, Katzir Z, Green M, Fainaru M, et al. Serum malondialdehyde and prevalent cardiovascular disease in hemodialysis. *Kidney Int.* (1999) 56:1078–83. doi: 10.1046/j.1523-1755.1999.00613.x
23. Ben Omrane Sioud O, El Ati Z, Bouzidi H, Kerkeni M, Hammami M. Lipid and oxidative profile in hemodialysis patients: clinical follow-up for three years. *Tunis Med.* (2019) 97:551–55.
24. Lucchi L, Iannone A, Bergamini S, Stipo L, Perrone S, Uggeri S, et al. Comparison between hydroperoxides and malondialdehyde as markers of acute oxidative injury during hemodialysis. *Artif Organs.* (2005) 29:832–37. doi: 10.1111/j.1525-1594.2005.00136.x
25. Kang ES, Tevlin MT, Wang YB, Chiang TM, Cardenas R, Myers LK, et al. Hemodialysis hypotension: interaction of inhibitors, iNOS, and the interdialytic period. *Am J Med Sci.* (1999) 317:9–21. doi: 10.1097/00000441-199901000-00003
26. Noris M, Benigni A, Boccardo P, Aiello S, Gaspari F, Todeschini M, et al. Enhanced nitric oxide synthesis in uremia: Implications for platelet dysfunction and dialysis hypotension. *Kidney Int.* (1993) 44:445–50. doi: 10.1038/ki.1993.264
27. Raj DSC, Vincent B, Simpson K, Sato E, Jones KL, Welbourne TC, et al. Hemodynamic changes during hemodialysis: role of nitric oxide and endothelin. *Kidney Int.* (2002) 61:697–704. doi: 10.1046/j.1523-1755.2002.00150.x
28. McGuire BB, Watson RW, Pérez-Barriocal F, Fitzpatrick JM, Docherty NG. Gender differences in the renin-angiotensin and nitric oxide systems: relevance in the normal and diseased kidney. *Kidney Blood Press Res.* (2007) 30:67–80. doi: 10.1159/000099150
29. Assmann TS, Brondani LA, Bouças AP, Rheinheimer J, de Souza BM, Canani LH, et al. Nitric oxide levels in patients with diabetes mellitus: a systematic review and meta-analysis. *Nitric Oxide.* (2016) 69:1–9. doi: 10.1016/j.niox.2016.09.009
30. Tsapenko MV, d'Uscio LV, Grande JP, Croatt AJ, Hernandez MC, Ackerman AW, et al. Increased production of superoxide anion contributes to dysfunction of the arteriovenous fistula. *Am J Physiol Renal Physiol.* (2012) 303:F1601–7. doi: 10.1152/ajprenal.00449.2012
31. Meyer C, Heiss C, Drexhage C, Kehmeier ES, Balzer J, Mühlfeld A, et al. Hemodialysis-induced release of hemoglobin limits nitric oxide bioavailability and impairs vascular function. *J Am Coll Cardiol.* (2010) 55:454–9. doi: 10.1016/j.jacc.2009.07.068
32. Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol.* (2006) 147:S193–201. doi: 10.1038/sj.bjp.0706458
33. Gedik CM, Boyle SP, Wood SG, Vaughan NJ, Collins AR. Oxidative stress in humans: validation of biomarkers of DNA damage. *Carcinogenesis.* (2002) 23:1441–46. doi: 10.1093/carcin/23.9.1441
34. Kasai H. Analysis of a form of oxidative DNA damage, 8-hydroxy-2-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res.* (1997) 387:147–63.
35. Xu H, Watanabe M, Qureshi AR, Heimbürger O, Bárány P, Anderstam B, et al. Oxidative DNA damage and mortality in hemodialysis and peritoneal dialysis patients. *Perit Dial Int.* (2015) 32:206–15. doi: 10.3747/pdi.2013.00259
36. Mastalerz-Migas A, Steciwko A, Pokorski M, Pirogowicz I, Drobnik J, Bunio A, et al. What influences the level of oxidative stress as measured by 8-hydroxy-2'-deoxyguanosine in patients on hemodialysis? *J Physiol Pharmacol.* (2006) 57:199–205.
37. Tarng DC, Liu TY, Huang TP. Protective effect of vitamin C on 8-hydroxy-2'-deoxyguanosine level in peripheral blood lymphocytes of chronic hemodialysis patients. *Kidney Int.* (2004) 66:820–31. doi: 10.1111/j.1523-1755.2004.00809.x
38. Pastor S, Coll E, Rodríguez-Ribera L, Stoyanova E, Corredor ZF, Marcos R. Influence of carnicor, venofer, and sevelamer on the levels of genotoxic damage in end-stage renal disease patients. *Environ Mol Mutagen.* (2018) 59:302–11. doi: 10.1002/em.22170
39. Brønden A, Larsen EL, KarstoftK, Henriksen T, Vilsbøll T, Poulsen HE, et al. Changes in oxidative nucleic acid modifications and inflammation following one-week treatment with the bile acid sequestrant sevelamer: two randomized, placebo-controlled trials. *J Diabetes Complications.* (2020) 34:107446. doi: 10.1016/j.jdiacomp.2019.107446

40. Bravard A, Vacher M, Gouget B, Coutant A, de Boisferon FH, Stéphanie Marsin, et al. Redox regulation of human OGG1 activity in response to cellular oxidative stress. *Mol Cell Biol.* (2006) 26:7430–36. doi: 10.1128/MCB.00624-06
41. Tarng DC, Tsai TJ, Chen WT, Liu TY, Wei YH. Effect of human OGG1 1245C–>G gene polymorphism on 8-hydroxy-2'-deoxyguanosine levels of leukocyte DNA among patients undergoing chronic hemodialysis. *J Am Soc Nephrol.* (2001) 12:2338–47. doi: 10.1681/ASN.V12112338
42. Pan G, Deshpande M, Pang H, Stemmer PM, Carruthers NJ, Shearn CT, et al. 4-Hydroxy-2-nonenal attenuates 8-oxoguanine DNA glycosylase 1 activity. *J Cell Biochem*. (2020) 121:4887–97. doi: 10.1002/jcb.29814
43. Gutiérrez-Prieto JA, Soto-Vargas J, Parra-Michel R, Pazarín-Villaseñor HL, García-Sánchez A, Miranda-Díaz AG. The Behavior of the type of peritoneal transport in the inflammatory and oxidative status in adults under peritoneal dialysis. *Front Med.* (2019) 6:210. doi: 10.3389/fmed.2019.00210
44. Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem.* (1995) 64:97–112. doi: 10.1146/annurev.bi.64.070195.000525
45. Kimura F, Hasegawa G, Obayashi H, Adachi T, Hara H, Ohta M, et al. Serum extracellular superoxide dismutase in patients with type 2 diabetes: relationship to the development of micro- and macrovascular complications. *Diabetes Care.* (2003) 26:1246–50. doi: 10.2337/diacare.26.4.1246
46. Tajbakhsh R, Qorbani M, Mehrpour G, Rahimzadeh M, Azimzadeh MM, Mirmiranpour H. Effect of hemodialysis on oxidants and antioxidant factors in chronic renal failure. *Saudi J Kidney Dis Transpl.* (2017) 28:507–16. doi: 10.4103/1319-2442.206446
47. Rezaie A, Parker RD, Abdollahi M. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Dig Dis Sci.* (2007) 52:2015–21. doi: 10.1007/s10620-006-9622-2
48. Yacoub R, Nugent M, Cai W, Nadkarni GN, Chaves LD, Abyad S, et al. Advanced glycation end products dietary restriction effects on bacterial gut microbiota in peritoneal dialysis patients; a randomized open label-controlled trial. *PLoS ONE.* (2017) 12:e0184789. doi: 10.1371/journal.pone.0184789
49. Yubero-Serrano EM, Woodward M, Poretsky L, Vlassara H, Strike GE. AGE-less Study Group. Effects of sevelamer carbonate on advanced glycation end products and antioxidant/pro-oxidant status in patients with diabetic kidney disease. *Clin J Am Soc Nephrol*. (2015) 10:759–66. doi: 10.2215/CJN.07750814
50. Ogunleye A, Akinbodewa AA, Adejumo OA, Oluwafemmi TT, Akinfaderin DA. Changes in antioxidant status associated with haemodialysis in chronic kidney disease. *Ghana Med J.* (2018) 52:29–33. doi: 10.4314/gmj.v52i1.6
51. Arena A, Stassi G, Iannello D, Gazzara D, Calapai M, Bisignano C, et al. Both IL-1β and TNF-α regulate NGAL expression in polymorphonuclear granulocytes of chronic hemodialysis patients. *Mediators Inflamm.* (2010) 2010:613937. doi: 10.1155/2010/613937
52. Ruggiero B, Trillini M, Tartaglione L, Rotondi S, Perticucci E, Tripepi R, et al. Effects of sevelamer carbonate in patients with CKD and proteinuria: the ANSWER randomized trial. *Am J Kidney Dis.* (2019) 74:338–50. doi: 10.1053/j.ajkd.2019.01.029
53. Kubotera N, Prokopienko AJ, Garba AO, Pai AB. Endotoxin binding by sevelamer: potential impact on nutritional status. *Int J Nephrol.* (2013) 2013:954956. doi: 10.1155/2013/954956
54. Perianayagam MC, Jaber BL. Endotoxin-binding affinity of sevelamer hydrochloride. *Am J Nephrol.* (2008) 285:802–7. doi: 10.1159/000135691
55. Shantouf R, Budoff MJ, Ahmadi N, Tiano J, Flores F, Kalantar-Zadeh K. Effects of sevelamer and calcium-based phosphate binders on lipid and inflammatory markers in hemodialysis patients. *Am J Nephrol.* (2008) 28:275–79. doi: 10.1159/000111061
56. Navarro-González JF, Mora-Fernández C, Muros de Fuentes M, Dontate-Correa J, Cazaña-Pérez V, García-Pérez J. Effect of phosphate binders on serum inflammatory profile, soluble CD14, and endotoxin levels in hemodialysis patients. *Clin J Am Soc Nephrol.* (2011) 6:2272–79. doi: 10.2215/CJN.01650211
57. Cachofeiro V, Goicochea M, de Vinuesa SG, Oubiña P, Lahera V, Luño J. Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease. *Kidney Int.* (2008) 111:S4–9. doi: 10.1038/ki.2008.516
58. Kuo WH, Lee YT, Ng HY, Wang CY, Wu CH, Lee CT. C-reactive protein variability is associated with vascular access outcome in hemodialysis patients. *J Clin Lab Anal.* (2018) 32:e22213. doi: 10.1002/jcla.22213
59. Kuragano T, Matsumura O, Matsuda A, Hara T, Kiyomoto H, Murata T, et al. Association between hemoglobin variability, serum ferritin levels, and adverse events/mortality in maintenance hemodialysis patients. *Kidney Int.* (2014) 86:845–5. doi: 10.1038/ki.2014.114

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Inhibition of Bruton's Tyrosine Kinase Protects Against Burn Sepsis-Induced Intestinal Injury

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This study aimed to investigate the role and molecular mechanisms of Bruton's tyrosine kinase (BTK), a member of the Tec family in burn sepsis-induced intestinal injury. Eighty C57BL/6 mice were randomly divided into four groups: the sham group, the burn group, the burn + sepsis group, and the burn + sepsis + LFM-A13 (a selective BTK inhibitor) group. The dynamic expression profiles of BTK and p-BTK in the intestine were measured by Western blot analysis. Intestinal histopathological changes and cellular apoptosis were determined. Inflammatory cytokines in serum and intestinal tissue were examined through enzyme-linked immunosorbent assay. Myeloperoxidase (MPO) activity was determined *via* a colorimetric assay. Intestinal p-BTK expression in the burn+sepsis group was significantly increased compared with that in the sham and burn groups. In the burn + sepsis group, the p-BTK expression level increased over time, peaked at 12, and then decreased at 24 h. LFM-A13 administration significantly inhibited p-BTK expression in the intestine. In contrast to the sham and burn groups, the burn + sepsis group exhibited obvious histopathological changes, which gradually aggravated over time. LFM-A13 also reduced the histopathological changes and cellular apoptosis in intestinal tissues, inhibited the inflammatory cytokines IL-4, IL-6, and TNF-α in serum and intestinal tissues, and significantly inhibited the increase in intestinal MPO activity induced by burn sepsis. BTK activation is one important aspect of the signaling event that may mediate the release of the anti-inflammatory cytokine IL-4 and the pro-inflammatory cytokines IL-6 and TNF-α; oxidative stress; and intestinal cell apoptosis. Thus, it contributes to burn sepsis-induced intestinal injury.

Keywords: Bruton's tyrosine kinase, intestinal injury, burns, sepsis, oxidative stress

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INTRODUCTION

Burn injuries, as one of the common clinical critical illnesses worldwide caused by thermal aggressions, have caused high morbidity and mortality rates, especially in low and middle-income countries (1). Burns affect the integrity of the skin, which acts as the protective organ that prevents the body from experiencing microbial invasion; extensive burn injuries are highly prone to inflicting deep damages, such as severe infections, complications, and sepsis, to the body (2). Sepsis is a multifactorial and complex pathophysiological syndrome caused by systemic infection with exaggerated inflammatory response and leads to severe consequences, such as shock, multiple organ dysfunction, and even death (3). According to the recent statistics of the United States, the mortality rate among patients with sepsis is approximately 20–50%, making sepsis a major health

and economic concern (4, 5). A series of diagnostic and treatment measures, including appropriate antimicrobial treatment, infection control measures, fluid and nutritional support, debridement, and wound closure, have been gradually improved in recent years, considerably enhancing the prevention and treatment of burn sepsis and improving the taking rate (6, 7). However, the pathogenesis and knowledge of sepsis remain insufficient even after decades of exploration by researchers, resulting in the lack of theoretical guidance for the further treatment of this disease.

As generally known, the main functions of the intestinal tract are digestion and absorption to provide essential energy to organisms, and the intestinal tract is often considered to play a passive role in the development of sepsis and the actions of the body against pathogenic microorganisms (8). However, several scholars have found that during the early stage of sepsis, some abnormal phenomena occur during intestinal metabolism, that is, the intestinal tract is considered to be the "engine" in the process of sepsis-induced multiorgan dysfunction syndrome (MODS) (9). Therefore, exploring the relationship between the dysfunction of the intestinal mucosal barrier and the occurrence of sepsis is essential (10). With the deepening of research in recent years, researchers have discovered that the dysfunction of the intestinal mucosal barrier leads to the imbalance of intestinal microbiota; the entry of endotoxins into the blood through the barrier may induce the release of large amounts of inflammatory factors and thus accelerate the development of sepsis (11). In addition, intestinal epithelial and endothelial cells are further disrupted when sepsis-induced excessive inflammatory responses occur, further exacerbating intestinal permeability and intestinal flora imbalance and thus inducing a vicious cycle of sepsis (12). Moreover, these interactions lead to the activation of large numbers of genes, inflammatory cytokines (e.g., the proinflammatory cytokines TNF-α andIL-6 and the anti-inflammatory cytokine IL-4), and chemokines that are released by macrophages under the mediation of downstream signaling molecules involved in inflammatory responses (13). These processes enhance the systemic inflammatory response and further aggravate damage to the viscera. Therefore, the treatment of sepsis by blocking these signaling molecules is considered as a promising approach.

Recent studies have shown that Gram-negative bacteria are one of the prominent pathogens that cause sepsis, and the key molecule that can induce sepsis is the lipopolysaccharide (LPS) present on their surfaces (14, 15). As a Toll-like receptor (TLR), an important protein molecule involved in non-specific immunity, the TLR4 molecule could bind with the antigen molecule LPS, activate the signaling pathways of TRIF and MyD88, and then stimulate their downstream signaling pathways, this causing systemic or local inflammatory reactions in the body (16, 17). Therefore, TLR4 is a promising target in the treatment and intervention of sepsis. However, blocking the whole TLR4 pathway may result in the failure of follow-up anti-infective treatment given that burn sepsis is an aggravation of systemic inflammatory response.

The Tec family, which has been widely studied in recent years, is a series of cytoplasmic tyrosine protein kinase molecules and non-receptor tyrosine kinases (TKs) that are mainly expressed in lymphocytes and myeloid cells (18). BTK, a member of the Tec family, is involved in the growth and function of B cells; it is closely associated with a variety of B-cell lymphoid tissue disorders and is also a key kinase in the B-cell antigen receptor signaling pathway (19). In addition, in human mono-nuclear cell lines and primary human mono-nuclear cells, BTK is one of the important signaling molecules in the TLR signal pathway and is phosphorylated after LPS stimulation and interacts with multiple components of the TLR pathway (20). Therefore, the selective blocking of BTK may provide greater benefits to patients than blocking the whole TLR4 pathway, and BTK may be an ideal target for intervention in patients under the condition of systemic inflammatory response and organ dysfunction caused by burn sepsis. LFM-A13, the first inhibitor of BTK, was used as the selective blocking of BTK in this study (21).

Therefore, the present study was designed to determine the effects of LFM-A13 on burn-sepsis-induced intestinal injury; the intestinal activation of BTK; the expression changes of the anti-inflammatory cytokine IL-4 and the proinflammatory cytokines IL-6 and TNF-α; oxidative stress; and intestinal cell apoptosis in the intestinal injury of mice induced by burn sepsis.

MATERIALS AND METHODS

Animals and Reagents

Male C57BL/6 mice with average weights of 23–26 g were purchased from the Corporation of Lingchang Biological Technology (Shanghai, China). The animals were allowed to acclimate to their surroundings for 1 week. LPS was purchased from Sigma (USA). LFM-A13 was purchased from Topscience (Shanghai, China). Kits for protein extraction, BCA protein assay, SDS-PAGE gel preparation, Western blot analysis, hematoxylin and eosin (H&E staining), TUNEL apoptosis assay, mouse IL-4 enzyme-linked immunosorbent assay (ELISA), mouse IL-6 ELISA, mouse TNF-α ELISA, and myeloperoxidase (MPO) colorimetric activity assay were purchased from KeyGEN (Nanjing, China). Neutral gum, methanol, ethanol, and xylene were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Triton-X100 was purchased from KeyGEN (Nanjing, China). Rabbit anti-GAPDH (10B8) and goat antirabbit IgG-HRP were purchased from KeyGEN (Nanjing, China). Rabbit anti-p-BTK (ab52192) was purchased from Abcam (Shanghai, China). Rabbit anti-BTK (DF6472) was purchased from Affinity Biosciences (Cincinnati, USA).

Animal Model Preparation and Groups

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Anhui Medical University Ethics Committee of Animal Experiments in Hefei city, China (Permit Number: 20160188).

Mice were anesthetized intraperitoneally with pentobarbital sodium (30 mg/kg), and their back skin were shaved. Eighty animals were randomly divided into four groups: sham group ($n = 8$), burn group ($n = 8$), burn + sepsis group ($n = 32$), and burn + sepsis + LFM-A13 group ($n = 32$). Animals in the

sham group were sacrificed after anesthesia. The mice received full-thickness scald burns on 10% of their total body surface area on their dorsal area by immersion for 15 s in a 100°C water bath. Animals in the burn group were resuscitated immediately with the intraperitoneal administration of normal saline (10 mg/kg). Animals in the burn + sepsis group were injected with LPS (10 mg/kg) intraperitoneally after scalding to establish the sepsis model. Animals in the burn + sepsis + LFM-A13 group received intervention through the intraperitoneal administration of LFM-A13 immediately after scalding before LPS injection. According to our previous study, the dose of LFM-A13 was set at 10 mg/kg (22). Animals in the burn + sepsis and burn + sepsis + LFM-A13 groups were sacrificed at 0, 8, 12, and 24 h (8 animals per time-point) after dosing.

Sample Collection

A portion of intestinal tissue was removed from each mouse and shock frozen in liquid nitrogen for Western blot analysis. Another portion of intestinal tissue was collected for histopathological examination (H&E staining), cellular apoptosis (TUNEL assay), inflammatory cytokine detection (ELISA), and MPO activity determination (MPO colorimetric activity assay).

Measurement of Total BTK and p-BTK Protein Levels *via* Western Blot Analysis

Total proteins were extracted from intestinal tissue samples for Western blot analysis. The concentration of protein was determined by using the Bradford method established by Bradford in 1976. The protein samples were separated through SDS-PAGE, transferred to nitrocellulose membranes, and oscillated in 5% blocking solution at room temperature. The membranes were incubated with primary antibodies on a shaker at 4°C overnight. The membranes were washed three times with TBST, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, and washed three times with TBST for color development. Blots were imaged by using a G:BOX ChemiXR5 imaging system, and the gray values were analyzed by using Gel-pro 32 analysis software.

Histopathological Examination *via* H&E Staining

Intestinal tissues were fixed, dehydrated, and embedded in paraffin and sectioned to a thickness of 5 μm for H&E staining. The sections were observed through optical microscopy (×400) with three fields of vision that were randomly selected from each section; photographed; and scored for histopathological changes. The scoring criteria were as follows: 0 represents neatly arranged and complete glandular architecture, columnar-lined epithelia, and abundant goblet cells, and each one added point represents glandular atrophy, epithelial thinning, and absent or shedding goblet cells. Gland absence is represented by a score of three points.

Measurement of Cellular Apoptosis *via* TUNEL Assay

Cellular apoptosis was detected through TUNEL assay by using a TUNEL apoptosis assay kit. Paraffin-embedded sections were dewaxed, dehydrated, and rehydrated for the TUNEL assay. Slides containing cells were treated with 0.1% Triton X-100 for 15 min at room temperature. Next, each sample was incubated with 10 μl of proteinase K solution and 90 μl of PBS for 5 min and washed with PBS. Each sample was incubated with 50 μl of the TUNEL reaction mixture for 1 h at 37°C and washed with PBS. DAB solution was utilized for color development. The slides were observed by using an optical microscope, and the apoptotic index (AI) was calculated as follows:

$$\mathrm{AI} = \frac{\text{number of apoptotic cells}}{\text{number of total cells}} \times 100\%$$

Determination of Inflammatory Cytokines *via* Standard Sandwich ELISA

Serum and intestinal tissue samples were collected from the mice, and the expression levels of inflammatory cytokines (IL-4, IL-6, and TNF-α) were detected by using specific ELISA kits. A total of 100 μl of the sample or the standard was added to each well of the ELISA plate. The plates were sealed and incubated at 37°C for 1.5 h. After five cycles of washing, the biotin–antibody diluent and biotinylation antibody working solution were added to the ELISA plates. The plates were sealed and incubated at 37°C for 0.5 h. After five cycles of washing, the substrate solution was added for color development, and the plates were incubated at 37°C for 0.25 h under protection from light. Finally, the termination solution was added and mixed well to terminate the reaction, and the OD450 was measured and recorded immediately.

Examination of MPO Activity in Intestinal Tissues *via* MPO Colorimetric Assay

MPO activity in intestinal tissues was detected by using a MPO colorimetric activity assay kit. Mouse intestinal tissues were homogenized, mixed thoroughly with buffer, and placed in a water bath at 60°C for 10 min to detect MPO activity. The MPO activity of intestinal tissues was evaluated by measuring the absorbance of the samples at 460 nm (OD). MPO activity was expressed in milliunits per gram weight of wet tissue with the following calculation formula:

$$\mathrm{MPO(U/g)} = \frac{\text{determination OD value} - \text{control OD value}}{11.3 \times \text{sample volume (g)}}$$

Statistical Analysis

Data was expressed as the mean ± standard deviation ($\bar{x} \pm s$). Normality test and homogeneity test of variance were performed. SPSS25.0 statistical software was used for data analysis. Data were analyzed with independent-sample *t*-test and one-way analysis of variance followed by Bonferroni. $P < 0.05$ was regarded as statistically significant.

RESULTS

Expression Levels of Total BTK and p-BTK in Intestinal Tissue

As shown in **Figure 1**, the expression levels of total BTK protein in the intestinal tissues of mice in each group did not significantly differ ($P > 0.05$). Meanwhile, the Western blot analysis results indicated that the p-BTK level in the sham and burn groups were low, whereas that in the burn + sepsis group increased over time, peaked at 12 h, and then decreased at 24 h ($P < 0.05$). The Western blot analysis results of the burn + sepsis + LFM-A13 and burn + sepsis group exhibited similar trends ($P < 0.05$). However, at 8, 12, and 24 h, the expression levels of p-BTK in the burn +sepsis+ LFM-A13 group were significantly decreased relative to those in the burn + sepsis group ($P < 0.05$), indicating that LFM-A13 had a significant inhibitory effect on the activation and expression of BTK in the intestinal tissues of burn sepsis mice.

Histopathological Analysis and Scores of Intestinal Tissue

As shown in **Figure 2**, the sham and burn groups received histopathological scores of 0 because they exhibited neatly arranged and intact glands, columnar-lined epithelia, and abundant goblet cells. However, the burn + sepsis group demonstrated obvious histopathological changes, including glandular atrophy, epithelial thinning, and goblet cell reduction or absence that gradually aggravated over time. Meanwhile, the histopathological scores of the burn + sepsis group increased gradually over time (1.44 ± 0.51 vs. 2.22 ± 0.39 vs. 2.77 ± 0.36 vs. 2.89 ± 0.19 vs., $P > 0.05$). Compared with those or the burn+sepsis group at the corresponding time points, the degree of intestinal histopathological changes and pathological scores or the burn+sepsis+LFM-A13 group were significantly lower (1.11 ± 0.19 vs. 1.33 ± 0.33 vs. 1.89 ± 0.19 vs. 1.66 ± 0.34, $P < 0.05$) but were not significantly different at 0 h ($P > 0.05$).

Apoptosis and AI of Intestinal Epithelial Cells

Cells with brown or dark brown granules in their nuclei were considered to be apoptotic cells (TUNEL-positive cells). As shown in **Figure 3**, in the sham group, almost no apoptotic cells were observed in the sections of intestinal tissues, and the AI was 1.06 ± 1.16%. In the burn group, a few apoptotic cells were observed, and the corresponding AI was 1.78 ± 1.25%. However, the apoptotic cells in the burn + sepsis group increased sharply and increased over time ($P < 0.05$). The AIs at 0, 8, and 12 h were 6.77 ± 1.21%, 15.47 ± 0.78%, and 22.71 ± 3.13%, respectively. The corresponding AI of apoptotic cells at 24 h was 23.99 ± 2.14%, and no significant difference was found between the AI at 12 h and that at 24 h. Similarly, compared with that of the burn + sepsis group, the AI of intestinal epithelial cells in the burn + sepsis + LFM-A13 group were significantly lower at the corresponding time points ($P < 0.05$) but did not significantly differ at 0 h ($P > 0.05$).

Levels of IL-4, IL-6, and TNF-α in Serum and Intestinal Tissue

The levels of three kinds of inflammatory cytokines, namely, IL-4, IL-6, and TNF-α, in serum were determined through ELISA. The expression levels of the inflammatory cytokines in each group showed similar trends. As shown in **Figures 4A–C**, the sham group had the lowest levels of inflammatory cytokines, i.e., IL-4 levels of 25.29 ± 3.07 pg/ml, IL-6 levels of 81.06 ± 8.60 pg/ml, and TNF-α levels of 28.37 ± 1.99 pg/ml. In the burn group, the levels of inflammatory cytokines increased, and the level of IL-4 was 32.27 ± 3.48 pg/ml, that of IL-6 was 125.76 ± 14.29 pg/ml, and that of TNF-α was 37.03 ± 1.40 pg/ml. The levels of the three kinds of inflammatory cytokines in the serum of the burn + sepsis group significantly increased and increased over time ($P < 0.05$). Specifically, IL-4 levels increased from 41.24 ± 4.03 pg/ml (0 h) to 88.19 ± 3.04 pg/ml (24 h); IL-6 levels increased from 159.06 ± 12.84 pg/ml (0 h) to 374.98 ± 19.48 pg/ml (24 h); and TNF-α levels increased from 53.66 ± 16.43 pg/ml (0 h) to 315.49 ± 27.10 pg/ml (24 h). By contrast, the levels of the three inflammatory cytokines, except for those at 0 h ($P > 0.05$), in the burn+sepsis+LFM-A13 group were significantly lower than those in the burn + sepsis group ($P < 0.05$). As shown in **Figures 4D–F**, the variation trends of the expression levels in each group were consistent with those of the expression levels in serum. Specifically, the expression levels of the inflammatory cytokines in the sham group were the lowest and increased in the burn group. In the burn + sepsis group, the levels of the three inflammatory cytokines in intestinal tissues were also significantly increased and increased over time ($P < 0.05$), whereas those in the burn + sepsis + LFM-A13 group were significantly lower than those in the burn + sepsis group ($P < 0.05$).

Activity of MPO in Intestinal Tissue

MPO is a functional and activation marker of neutrophils (PMNs), and PMN infiltration in intestinal tissues can be evaluated by assessing MPO activity in intestinal tissues. As shown in **Figure 5**, the activity of MPO in the burn group was slightly increased compared with that in the sham group, and that in the burn+sepsis group was significantly higher than that in the other two groups and gradually increased over time. It was 1.49 ± 0.05, 1.96 ± 0.04, and 2.27 ± 0.07 U/g at 0, 8, and 12 h, respectively ($P < 0.05$). No significant difference was found between the activity of MPO at 24 h and that at 12 h ($P > 0.05$). Compared with that in the burn+sepsis group, the activity of MPO in the burn + sepsis + LFM-A13 group was significantly lower ($P < 0.05$) but did not significantly differ at 0 h ($P > 0.05$).

DISCUSSION

Sepsis is a systemic disease that is caused by the dysregulated immune response to infection. It can lead to multisystem organ failure. It involves complex and dynamic processes, including immunity and inflammation, and other aspects with physiological, pathological, and biochemical abnormalities. Burn sepsis, as a type of sepsis, is likely to cause local or systemic

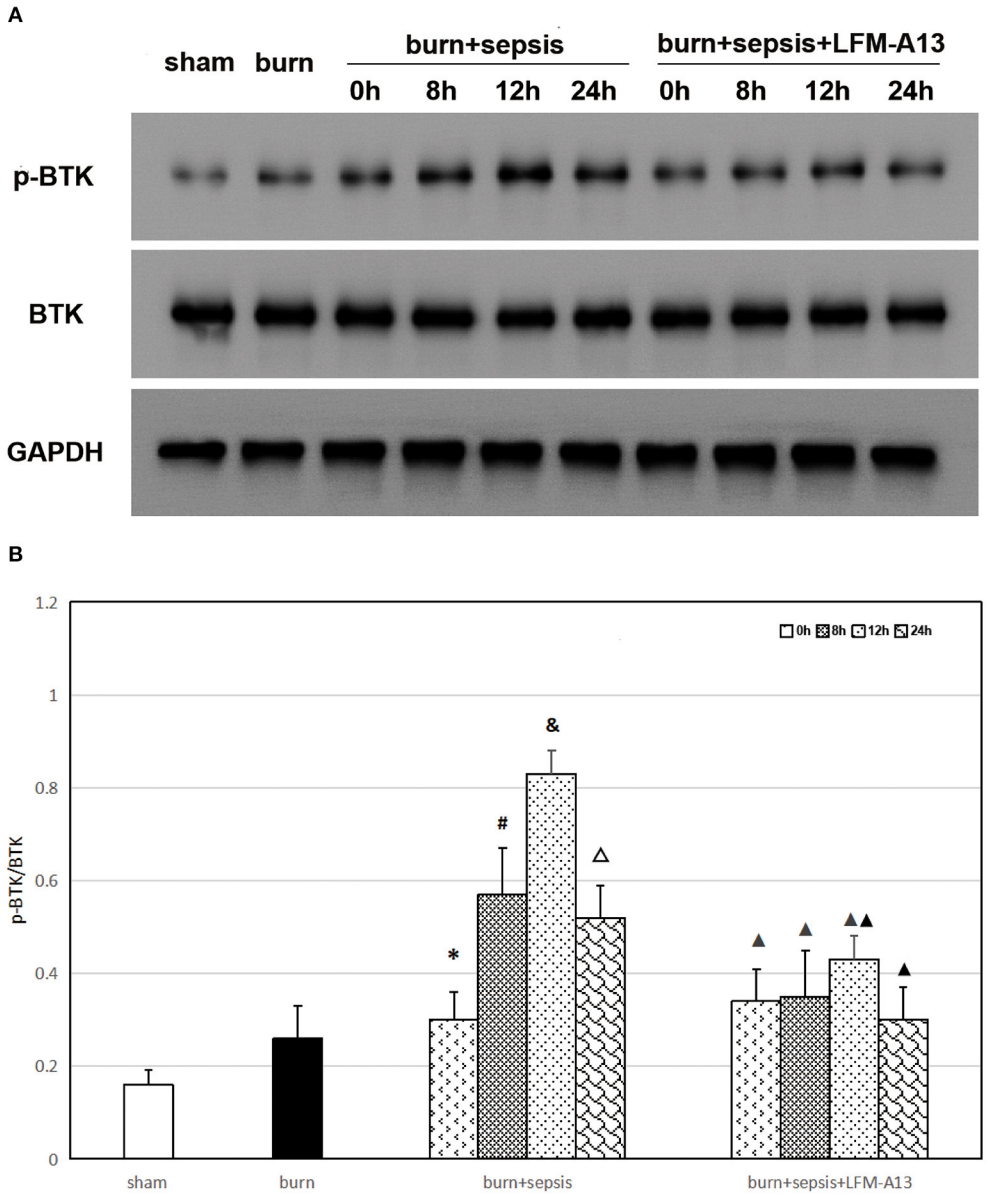

FIGURE 1 | Expression levels of total BTK and p-BTK in intestinal tissue. **(A)** A representative western blot image of total BTK protein and p-BTK in each group. **(B)** The expression level of p-BTK (p-BTK /BTK) in each group at different time points of postburn. $^{*}P < 0.05$, vs. the sham group and burn group; in the burn + sepsis group, $^{\#}P < 0.05$, vs. 0h; $^{\&}P < 0.05$, vs. 8h; $^{\triangle}P < 0.05$, vs. 12h; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, vs. the burn + sepsis group at corresponding time points except 0 h of postburn.

inflammatory response syndrome and MODS. In this study, we explored the activation of BTK in the intestinal tissue of burn sepsis mice. Then, we used LFM-A13 to intervene in mice to explore the roles and molecular mechanisms of BTK in burn-sepsis-induced intestinal injury.

Previous studies have shown that TKs are involved in a variety of inflammatory responses in the body and have an important role in autoimmune diseases and tumors (23). As a member of TKs, BTK is involved in the growth and function of B cells and is a key kinase in the B-cell antigen receptor signaling pathway. In this experiment, Western blot analysis was performed to detect the activation of the BTK protein quantitatively. The expression level of total BTK protein in the intestinal tissue of mice in each group clearly had no significant change. The p-BTK protein was expressed at low levels in intestinal tissues of mice in the sham and burn groups. However, the corresponding results of the burn + sepsis group showed that the expression level of p-BTK increased over time, peaked at 12 h, then decreased at 24 h. Similarly, the histopathological results also showed no obvious pathological changes in the sham and burn groups, whereas

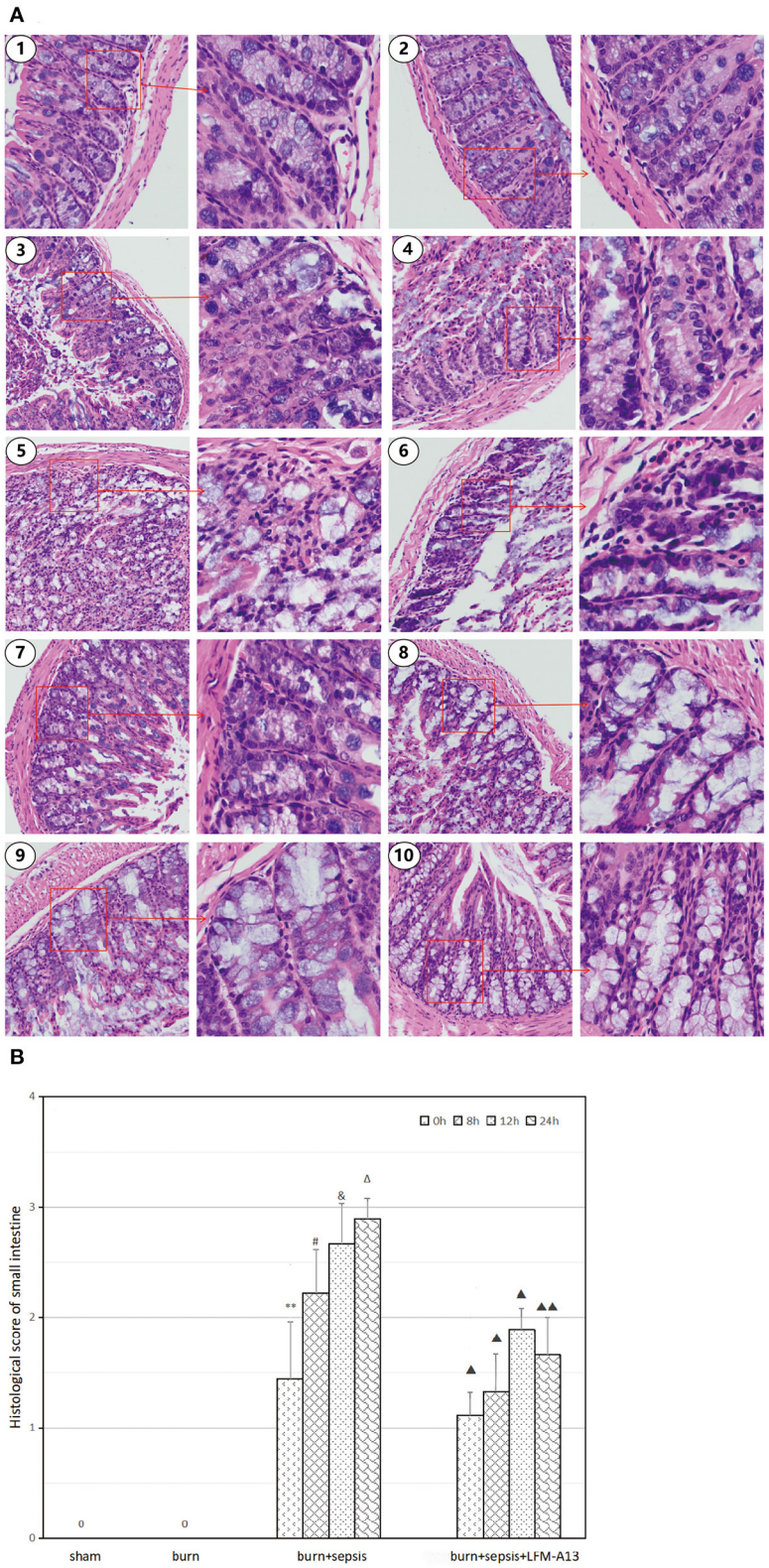

FIGURE 2 | Histopathological analysis and scores of intestinal tissue. **(A)** Histopathological changes in each group. (1) The sham group (×100 and × 400), (2) the burn group (×100 and × 400), (3) the burn+sepsis group at 0 h (×100 and × 400), (4) the burn + sepsis group at 8 h (×100 and × 400), (5) the burn + sepsis group

(Continued)

FIGURE 2 | at 12 h (×100 and × 400), (6) the burn + sepsis group at 24 h (×100 and ×400), (7) the burn+sepsis+LFM-A13 group at 0 h (×100 and ×400), (8) the burn+sepsis+LFM-A13 group at 8 h (×100 and × 400), (9) the burn+sepsis+LFM-A13 group at 12 h (×100 and × 400), (10) the burn + sepsis + LFM-A13 group at 24 h (×100 and × 400). **(B)** Histopathology scores in each group at different time points of post-burn. $^{**}P < 0.01$, vs. the sham group and burn group; in the burn+sepsis group, $^{\#}P > 0.05$, vs. 0 h; $^{\&}P > 0.05$, vs. 8h; $^{\Delta}P > 0.05$, vs. 12h; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, vs. the burn + sepsis group at corresponding time points except 0 h of post-burn.

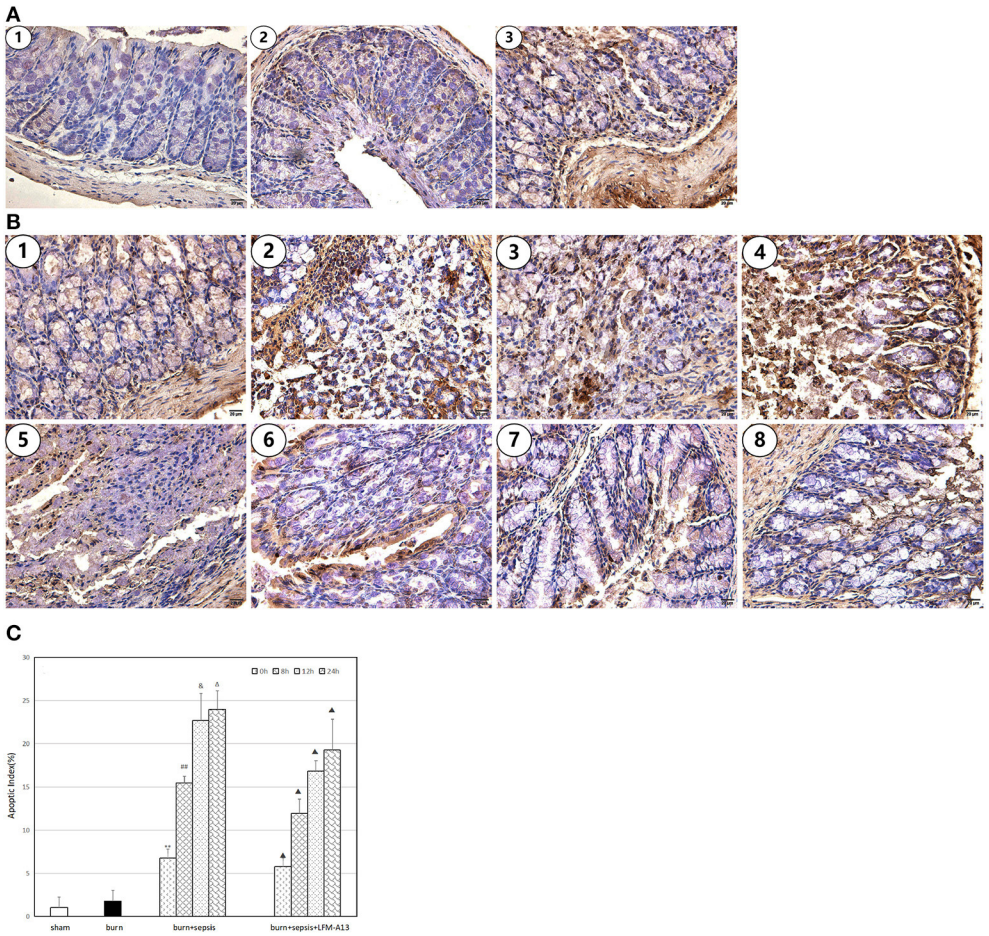

FIGURE 3 | Apoptosis and AI of intestinal epithelial cells. **(A)** Cell apoptosis at 0 h postburn in each group. (1) The sham group, (2) the burn group, (3) the burn + sepsis group. **(B)** Cell apoptosis at 4 different time points of postburn in the burn + sepsis group and burn + sepsis + LFM-A13 group. (1–4): The burn + sepsis group at 0; 8; 12; 24 h, (5–8): the burn + sepsis + LFM-A13 group at 0 h; 8; 12; 24 h. **(C)** AI in each group at different time points of postburn. $^{**}P < 0.01$, vs. the sham group and burn group; in the burn + sepsis group, $^{\#\#}P < 0.01$, vs. 0 h; $^{\&}P < 0.05$, vs. 8 h; $^{\Delta}P > 0.05$, *vs* 12 h; $^{\blacktriangle}P < 0.05$ vs. the burn + sepsis group at corresponding time points except 0 h of post-burn.

the degree of histopathological damage in the burn + sepsis group gradually intensified over time, peaked at 12 h, and then decreased at 24 h. Therefore, these results demonstrated that BTK activation was closely associated with the degree of intestinal injury and played a destructive role in intestinal injury.

TK inhibitors can be used to inhibit the abnormal pathways of cell signal transduction and participate in cell growth and proliferation. LFM-A13 is an inhibitor with relatively good targeting capability; it can inhibit some members of the Tec family, such as BTK, by inhibiting the activation of BTK in cells and thus inhibits excessive inflammatory responses in the body (24). In this study, one of the groups of mice received LFM-A13 intervention immediately after burn sepsis modeling, and their BTK expression levels were observed at 0, 8, 12, and 24 h after injury. The results showed that the expression levels of the total BTK protein in the intestinal tissue of mice in each group did not significantly differ. Compared with those in the burn+sepsis group at the corresponding time points (8, 12, and 24 h), the expression levels of the p-BTK protein in the burn + sepsis + LFM-A13 group had significantly decreased. H&E staining also showed that at corresponding time points (8, 12, and 24 h), the degree of histopathological changes in the burn + sepsis + LFM-A13 group was significantly reduced compared with that in the burn + sepsis group, and the pathological scores were significantly reduced. These results further demonstrated that in the burn sepsis-induced intestinal injury of mice, the

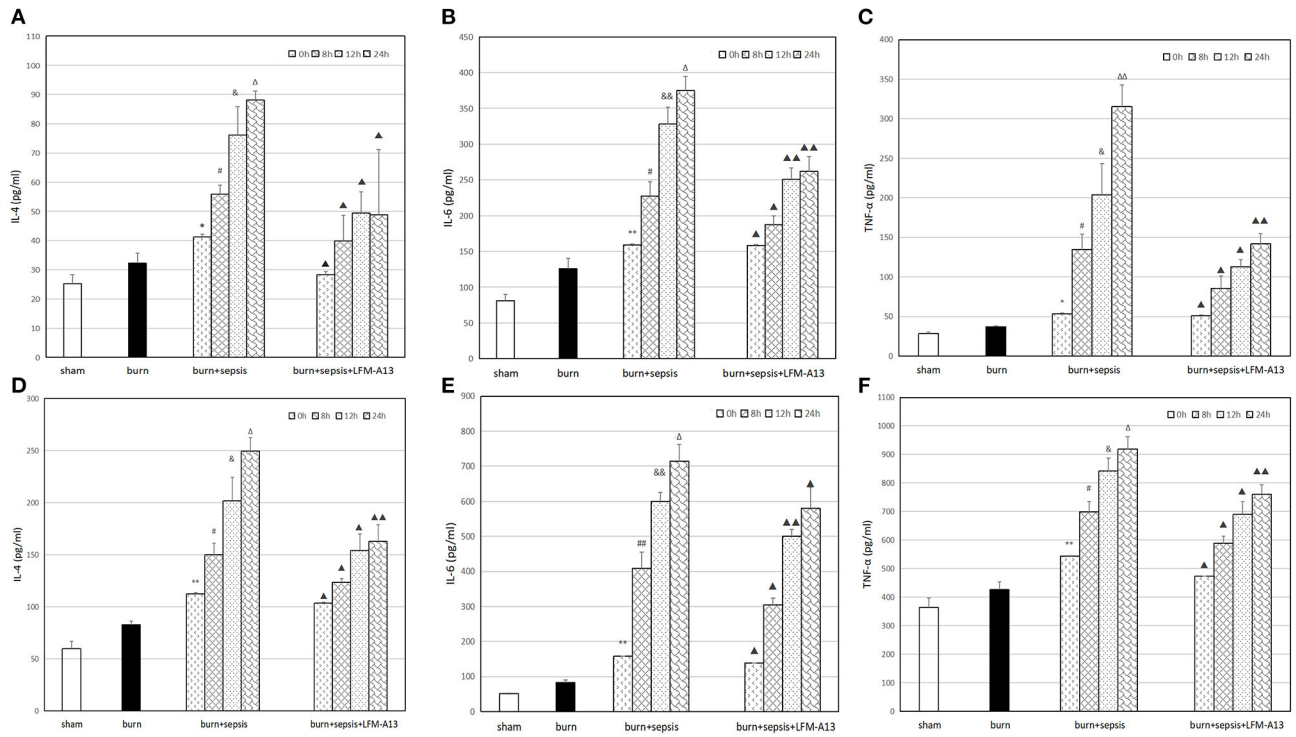

FIGURE 4 | Levels of IL-4, IL-6, and TNF-α in serum and intestinal tissue. **(A–C)**: Levels of IL-4 **(A)**, IL-6 **(B)**, and TNF-α **(C)** levels in serum at different time points in each group. $^{**}P < 0.01$ **(A,B)**, $^{*}P < 0.05$ **(C)**, vs. the sham group and burn group; in the burn+sepsis group, $P < 0.05$, $^{\#}P < 0.05$ **(A)**,$^{\#}P < 0.05$ **(B,C)** vs. 0 h, $^{\&}P < 0.05$ **(A)**, $^{\&\&}P < 0.01$ **(B)**, $^{\&}P > 0.05$ **(C)** vs. 8 h, $^{\triangle}P > 0.05$ **(A,B)**, $^{\triangle\triangle}P < 0.01$ **(C)** vs. 12 h; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ vs. the burn+sepsis group at corresponding time points except 0 h postburn. **(D–F)**: Levels of IL-4 **(D)**, IL-6 **(E)** and TNF-α **(F)** levels in intestinal tissue at different time points in each group. $^{**}P < 0.01$, vs. the sham group and burn group; in the burn+sepsis group, $^{\#}P > 0.05$ **(D)**, $^{\#\#}P < 0.01$ **(E)**, $^{\#}P < 0.05$ **(F)** vs. 0 h, $^{\&}P < 0.05$ **(D,F)**, $^{\&\&}P < 0.01$ **(E)**, vs. 8 h, $^{\triangle}P < 0.05$ **(D,E)**, $^{\triangle}P > 0.05$ **(F)**, vs. 12 h; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, vs. the burn + sepsis group at corresponding time points except 0 h post-burn.

activation of BTK could be inhibited significantly by LFM-A13. Our previous study also showed that LFM-A13 inhibited BTK activation and inhibited the burn sepsis-induced pyroptosis of intestinal cells (22).

In the present study, the apoptosis of intestinal epithelial cells and AIs were detected by using the TUNEL assay. As can be clearly seen, apoptotic cells were rare in the sections of intestinal tissues from the sham and burn groups. However, the number of apoptotic cells increased sharply in the burn + sepsis group, and the AIs increased accordingly. These results indicated that in burn sepsis mice, the activation of BTK was closely associated with the apoptosis of intestinal epithelial cells. The TUNEL assay was also used to detect the apoptotic cells in the intestinal tissues of mice in the burn + sepsis + LFM-A13 group to illustrate this point. At the corresponding time points, the number of apoptotic cells significantly decreased and the AI decreased accordingly in the burn + sepsis + LFM-A13 group compared with those in the burn + sepsis group. These results also indicated that LFM-A13 could significantly reduce the apoptosis of intestinal epithelial cells in burn sepsis mice by inhibiting the activation and expression of BTK during intestinal injury and thus played a protective role against intestinal injury in burn sepsis mice.

Previous studies have shown that cell apoptosis is mainly induced by the activation of Caspase-3, which acts as the final downstream protein required for cell apoptosis (25). Bcl-2 and Bax, the main antiapoptotic proteins of the Bcl family, could execute apoptotic programs by regulating Caspase-3 during apoptosis (26, 27). This study found that LFM-A13 could significantly reduce the apoptosis of intestinal epithelial cells in burn sepsis mice. Thus, whether LFM-A13 could reduce the apoptosis of cells by inhibiting the activation of Caspase-3, by activating Bcl-2 and Bax, or by doing both remains unclear. The mechanisms of LFM-A13 in inhibiting apoptosis in intestinal tissues warrant further studies.

Previous studies have shown that the main role of the gastrointestinal tract in the body is digestion and absorption and is often regarded as a passive organ during the occurrence and development of sepsis and resistance to invasion by pathogenic microorganisms (28). However, with the deepening of research in recent years, the intestinal metabolism of the body has been found to be abnormal in the early stage of sepsis and that the intestine is vulnerable to damage during sepsis, causing multiple organ dysfunction (29). In a normal organism, the intestinal mucosal barrier could keep endotoxins and bacteria in the gut away from the blood and allow the body to function normally (30). Severe burns cause a wide range of immune responses and extensive cell damage, which is considered a precursor to organ dysfunction. Then, excessive inflammatory reaction would

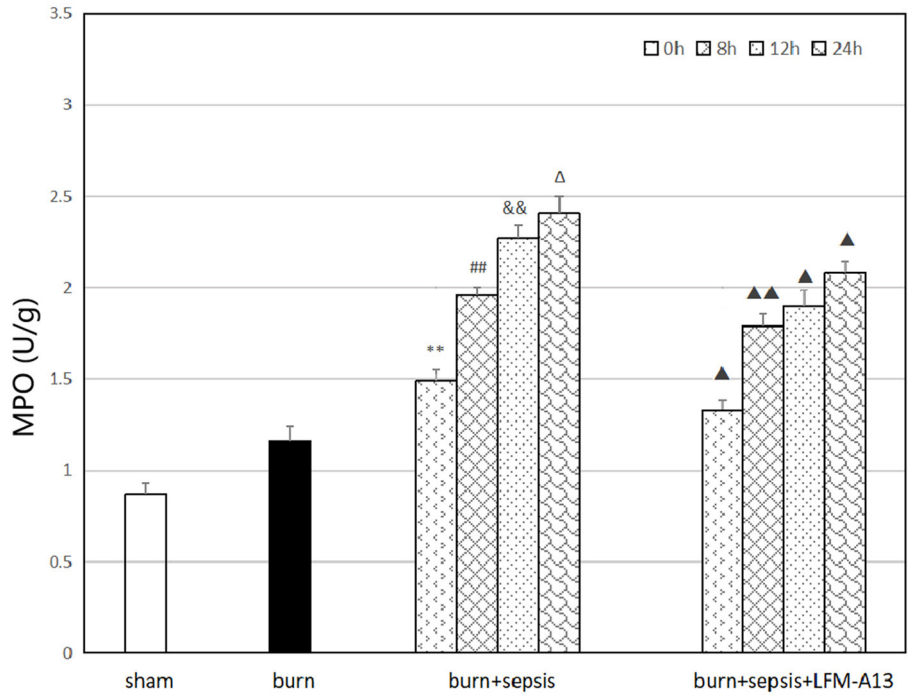

FIGURE 5 | Activity of MPO in intestinal tissue at different time points in each group. $^{**}P < 0.01$, vs. the sham group and burn group; in the burn + sepsis group, $^{\#\#}P < 0.01$, vs. 0 h, $^{\&\&}P < 0.01$, vs. 8 h, $^{\Delta}P > 0.05$, vs. 12 h; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, vs. the burn + sepsis group at corresponding time points.

destroy endothelial and intestinal epithelial cells and lead to their apoptosis, resulting in increased intestinal permeability and aggravating intestinal mucosal barrier dysfunction; these effects are followed by endotoxin and bacterial migration and further intestinal flora imbalance (31). As a result, sepsis in patients progressively develops and deteriorates. Among the many known inflammatory mediators, three inflammatory factors (IL-4, IL-6, and TNF-α) play an important role in local or systemic inflammatory response (32). They could release secondary inflammatory factors by stimulating immunoreactive cells, such as monocytes and macrophages, to cause intestinal tissue inflammation. They could also promote the release of proteases and oxygen free radicals from PMNs, causing inflammatory injury (33).

ELISA results showed that the levels of inflammatory factors in the burn sepsis group were significantly higher than those in the other two groups and that IL-4, IL-6, and TNF-α continuously increased over time. Therefore, these results showed that the activation of BTK was closely related to the release of inflammatory factors in the serum and intestinal tissue of burn sepsis mice. Compared with those in the burn+sepsis group at the corresponding time points (8, 12, and 24 h), the levels of inflammatory cytokines in serum and intestinal tissue were significantly decreased in the group treated with LFM-A13. These results indicated that in burn sepsis mice, LFM-A13 could significantly inhibit the expression of inflammatory cytokines by inhibiting the activation and expression of BTK during intestinal injury, thereby reducing the excessive inflammatory response and exerting a protective effect.

MPO, a peroxidase present in myeloid cells, is a specific marker of myeloid cells and a marker of the function and activation of PMNs; changes in its expression level and activity represent the function and activity of PMNs (34). The detection of MPO activity in the intestinal tissues of mice showed that the activity of MPO in the burn+sepsis group was significantly higher than that in the sham and burn groups and gradually increased over time. Therefore, the results suggested that in the intestinal tissues of burn sepsis mice, the activation of BTK was closely associated with the activity of MPO. MPO catalyzes the conversion of H_2O_2 and Cl^- to form OCl^- and free radicals with oxidative capacity; the MPO–H_2O_2-Cl^- system is then formed to regulate the immune response of the body. Meanwhile, MPO can activate signaling pathways, such as the NF-κB pathway, to promote the development of inflammatory response. At the corresponding time points (8, 12, and 24 h), the activity of MPO in the burn + sepsis + LFM-A13 group was significantly lower than that in the burn + sepsis group. These results indicated that in burn sepsis mice, the BTK-specific inhibitor LFM-A13 could significantly inhibit the expression of MPO in intestinal tissues by inhibiting the activation and expression of BTK during intestinal injury, thus mitigating the oxidative stress response.

CONCLUSION

BTK signaling pathway mediated intestinal cell apoptosis; regulated the production of the anti-inflammatory cytokine IL-4 and the proinflammatory cytokines IL-6 and TNF-α; and participated in intestinal oxidative stress. Thus, it played an

important regulatory role in the intestinal injury induced by burn sepsis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Anhui Medical University Ethics Committee of Animal Experiments.

AUTHOR CONTRIBUTIONS

X-LC developed the idea, designed the study, and provided financial support for the study. JW performed the experiment, drafted the manuscript, summarized the data, and contributed to data interpretation. XY, J-QN, LQ, and FW were involved in the acquisition of the data. The corresponding author had full access to all the data in the study and was responsible for submission for publication. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Sasor SE, Chung KC. Upper extremity burns in the developing world: a neglected epidemic. *Hand Clin*. (2019) 35:457–66. doi: 10.1016/j.hcl.2019.07.010
2. Greenhalgh DG. Sepsis in the burn patient: a different problem than sepsis in the general population. *Burns Trauma*. (2017) 5:23. doi: 10.1186/s41038-017-0089-5
3. Fernando SM, Rochwerg B, Seely AJE. Clinical implications of the third international consensus definitions for sepsis and septic shock (Sepsis-3). *CMAJ*. (2018) 190:E1058–9. doi: 10.1503/cmaj.170149
4. Rhee C, Dantes R, Epstein L, Murphy DJ, Seymour CW, Iwashyna TJ, et al. Incidence and trends of sepsis in US hospitals using clinical vs. claims data, (2009–2014). *JAMA*. 2017 318:1241–9. doi: 10.1001/jama.2017.13836
5. Kadri SS, Rhee C, Strich JR, Morales MK, Hohmann S, Menchaca J, et al. Estimating 10-year trends in septic shock incidence and mortality in united states academic medical centers using clinical data. *Chest*. (2017) 151:278–85. doi: 10.1016/j.chest.2016.07.010
6. Yao RQ, Ren C, Wang JN, Wu GS, Zhu XM, Xia ZF, et al. Publication trends of research on sepsis and host immune response during 1999–2019: a 20-year bibliometric analysis. *Int J Biol Sci*. (2020) 16:27–37. doi: 10.7150/ijbs.37496
7. Dvorak JE, Ladhani HA, Claridge JA. Review of sepsis in burn patients in 2020. *Surg Infect (Larchmt)*. (2021) 22:37–43. doi: 10.1089/sur.2020.367
8. Knackstedt R, Gatherwright J. The role of thermal injury on intestinal bacterial translocation and the mitigating role of probiotics: a review of animal and human studies. *Burns*. (2020) 46:1005–12. doi: 10.1016/j.burns.2019.07.007
9. Yang XJ, Liu D, Ren HY, Zhang XY, Zhang J, Yang XJ. Effects of sepsis and its treatment measures on intestinal flora structure in critical care patients. *World J Gastroenterol*. (2021) 27:2376–93. doi: 10.3748/wjg.v27.i19.2376
10. Wang ZE, Peng J, Wu D, Zheng JJ, Peng X. Effects of intestinal trefoil factor on intestinal mucus barrier in burned mice. *Am J Transl Res*. (2020) 12:7187–98.
11. Brinkhoff A, Zeng Y, Sieberichs A, Dolff S, Shilei X, Sun M, et al. B-cell dynamics during experimental endotoxemia in humans. *Biosci Rep*. (2019) 39. doi: 10.1042/BSR20182347
12. Tian X, Li L, Fu G, Wang J, He Q, Zhang C, et al. miR-133a-3p regulates the proliferation and apoptosis of intestinal epithelial cells by modulating the expression of TAGLN2. *Exp Ther Med*. (2021) 22:824. doi: 10.3892/etm.2021.10256
13. McBride MA, Owen AM, Stothers CL, Hernandez A, Luan L, Burelbach KR, et al. The metabolic basis of immune dysfunction following sepsis and trauma. *Front Immunol*. (2020) 11:1043. doi: 10.3389/fimmu.2020.01043
14. Abe R, Oda S, Sadahiro T, Nakamura M, Hirayama Y, Tateishi Y, et al. Gram-negative bacteremia induces greater magnitude of inflammatory response than Gram-positive bacteremia. *Crit Care*. (2010) 14:R27. doi: 10.1186/cc8898
15. Alexandraki I, Palacio C. Gram-negative vs. Gram-positive bacteremia: what is more alarmin(g)? *Crit Care*. (2010) 14:161. doi: 10.1186/cc9013
16. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine*. (2008) 42:145–51. doi: 10.1016/j.cyto.2008.01.006
17. Molteni M, Gemma S, Rossetti C. The role of toll-like receptor 4 in infectious and non-infectious inflammation. *Mediators Inflamm*. (2016) 2016:6978936. doi: 10.1155/2016/6978936
18. Rip J, Van Der Ploeg EK, Hendriks RW, Corneth OBJ. The role of Bruton's tyrosine kinase in immune cell signaling and systemic autoimmunity. *Crit Rev Immunol*. (2018) 38:17–62. doi: 10.1615/CritRevImmunol.2018025184
19. Lee E, Cho H, Lee DK, Ha J, Choi BJ, Jeong JH, et al. Discovery of 5-Phenoxy-2-aminopyridine derivatives as potent and selective irreversible inhibitors of bruton's tyrosine kinase. *Int J Mol Sci*. (2020) 21:8006. doi: 10.3390/ijms21218006
20. Jefferies CA, Doyle S, Brunner C, Dunne A, Brint E, Wietek C, et al. (2003). Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor kappaB activation by Toll-like receptor 4. *J Biol Chem*. (2003) 278, 26258–64. doi: 10.1074/jbc.M301484200
21. Mahajan S, Ghosh S, Sudbeck EA, Zheng Y, Downs S, Hupke M, et al. Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2, 5-dibromophenyl)propenamide]. *J Biol Chem*. (1999) 274:9587–99. doi: 10.1074/jbc.274.14.9587
22. Jin X, Wan J, Duan SF, Gong YZ, Wang F, Chen XL. Role of Bruton's tyrosine kinase in endotoxin/lipopolysaccharide-induced pyroptosis of intestinal cells in scalded mice. *Zhonghua Shao Shang Za Zhi*. (2021) 37:546–54. doi: 10.3760/cma.j.cn501120-20210119-00027
23. Szilveszter KP, Nemeth T, Mocsai A. Tyrosine kinases in autoimmune and inflammatory skin diseases. *Front Immunol*. (2019) 10:1862. doi: 10.3389/fimmu.2019.01862
24. Abdel-Magid AF. Inhibitors of Bruton's tyrosine kinase (Btk) May treat inflammation, immunological disorders, and cancer. *ACS Med Chem Lett*. (2013) 4:690–1. doi: 10.1021/ml400220w
25. Su T, Huang L, Zhang N, Peng S, Li X, Wei G, et al. FGF14 Functions as a tumor suppressor through inhibiting PI3K/AKT/mTOR pathway in colorectal cancer. *J Cancer*. (2020) 11:819–25. doi: 10.7150/jca.36316
26. Zhang H, Liu M, Zhang Y, Li XJFip. Trimetazidine attenuates exhaustive exercise-induced myocardial injury in rats *via* regulation of the Nrf2/NF-κB signaling pathway. Front Pharmacol. (2019) 10:175. doi: 10.3389/fphar.2019.00175
27. Du Z, Chen S, Cui G, Yang Y, Zhang E, Wang Q, et al. Silica nanoparticles induce cardiomyocyte apoptosis *via* the mitochondrial pathway in rats following intratracheal instillation. *Int J Mol Med*. (2019) 43:1229–40. doi: 10.3892/ijmm.2018.4045
28. Nguyen T, Le H, Kim T, The H, Nguyen T, Cornet V, et al. Clerodendrum cyrtophyllumanti-inflammatory and antioxidant properties of the

ethanol extract of turcz in copper sulfate-induced inflammation in zebrafish. *Antioxidants*. (2020) 9:192. doi: 10.3390/antiox9030192
29. Lin P, Chen C, Wallace C, Chen K, Chang C, Chen H, et al. Therapeutic effect of rosuvastatin and propylthiouracil on ameliorating high-cholesterol diet-induced fatty liver disease, fibrosis and inflammation in rabbit. *Am J Transl Res*. (2017) 9:3827–41.
30. Tian P, Lu X, Jin N, Shi JJ. Knockdown of ghrelin-O-acyltransferase attenuates colitis through the modulation of inflammatory factors and tight junction proteins in the intestinal epithelium. *Cell Biol Int*. (2020) 44:1681–90. doi: 10.1002/cbin.11362
31. Quan R, Chen C, Yan W, Zhang Y, Zhao X, Fu YJ. BAFF Blockade attenuates inflammatory responses and intestinal barrier dysfunction in a murine endotoxemia model. *Int J Biol Macromol*. (2020) 11:570920. doi: 10.3389/fimmu.2020.570920
32. Xu L, Sang R, Yu Y, Li J, Ge B, Zhang XJIjobm. The polysaccharide from inonotus obliquus protects mice from Toxoplasma gondii-induced liver injury. *Int J Biol Macromol*. 2019;125:1–8. doi: 10.1016/j.ijbiomac.2018.11.114
33. Dispenza M, Regan J, Bochner BJ. Eroci potential applications of Bruton's tyrosine kinase inhibitors for the prevention of allergic reactions. (2017) 13:921–23. doi: 10.1080/1744666x.2017.1370374
34. Wang J, Chen X, Zhang L, Pan Y, Gu Z, Yuan YJ. Anti-inflammatory effects of Eucommia ulmoides Oliv. male flower extract on lipopolysaccharide-induced inflammation. *Chin Med J*. (2019) 132:319–28. doi: 10.1097/cm9.0000000000000066

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Immune dysfunction following severe trauma: A systems failure from the central nervous system to mitochondria

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When a traumatic injury exceeds the body's internal tolerances, the innate immune and inflammatory systems are rapidly activated, and if not contained early, increase morbidity and mortality. Early deaths after hospital admission are mostly from central nervous system (CNS) trauma, hemorrhage and circulatory collapse (30%), and later deaths from hyperinflammation, immunosuppression, infection, sepsis, acute respiratory distress, and multiple organ failure (20%). The molecular drivers of secondary injury include damage associated molecular patterns (DAMPs), pathogen associated molecular patterns (PAMPs) and other immune-modifying agents that activate the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic stress response. Despite a number of drugs targeting specific anti-inflammatory and immune pathways showing promise in animal models, the majority have failed to translate. Reasons for failure include difficulty to replicate the heterogeneity of humans, poorly designed trials, inappropriate use of specific pathogen-free (SPF) animals, ignoring sex-specific differences, and the flawed practice of single-nodal targeting. Systems interconnectedness is a major overlooked factor. We argue that if the CNS is protected early after major trauma and control of cardiovascular function is maintained, the endothelial-glycocalyx will be protected, sufficient oxygen will be delivered, mitochondrial energetics will be maintained, inflammation will be resolved and immune dysfunction will be minimized. The current challenge is to develop new systems-based drugs that target the CNS coupling of whole-body function.

KEYWORDS

trauma, hemorrhage, immune, inflammation, mitochondria, system, ALM, cytokines

Introduction

Globally, over one billion people sustain traumatic injuries, and over six million die annually (1). Mortality is twofold higher in low- and middle-income countries compared to high-income countries, and up to 5-times higher in resource-limited rural and remote regions (1, 2). In patients who survive the first few hours of hospital admission, complications can occur at different times (**Figure 1**) (3). The first window is 3–6 to 24 h where CNS dysfunction (∼50% of early deaths) and circulatory collapse (usually manifesting as shock) lead to early deaths (40% of early deaths) (**Figure 1**) (4–6). The second window occurs over the next few weeks and typically involves infectious complications with a prolonged indolent form of multiple organ failure, immunosuppression and sepsis, referred to as Persistent Inflammation, Immunosuppression and Catabolism Syndrome (PIICS) (∼20% of deaths) (**Figure 1**) (4, 7, 8). Sepsis develops in ∼10% of these patients and multiple organ dysfunction syndrome (MODS) in around 70% (3, 9, 10). Despite decades of research, little progress has been made in the development of effective drugs to treat the polytrauma patient (2, 11, 12). The lack of progress in drug development may reflect the way we think about the problem (13, 14). In this review, our aim is to discuss the inflammatory and immune mechanisms that are believed to be responsible for early and late secondary injuries and death following traumatic injury, and possible ways to reduce morbidity and mortality from a systems-based perspective. Before doing so, we will briefly discuss the physiological importance of the system.

Challenging the steady-state and evolutionary internal tolerances

After a traumatic injury, defined as one or more sudden injuries requiring immediate medical attention, the body activates a series of defense mechanisms to restore homeostatic balance. The concept of homeostatic balance was introduced into medicine in 1916 by Cannon (15). Cannon's genius was to combine the ideas of Pfluger's "*natural adjustments*" (1877), Bernard's concept of "*milieu intérieur*" (1878), and Richet's "*living beings were stable but modifiable*" (1900) into a unified scheme (16). Cannon proposed that every living organism was in a *dynamic state of constancy*, with its constituent parts and processes being *actively* maintained in balance despite external fluctuations (15). The system is not an equilibrium system as it requires a continual flow of matter, energy and exchange with the environment (16, 17). In the mid-1930s, Cannon's concept was refined to include negative and positive feedback circuits (18), and the system's steady-state was now viewed as the net sum of negative and positive feedback mechanisms that operate within a range of tolerances, which differ from person to person, and from species to species. The system has evolved such that small injury perturbations are self-limiting and quickly resolved. However, when the trauma overwhelms the system, it triggers a CNS stress response that typically involves excessive sympathetic and neuroendocrine outflows from the brain's central control, hyperinflammation, immune dysregulation, coagulopathy, endothelial activation and metabolic dysfunction (13, 19, 20). *If homeostatic balance is not restored early, secondary injury processes will amplify and may become life-threatening* (14).

Early and Late Deaths after Major Traumatic Injury

Early deaths: 3-6 to 24 h	Late deaths: 1 to 7-14 days
(~30% of deaths after admission)	**(~20% of deaths after admission)**
• Of 30% early deaths, ~50% are from complex CNS, ~40% from cardiovascular, shock, and ~10% other. • Unresponsive to fluids and support. • Lactate and creatine kinase rises. • CNS swelling in non-TBI patients. • Profound cardiac and vascular failure.	• Prolonged indolent form of multiple organ failure different from the past. • Immunosuppression. • Multiple episodes of sepsis. • Excessive or dysfunctional immune response to trauma.

FIGURE 1
After hospital admission, complications occur at different times and depend upon type and severity of injury. These have been characterized as early and late Deaths. Modified after Rauf et al. (3) and Brohi et al. (4). Patients who died at the point-of-injury or in the first few hours upon arrival to hospital have not been included. CNS, central nervous system; TBI, traumatic brain injury.

First line-of-defense: The innate immune system

> When I first put forward the biological theory of inflammation 8 years ago, I expressed the idea that this reaction is affected by the intermediation of a physiological continuity between "the cells of the connective tissue, those of the endothelial wall and the leucocytes, which form a complete chain and play the principal part in the inflammation of vertebrates." The connective tissue cells which are first attacked, would, I thought, transmit the action to the vascular wall, the cells of which would contract to facilitate the passage of the white corpuscles.
>
> Metchnikoff (21) p. 191.

Any trauma to the body inflicts a barrier breach in three-dimensional space and one in time. Damage signals from cellular, vascular and nerve injury are sent around the body, and to the CNS *via* nerve afferents and resident damage control mechanisms to begin the process of tissue repair and remodeling (14, 22). Recovery begins by rapidly closing the breach, activating immuno-inflammatory processes, removing damaged cells and killing any invading microbes (**Figure 2**).

Immune defense occurs in two parts: First, there is a local frontline defense from patrolling resident immune cells in tissues, and second, from deployment of additional leukocyte subsets from the circulation. Early defense includes activation of tissue resident macrophages, dendritic cells (DCs), neutrophils (PMNs), mast cells, a subset of memory B cells, natural killer (NK) cells, complement (22–24) and recently characterized resident T cells, referred to as innate lymphoid cells (ILCs), which are believed to interact with other resident cells, and trigger the early adaptive immune response and recruitment of cells from the circulation to repair and restore tissue function (25–29) (**Figure 2**).

This diverse group of resident innate cells have evolved different pattern-recognition receptors that detect and respond to changes in the local environment, including damage associated molecular patterns (DAMPS), pathogen associated molecular patterns (PAMPs) and other immune-modifying triggers (**Figure 2**) (30–32). DAMPs are released from damaged, stressed or dying cells, including extracellular and cell membrane, cytosolic, cytoskeleton, nuclear mitochondrial, endothelial and blood components (30, 33), while PAMPs are signature proteins, lipoproteins, nucleic acids and saccharides located on the cell surface or released from invading pathogens. Together, they activate the body's early immune and inflammatory systems to dial in the right response to repair and restore function (**Figure 2**). Early post-traumatic DAMP markers include high mobility group box protein 1 (HMGB1), mitochondrial DNA (mtDNA), S100, cell fragments, and many other molecules from injured or dying cells and proteoglycans and glycoproteins from endothelial-glycocalyx shedding (34). Importantly, DAMPs and PAMPs are not mutually exclusive and may share co-receptors and accessory molecules, and form partnerships to coordinate the right response (35).

A 2011 landmark study of Xiao and collaborators shed light on the early activation patterns of the immune system following severe blunt trauma and *burn injuries*. The group reported there was ∼80% activation of the leukocyte transcriptome in the circulation, which they termed a genomic storm (36). This storm developed within 4–12 h and lasted days to weeks. Importantly, in Xiao's study, what separated patients who developed secondary complications was not the magnitude of the storm, rather the time to resolve it (36). Prolonged resolution times led to worse outcomes. Moreover, both pro-inflammatory and anti-inflammatory pathways were activated early, which challenges the older two-hit and other sequential pro-inflammatory and compensatory anti-inflammatory models of trauma (37). On a cautionary note, although transcriptomic analysis establishes early temporal patterns of change, it provides little or no knowledge into the molecular mechanisms. Future studies should include proteomic and pathway-level analysis to establish the different roles of the early innate (and adaptive systems) to amplify inflammation after severe trauma.

Early drivers of inflammation and immune dysfunction

Inflammation is universal, beneficial and restorative. However, after major trauma, it can be lethal. As mentioned earlier, the massive release of DAMPs can overwhelm the system and trigger a hyperinflammatory state that, if not resolved in a timely manner, can lead to immune dysfunction, immunosuppression, infection, sepsis and MODS (4, 13, 19, 38–41). The disruption can lead to pathological interactions between monocyte, macrophage, NK and DCs, T cell dysfunction, and the development of persistent lymphopenia (8–10, 34, 38, 40, 42–45). Persistent lymphopenia carries a high mortality. Brohi's group recently reported a 45% mortality rate in trauma patients when the lymphocyte count was $\leq 0.5 \times 10^9$/L at 48 h after hospital admission (38). In addition, the type of trauma determines a patient's susceptibility to persistent lymphopenia and infection, with traumatic brain injury (TBI) patients having disproportionally worse outcomes compared to those with burns, polytrauma or major surgery (43). A recent study of Campbell et al. reported that 37% of TBI patients were lymphopenic on hospital admission, and its persistence was associated with increased risk of mortality and pneumonia (46). Wang further reported that up to 83% of severe TBI patients contracted a respiratory infection within 3 days following injury (43, 47).

The mechanisms responsible for persistent lymphopenia and immunosuppression are not well understood (38, 48). The difficulty is that immunosuppression is a highly heterogeneous response involving differential T cell loss, T-cell exhaustion, T-helper 1 (Th1) depression, receptor shedding, and expansion of myeloid-derived suppressor cells (MDSCs) that have suppressive activity (44, 49). Separating the relative contributions of different immune cell subsets to post-traumatic immunosuppression has been a challenge. In a ground-breaking study, Mansen and colleagues examined early changes in circulating lymphocytes and showed that trauma patients who developed MODS within 24 h had nearly 2-fold higher $CD56^{dim}$ NK cells, 80% lower gamma delta (γδ)-low T cells and 4-fold higher IFN-γ upon hospital admission, compared to patients who did not (38). $CD56^{dim}$ NK cells are potent mediators of natural and antibody-dependent cytotoxicity and only weakly secrete cytokines (50). Moreover, the group showed that the patients who developed MODS also developed lymphopenia within 24 h of injury, which if persisted to 48 h led to high mortality (38). The association between lymphopenia, MODS and decreased frequencies and functional responses of innate T cells in trauma patients suggests that early immuno-inflammatory events may "predetermine" late secondary complications. The rise in NK cells and early fall in γδ-low T cells seen in patients who developed MODS may be clinically significant and predict risk for late complications, however, further studies are required (38, 48).

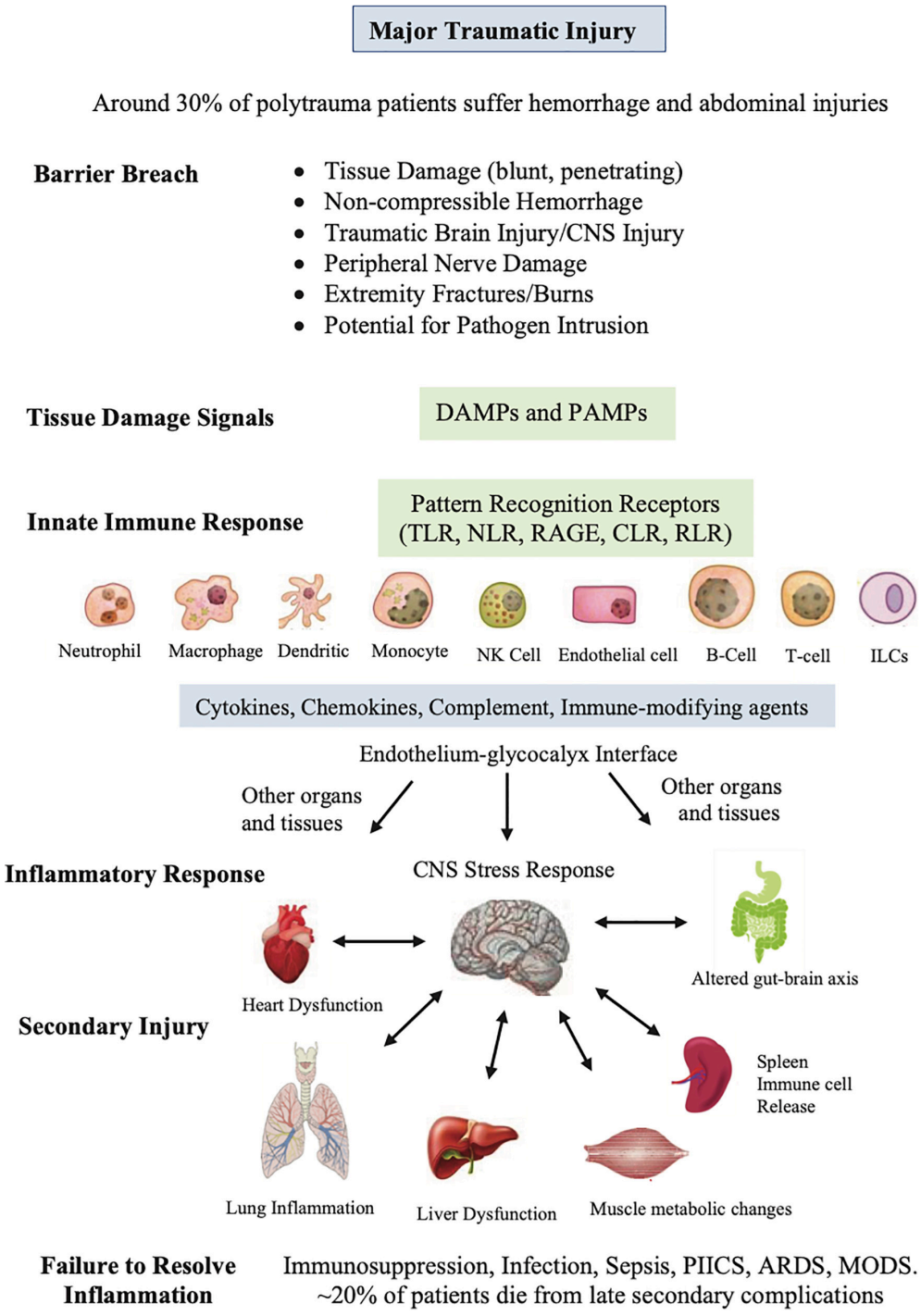

FIGURE 2
Sequence of events that occur after major traumatic injury. This diverse group of innate cells resident in the tissues detect and respond to changes in the local environment, including damage associated molecular patterns (DAMPs), pathogen associated molecular patterns (PAMPs), neural signals, and other immune-modifying triggers. The pattern recognition receptors on these cells include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic-acid-inducible gene-I (RIG-I)-like receptors (RLRs), and receptor for advanced glycation end products (RAGE). PAMPs can be derived from viruses, opportunistic bacteria, fungi, and protozoa and helminths. The innate cells orchestrate an immune response to response to the barrier breach by releasing different inflammatory factors. If dysregulated, the response can lead to secondary injury to the CNS and major organs of the body. The spleen has been included as it is reservoir of platelets, peripheral macrophages, undifferentiated monocytes and other immune cells. ILCs, innate lymphoid cells; NK, natural killer cells, PIICS; Persistent Inflammation, Immunosuppression and Catabolism Syndrome, ARDS; acute respiratory distress syndrome, MODS; multiple organ dysfunction.

TABLE 1 Possible mechanisms for T-cell apoptosis and immunosuppression after major trauma.

Pathway	Mechanisms	Comment	References
Cell-autonomous T cell death (ACAD)	• Intrinsic "caspase" pathway • Independent of death signals • Regulated by declining Bcl-2 at level of mitochondria • Cytochrome C released • Activates caspases • T cells undergo apoptosis without TCR restimulation	Toward the end of the immune response, activated lymphocytes not restimulated can die by permeabilizing the mitochondrial membrane. Bcl-2 is an anti-apoptotic protein that blocks the release of cytochrome c from mitochondria.	(53–55)
Stress-induced activation-induced cell death (AICD)	• Extrinsic "caspase" pathway • Death receptors: TNFR1, Fas, DR3, DR6, Trail-R1. • Glucocorticoid receptors (GRs) may also be involved • Receptor-driven apoptosis • Bax, bak, and BH3 domain • Activate caspases 8 and 3	A death receptor-mediated apoptosis pathway. The ligands for death receptors form a family of related cytokines collectively named as the TNF family.	(42, 52, 53, 56)
Monocyte-T cell interaction	• Extrinsic pathway • Inflammasome activation in monocytes sense DAMPs • IL-1β induced Fas-mediated monocyte driven T cell death *via* apoptosis	Monocytes sense injury-released DNA (DAMPs) *via* the AIM2 inflammasome and induce the extrinsic cell death of T cells.	(57)

Another early driver of immune complications is HMGB1, which is a major DAMP that induces inflammation *via* TNF-α, IL-6, and IL-1β that in turn stimulate pattern recognition receptors TLR4 and RAGE on immune cells (**Figure 2**) (44, 51). In a rat polytrauma model (femoral osteotomy, blunt chest contusion and burn injury), Muire and colleagues showed that HMGB1 was an early contributor to the onset of lymphopenia and the loss of $CD4^+$, $CD8^+$, and γδ-T cells (34). Interestingly, the decrease in T cells was partly attenuated when HMGB1 was neutralized immediately post-trauma, however, the γδ-T cell population was not affected (51). The authors proposed that diminished levels of surface expression of RAGE and TLR4 on T cells, *via* ectodomain shedding, may be responsible for suppression *in vivo* (51). HMGB1 has also been shown to activate MDSCs after trauma and cancer (44), and is a late mediator of sepsis (44, 51), which further highlights the complexity of the system.

Apoptosis is believed to play a central role in persistent lymphopenia (52–58). Three main mechanisms for inducing lymphocyte apoptosis include: (1) cell-autonomous T-cell death (ACAD), (2) stress-related activation-induced cell death (AICD), and (3) newly discovered inflammasome-dependent monocyte activation (52–58) (**Table 1**). Persistently elevated plasma interleukin (IL)-10 levels have further been correlated with monocyte deactivation, reduced T cell activation and secondary infectious complications (8, 39, 40, 42, 59). Platelets also modulate T cell subsets *via* PAR4 that may link the innate and adaptive systems *via* pro-inflammatory cytokines (58). The interconnectedness of the T cell subsets and potential drivers of immunosuppression requires further research. Interestingly, post-injury immunosuppression shares many similarities with non-traumatic, sepsis-induced immunosuppression (41, 57).

Central nervous system and organ interconnectedness: A major overlooked factor

> The defense of the organism against deleterious agencies, which is at first confined to the phagocytic mechanisms and the somatic system of nerves, by and by spreads to and is undertaken by the psychical nervous apparatus . . . One function of these psychical cells has been to develop a complete science for the defense of the organism against hostile influences.
>
> Metchnikoff (21) p. 195.

Metchnikoff had it right over 130 years ago. Activation of the "psychical cells" of the CNS following severe trauma results are important, and involve the release of norepinephrine, epinephrine and hormones (ACTH and glucocorticoids) from the adrenal medulla into the circulation and from the postganglionic nerve endings innervating the heart, and other organs of the body (14, 60–65). Traditionally, this is known as the whole-body stress response which dates back to Cannon (20, 66). The link between CNS injury, the immune system and immunosuppression is less well known. Yang and colleagues recently showed in a rat model of TBI that activation of sympathetic nervous system upregulated the expression of programmed cell death-1 (PD-1) on $CD4^+$ and $CD8^+$ T cells, and subsequently contributed to immunosuppression (43). The group speculated that immunosuppression may be partly mediated by stress hormones targeting β-adrenergic receptors (β-AR) on T cells (and indirectly B cells), because propranolol, a β-AR blocker, restored dysfunction *in vitro,* although they acknowledge it was more complex in the intact animal (43).

CNS modulation of the immune system occurs *via* the central hypothalamic-pituitary-adrenal (HPA) axis and the brainstem's nucleus tractus solitarius (NTS) (67–70). After major trauma, the CNS balance switches to a sympathetic dominance and suppression of the parasympathetic system that normally counters inflammation *via* activation of the parasympathetic vagal cholinergic neurons and splanchnic/splenic nerves, known as the inflammatory reflex (71–73). The shift in CNS balance also impacts other organs,

such as the heart and vasculature, and the gut microbiome *via* the gut-brain axis, which can alter blood flow to the gut wall and cause ischemia and increased permeability, where bacteria and/or their active metabolic products (lipopolysaccharides, cytokines, neuropeptides, and protein messengers) can enter the blood stream or lymph vessels and increase PAMPs and a patient's susceptibility to infection (74, 75). Together, all these factors *may contribute to predetermining the extent and resolvability of the immuno-inflammatory response* after major trauma.

Another unappreciated fact in the polytrauma patient is that many undergo a second trauma from the corrective surgery itself (20). At all times, from the prehospital setting to after major surgery, the brain remains "wide awake" to changes in circulating DAMPs and PAMPs, inflammatory cytokines and immune cells circulating in the body (20, 76). Even the anesthetized brain remains "awake" because the blood brain barrier (BBB) is disrupted from the trauma and changes in cerebral blood flow and shear stress, which is part of the injury phenotype (77), and this is further amplified in the patient with a TBI (20, 74, 78, 79). Following any major trauma, the brain loses its "immune privilege" as it is no longer "separated" from the rest of the body (77). This is a research area in its infancy. We recently showed in a rat model of a laparotomy, designed to simulate a penetrating wound, that profound changes in gene expression occurred in brain, heart and other organs (80). Abdominal trauma was associated with 10–20-fold increases in plasma corticosterone, pro-inflammatory cytokines, endothelial injury markers, neutrophils (6 h), lactate (3 days), and coagulopathy (80). Lymphocytes decreased by ∼70% at 6 h and 3 days, and IL-10 dramatically increased from undetectable baseline levels to 483 pg/ml after 6 h and again at 3 days (1,149 pg/ml). Cortical excitability was high over 3 days with 30-fold increases in M1 muscarinic receptor expression and α-1A-adrenergic expression, and similar in heart with 8-fold increases in β-1-adrenergic receptor expression, and up to 6-fold increases in M2 and M1 muscarinic receptors after 6 h despite no changes in hemodymanics (80). These "silent" changes are remarkable given that there was only one incision, with no further injury to brain or heart. Unfortunately, we did not examine changes in the different T-cell subsets to further understand changes in immune activation.

Systems hypothesis of trauma

> Except on few occasions, the patient appears to die from the body's response to infection rather than from it.
>
> William Osler (81)

Osler's point cannot be overemphasized. It is not the infection that kills you it's the body's response to the trauma. The shift in homeostatic balance toward extreme limits and death led us to develop the Systems Hypothesis of Trauma (SHOT) (82) (**Figure 3**), which has undergone a number of iterations to include hemorrhagic trauma and the trauma of surgery (12, 13, 16, 20). SHOT has three pillars of protection.

1. CNS-cardiovascular coupling (Central Controller)
2. Endothelial glycocalyx material exchange (Systemic Integrator)
3. Mitochondrial integrity (Systemic Regulator)

First pillar: Central nervous system-cardiovascular coupling

If the CNS can be protected early after trauma and the "hyperdrive" response can be suppressed, we argue that the immuno-inflammatory storms and lymphopenia will be reduced (20). According to SHOT, shifting autonomic balance toward parasympathetic outflows in the first minutes to hours after trauma would assist to maintain ventriculo-arterial (VA) coupling close to unity. VA coupling is a metric rarely discussed or measured in major trauma patients. It is the ratio of arterial elastance (Ea) to left-ventricular (LV) elastance (Ees) and can be measured from routine echocardiography (83–88). When the ratio is close to unity, the efficiency of the system is considered optimal. If the ratio is excessively high or low, the heart as a pump and vascular load become uncoupled with adverse downstream clinical outcomes (86, 89, 90). *The clinical advantage of VA coupling over ejection fraction (EF) or cardiac output (CO) is that it provides arterial load properties in addition to LV function* (86, 87). If the proximal arterial vessels become stiff, as a result of the CNS stress response, it increases load on the pump (91), whereas if the heart becomes stiff it cannot relax optimally to fill and eject blood into the conduit vessels (87). If both occur, they lead to VA uncoupling, tissue hypoperfusion, mitochondrial damage (92, 93) and subsequent immuno-inflammatory dysfunction (**Figure 3**). In the case of a high VA coupling ratio, vasodilator therapies can lower Ea and reduce the Ea/Ees ratio toward 1.0, and in the case of a low ratio, inotropes can increases Ees to improve VA coupling (92).

We predict further that maintaining VA coupling would improve immune function by reducing gut-brain axis dysfunction and preventing the gut wall from becoming ischemic and leaky which exacerbates immuno-inflammatory conditions, coagulopathy, immunosuppression, infection and sepsis (75). Howard and colleagues reported in trauma patients rapid changes in the microbiome during resuscitation and stabilization (94), although further studies are required to understand the role of the gut in exacerbating

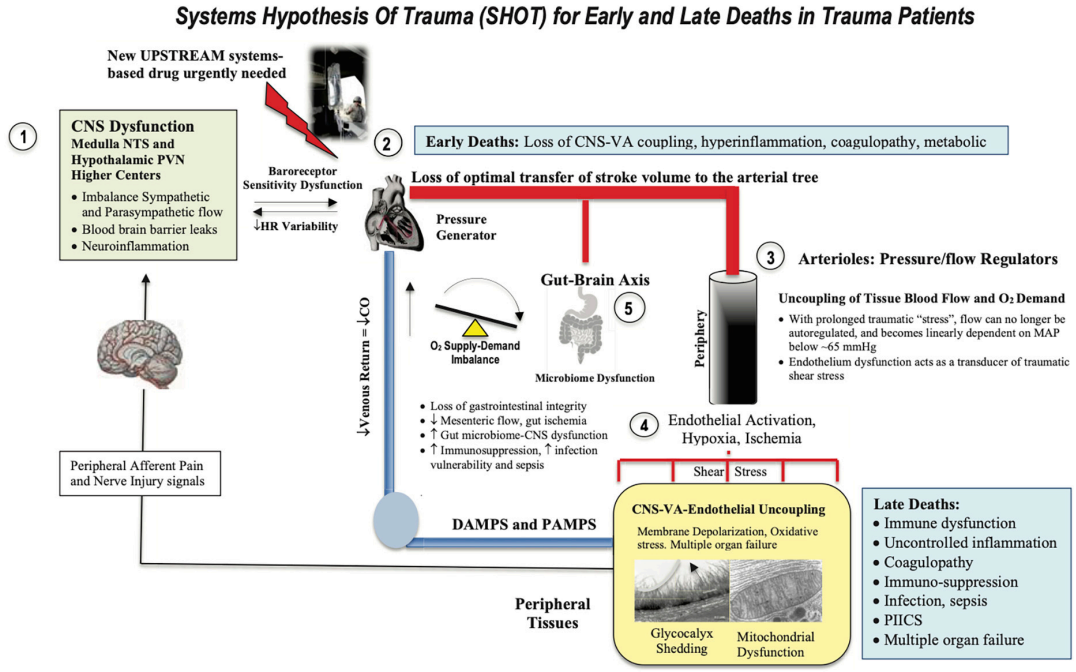

FIGURE 3
Systems Hypothesis Of Trauma (SHOT) showing key inter-connected sites of uncoupling during early and late deaths: (1) brain, (2) heart, (3) vasculature, (4) endothelial-glycocalyx-mitochondrial unit, and (5) gut barrier breaches. Our hypothesis is that if central and local control of cardiac output and ventriculo-arterial (VA) coupling are improved, endothelial and microvascular function will be improved and tissue O_2 delivery will be maintained. An uncoupling is reflected by an increase in stress hormones, sympathetic discharge, loss of baroreceptor sensitivity, reduced heart rate variability (183, 184), unresolved inflammation, immune dysfunction, coagulopathy and mitochondrial dysfunction. New drugs are required to prevent inflammatory hyperdrive and support a VA-coupled, high flow, vasodilatory system with endothelial-glycocalyx protection and tissue oxygenation (185). HR, heart rate; ATP, adenosine triphosphate; CNS, central nervous system; NTS, nucleus tractus solitarius; PVN, paraventricular nucleus of the hypothalamus; MAP, mean arterial pressure. PIICS, Persistent Inflammation, Immunosuppression and Catabolism Syndrome, see text for description of early and late deaths.

systemic inflammation and infectious complications after major trauma.

Second pillar: The endothelial glycocalyx

The second pillar of SHOT is to maintain the health of the endothelium (95). The endothelium is located at the nexus of the blood and tissues and controls the transfer of O_2, metabolic fuels, hormones, immune cells and factors, inflammatory regulators and fluids (96–102). Trauma-induced damage to this organ is termed the endotheliopathy of trauma (EoT), which is characterized by endothelial activation, vasoactivity, loss of barrier function, leukocyte adhesion, coagulopathy, inflammation and organ dysfunction (103–110). In addition, the endothelium, like the BBB, is highly sensitive to changes in blood flow and shear stress, which can alter vascular tone, tissue perfusion, exchange and permeability (111).

The endothelial surface area (SA) has been estimated to be 3,000–7,000 m^2 (98, 112). However, this estimate ignores the SA of the glycocalyx mesh that is synthesized and secreted by the endothelium and anchored to its cellular lining. As mentioned, the function of the endothelial glycocalyx is dynamic and diverse and it also acts as a vascular filter overlying the endothelial cell-cell junctions as it contains a large volume of non-circulating plasma (1–1.7 L) (113–115). We have estimated for the first time the SA of glycocalyx and found it was at least 10-fold higher than the endothelium ($SA_{glycocalyx}$ = 46,120 m^2) (see **Figure 4**). This is equivalent to a SA of over ~200 tennis courts or 8 USA football fields, and given its central role has major implications to immune function and secondary injury progression post-trauma.

When damaged by inflammatory mechanisms, the endothelium can rapidly shed its glycocalyx "fuzz" *via* sheddases, and release nanoscale bioactives and DAMPs, such as thrombomodulin, syndecan-1, heparan sulfate, hyaluronic acid, and other proteoglycans and glycoproteins, into the circulation (96, 104, 107, 116–119). This degradation is believed to perpetuate immuno-inflammation and coagulopathy (13, 120–125), immunosuppression (102, 126, 127) and mitochondrial dysfunction (115, 128, 129). SHOT predicts if VA coupling is close to unity and tissue perfusion and O_2 exchange

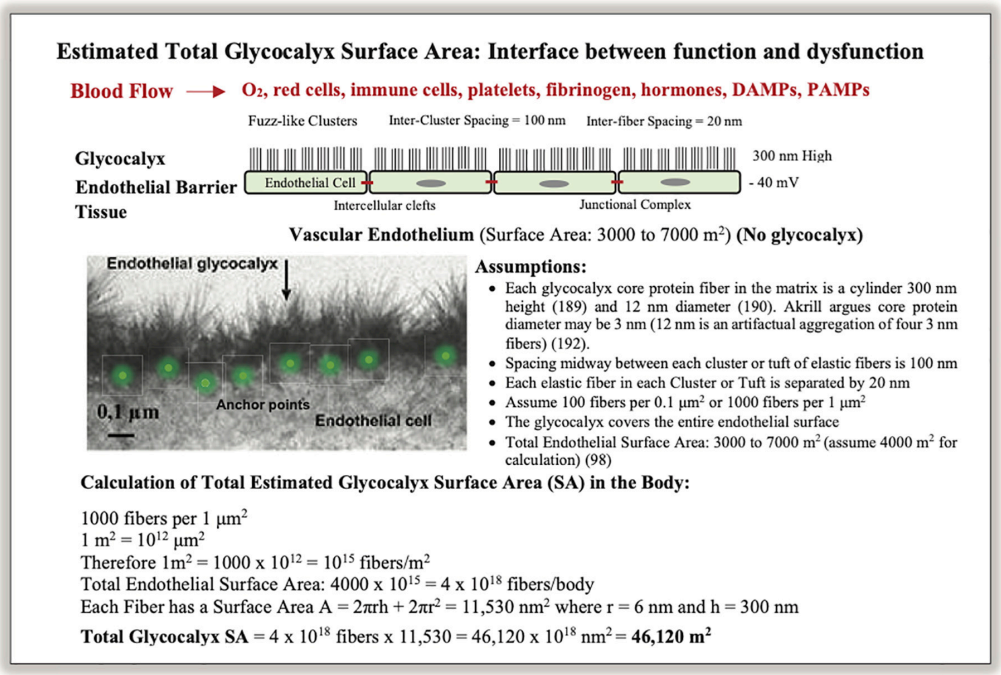

FIGURE 4
A schematic of the vascular endothelium and calculation of total glycocalyx surface area (SA) in humans. Photo insert was modified from Chappell et al. (186). Glycocalyx fibers appear in clusters composed of proteoglycans, glycosaminoglycans, and glycoproteins, which are anchored into endothelial cells by core-proteins (113, 115, 187, 188). Together they form a dynamic structure that participates in shear stress regulation, barrier protection, vascular permeability, inflammation, coagulopathy, fibrinolysis, mechanotransduction, immune function and cytokine signaling (97, 101, 104, 116). The glycocalyx is difficult to characterize because of its fragility and instability, and its structural dimensions critically depend on the method of ultrastructural visualization (97, 116, 189–193). The SA calculation should be viewed as approximate. A glycocalyx SA of 44,120 m^2 for material exchange equates to ~200 tennis courts or over 8 US football fields (see text).

can be maintained, damage to the endothelium-glycocalyx will be minimized and these secondary injury processes reduced (**Figure 3**). Remarkably, if adequate tissue perfusion can be restored, the damaged glycocalyx has the capacity to repair itself (130, 131). Timing of repair appears to depend upon the duration and extent of hypoperfusion and ischemia, and the type and severity of trauma (130, 131).

Third pillar: Mitochondrial integrity

Maintaining the functional integrity of mitochondria post-trauma is essential for a good outcome. Mitochondria are sensor organelles of ancient bacterial origins involved in ATP production, substrate regulation, immune cell signaling, calcium homeostasis, endoplasmic reticulum communication, and cell death regulation (32, 132–135). After severe trauma, prolonged hypoperfusion leads to mitochondrial damage. However, before damage occurs there is a switch from aerobic mitochondrial oxidative and to anaerobic glycolytic metabolism, which can only be sustained for short periods of time (136). Damage occurs from depletion of local glycogen stores, depolarization of the sarcolemma membrane, increased lactate, reduced pH, increases in cell Ca^{2+} loading, a fall in ATP phosphorylation and redox potentials, increased reactive oxygen species, reduced inner mitochondrial membrane proton pumping, opening of the permeability transition pore, collapse of mitochondrial membrane potential and finally the release of cytochrome C, and other DAMPs (32, 134, 135, 137–142). DAMPs from damaged mitochondria exacerbate CNS injury, cardiovascular dysfunction and secondary injury (143–145). According to SHOT, improving CNS protection and CNS-cardiovascular-endothelium coupling will improve tissue perfusion and protect mitochondrial integrity (146) (**Figure 3**).

Other unifying models of traumatic injury

In 2017, Johansson and colleagues introduced a model of SHock-INduced Endotheliopathy (SHINE) to better understand the underlying pathophysiological mechanisms for critically ill patients (147). Like SHOT, they propose that shock-induced sympatho-adrenal hyperactivation is a critical driver of endothelial cell and glycocalyx damage, hypoperfusion, and

subsequent hemostatic aberrations and multiorgan dysfunction (110). More recently, Henriksen reported that patients with identical trauma severity developed *significantly different degrees of endothelial dysfunction*, as measured by syndecan-1, and proposed a minimum of four shock-induced endotheliopathy phenotypes (148) with the differences most likely driven by a genetic component (148). Moreover, they introduced a new research tool in trauma by using metabolic systems biology, which should be encouraged. A major difference between SHOT and SHINE is the functional linkage between CNS and VA coupling, which is testable. SHINE does not include this key linkage, which describes the coupling of cardiac and arterial vascular reactivity to optimally propel blood to deliver sufficient oxygen from the lungs to tissue mitochondria and prevent and/or reduce ongoing immuno-inflammatory dysfunction (discussed above).

Urgent need for systems-based therapies: Heterogeneity vs. homogeneity in research

How do we switch the injury phenotype of a polytrauma patient to a survival one? Why are there no effective drugs to treat immune dysregulation in the early hours to days following major trauma or in the critically ill patient? We argue the main reasons for lack of progress in drug development include:

1. Failure to replicate the heterogeneity of humans.
2. Poorly designed trials lacking diversity.
3. Inappropriate use of pathogen-free animals.
4. Ignoring sex-specific differences.
5. The flawed practice of single-nodal targeting.

The heterogeneity of the human condition is a major variable when conducting animal experiments to solve a medical problem (149). Preclinical models typically use animals from relatively homogeneous breeding colonies whereas humans are genetically, epigenetically, biologically and physiologically heterogeneous (149, 150). Large animals, such as pigs and sheep, do have some advantages with similar physiologies and/or anatomies as humans, however, they are more costly than using rodents (149, 150). A second confounding variable are poorly designed human trials that are either not sufficiently powered or recruit patients who do not adequately represent the wider population for which the drug therapy is intended (151–153).

Similar problems apply to preclinical models that use specific pathogen-free (SPF) animals. SPF animals were introduced in the early 1960s to minimize disease or infection as an unwanted variable in experimental design (151, 154). However, SPF animals have different gut microbiota that can profoundly influence basic physiology, stress behaviors and the immuno-inflammatory response to trauma (151–153, 155). Beura et al. showed that SPF adult mice, for example, have "immature" immune systems that were more prone to infection than conventionally bred mice (156). SPF animals may be useful for studying specific questions in biochemical mechanisms, but they do not mimic the patient following trauma (152). The current consensus is that conventionally bred animals are the animals of choice if translation of a new drug therapy is the end-game (151). In addition, the mouse model may be problematic for trauma studies because unlike rats, guinea-pigs, pigs, sheep, dogs, and humans, mice can enter a dormant state, called torpor, when subjected to traumatic stress (157, 158). Torpor itself can profoundly change the animal's immune system by reducing the numbers of circulating leukocytes, lowering complement levels, and changing the animal's response to infection (159).

The other important variable in preclinical and clinical studies is sex. An increasing number of animal and human studies show sex-specific differences in pathophysiological responses to polytrauma, hemorrhagic shock, TBI and burns (160–162). Chaudry and colleagues have been emphasizing the importance of sex in biochemical research for over two decades. They showed that administration of female sex hormone 17β-estradiol in males and ovariectomized females after trauma-hemorrhage prevented the suppression of immune response (163, 164). On the basis of accumulated data, greater inclusion of females in preclinical modeling and translation has been earmarked by the National Institutes of Health (NIH), European Commission, US Department of Defense and FDA (151–153).

Lastly, the practice of single-nodal targeting is another factor for why there are no effective systems-acting drugs for the polytrauma patient. Past drug development efforts have focused more on alleviating symptoms rather than addressing an underlying problem. The current practice of treat-as-you-go using sequential, single-target therapies leads to what US surgeon William C. Shoemaker considered: "an uncoordinated and sometimes contradictory therapeutic outcome" (165). Targeting individual pro-inflammatory cytokines, or any single step along a signaling pathway, ignores the critical importance of the system. Single-nodal thinking rarely solves a medical problem unless the site is believed to be a central hub or upstream intersection point. The IL-1 receptor has been proposed to be such a target, and while *anakinra* (IL-1 antagonist) has an excellent safety record, further trials are required to demonstrate its clinical efficacy after trauma or infection (166, 167). Reductionism in scientific discovery is important in breaking a system into its constituent parts, however, *it does not do away with the system* (151–153). This flawed way of thinking, we believe, is a major contributor for the high failure rate of translating promising new drugs in clinical trials (168). Choosing the right model and experimental design, a systems approach is much more likely to increase animal-to-human translational success to improve trauma care.

Adenosine, lidocaine and magnesium (ALM): Toward a systems-based drug therapy

> If you control hemorrhage and infection, the patient will do the recovery, since every cell in his body is working hard in that direction already. But you must understand what those cells are doing so that you can help them.
>
> Walter B. Cannon [Moore, (169) p. 816].

We have been developing a small-volume intravenous (IV) ALM fluid therapy to treat polytrauma for civilian and military use (12, 16). In different animal models, ALM confers a survival advantage after hemorrhagic shock (12, 16, 170, 171), traumatic injury (170–174), sepsis (175, 176) and endotoxin insult (177). The ALM survival phenotype is not replicated with individual actives adenosine, lidocaine or magnesium (12, 16). ALM confers its benefit by shifting CNS function from sympathetic to parasympathetic dominance (178), blunting inflammation (172), correcting coagulopathy (179), maintaining VA coupling, improving tissue blood flow, lowering energy demand and protecting mitochondria (178). Studies carried out by US Army Institute of Surgical Research also showed that ALM therapy restored 97% of endothelial glycocalyx after severe hemorrhagic shock (180). Currently, we don't know how and when the "switch" from an injury phenotype to a survival phenotype occurs, however, we suspect it is early because the same 5 h therapy confers dual protection against trauma and infection (12, 14, 16). It is possible ALM may act in the first minutes to hours after administration to assist the body to develop a "normal" immune response with timely resolution of the immuno-inflammatory genomic storms. While the preclinical ALM data appear promising, translation to humans remains challenging given the failure rate of translating new drugs into humans exceeds 95% (181), and of those that do obtain FDA approval, around 30% show postmarket safety concerns (182). Understanding the underlying mechanisms of action of ALM is vital for safe translation.

Conclusion

Trauma is a leading cause of death and disability worldwide. Currently there are no effective drug therapies to reduce hyperinflammation and immune dysfunction, immunosuppression, infection and MODS following major trauma. The present treat-as-you-go approaches fail to appreciate that immuno-inflammatory complications are a systems failure, and not a single nodal failure. New therapies are required to target the CNS control of cardiovascular function, endothelial-glycocalyx shedding, tissue O_2 supply and its mitochondrial circuitry in both homeostatic and pathophysiological processes to prevent those complications.

Author contributions

GD: concept. GD, JM, and HL: data collection, data analyses, interpretation, and manuscript preparation and editing. All authors contributed equally to the design, implementation, literature analysis and writing of the manuscript.

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Conflict of interest

GD is the sole inventor of the ALM concept for cardiac surgery, trauma and sepsis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Bedard AF, Mata LV, Dymond C, Moreira F, Dixon J, Schauer SG, et al. A scoping review of worldwide studies evaluating the effects of prehospital time on trauma outcomes. *Int J Emerg Med.* (2020) 13:64. doi: 10.1186/s12245-020-00324-7

2. Dobson GP, Morris JL, Letson HL. Why are bleeding trauma patients still dying? Towards a systems hypothesis of trauma. *Front Physiol.* (2022) 13:990903. doi: 10.3389/fphys.2022.990903

3. Rauf R, Von Matthey F, Croenlein M, Zyskowski M, Van Griensven M, Biberthaler P, et al. Changes in the temporal distribution of in-hospital mortality in severely injured patients-an analysis of the TraumaRegister DGU. *PLoS One.* (2019) 14:e0212095. doi: 10.1371/journal.pone.0212095

4. Brohi K, Gruen RL, Holcomb JB. Why are bleeding trauma patients still dying? *Intensive Care Med.* (2019) 45:709–11. doi: 10.1007/s00134-019-05560-x

5. Lee C, Rasmussen TE, Pape HC, Gary JL, Stannard JP, Haller JM. The polytrauma patient: Current concepts and evolving care. *OTA Int.* (2021). 4:e108–14.

6. Lansink KW, Gunning AC, Leenen LP. Cause of death and time of death distribution of trauma patients in a Level I trauma centre in the Netherlands. *Eur J Trauma Emerg Surg.* (2013) 39:375–83. doi: 10.1007/s00068-013-0278-2

7. Rosenthal MD, Moore FA. Persistent Inflammation, Immunosuppression, and catabolism: evolution of multiple organ dysfunction. *Surg Infect (Larchmt).* (2016) 17:167–72. doi: 10.1089/sur.2015.184

8. Efron PA, Mohr AM, Bihorac A, Horiguchi H, Hollen MK, Segal MS, et al. Persistent inflammation, immunosuppression, and catabolism and the development of chronic critical illness after surgery. *Surgery.* (2018) 164:178–84. doi: 10.1016/j.surg.2018.04.011

9. Osuka A, Ogura H, Ueyama M, Shimazu T, Lederer JA. Immune response to traumatic injury: harmony and discordance of immune system homeostasis. *Acute Med Surg.* (2014) 1:63–9. doi: 10.1002/ams2.17

10. Mas-Celis F, Olea-Lopez J, Parroquin-Maldonado JA. Sepsis in trauma: a deadly complication. *Arch Med Res.* (2021) 52:808–16. doi: 10.1016/j.arcmed.2021.10.007

11. Kotwal RS, Staudt AM, Trevino JD, Valdez-Delgado KK, Le TD, Gurney JM, et al. A review of casualties transported to role 2 medical treatment facilities in Afghanistan. *Mil Med.* (2018) 183(Suppl. 1):134–45. doi: 10.1097/01.ccm.0000528322.39346.3a

12. Dobson GP, Letson HL. Far forward gaps in hemorrhagic shock and prolonged field care: an update of ALM fluid therapy for field use. *J Spec Oper Med.* (2020) 20:78–84. doi: 10.55460/06VT-9IH4

13. Dobson GP, Morris JL, Davenport LM, Letson HL. Traumatic-induced coagulopathy as a systems failure: a new window into hemostasis. *Semin Thromb Hemost.* (2020) 46:199–214. doi: 10.1055/s-0039-1701018

14. Dobson GP, Biros E, Letson HL, Morris JL. Living in a hostile world: inflammation, new drug development and coronavirus. *Front Immunol.* (2021) 11:610131. doi: 10.3389/fimmu.2020.610131

15. Cannon WB. Organisation for physiological homeostasis. *Physiol Rev.* (1929) 9:399–431. doi: 10.1152/physrev.1929.9.3.399

16. Dobson GP, Letson HL. Adenosine, lidocaine and Mg2+ (ALM): from cardiac surgery to combat casualty care: teaching old drugs new tricks. *J Trauma Acute Care Surg.* (2016) 80:135–45. doi: 10.1097/TA.0000000000000881

17. Dobson GP. Organ arrest, protection and preservation: natural hibernation to cardiac surgery: a review. *Comp Biochem Physiol Part B.* (2004) 139:469–85. doi: 10.1016/j.cbpc.2004.06.002

18. Egiazaryan GG, Sudakov KV. Theory of functional systems in the scientific school of P.K. Anokhin. *J Hist Neurosci.* (2007) 16:194–205. doi: 10.1080/09647040600602805

19. Carlson DL, Horton JW. Cardiac molecular signaling after burn trauma. *J Burn Care Res.* (2006) 27:669–75. doi: 10.1097/01.BCR.0000237955.28090.41

20. Dobson GP. Trauma of major surgery: a global problem that is not going away. *Int J Surg.* (2020) 81:47–54. doi: 10.1016/j.ijsu.2020.07.017

21. Metchnikoff E. Lectures on the comparative pathology of inflammation, delivered at the Pasteur institute in 1891. In: Metchnikoff E editor. *Lectures on the Comparative Pathology of Inflammation. (Translated by F. A. Starling and E. H. Starling.).* New York, NY: Dover Publications (1968).

22. Chou C, Li MO. Tissue-resident lymphocytes across innate and adaptive lineages. *Front Immunol.* (2018) 9:2104. doi: 10.3389/fimmu.2018.02104

23. Fan X, Rudensky AY. Hallmarks of tissue-resident lymphocytes. *Cell* (2016) 164:1198–211. doi: 10.1016/j.cell.2016.02.048

24. Roehr B. Tissue resident memory cells emerging as key player in health and disease. *Proc Natl Acad Sci USA.* (2017) 114:12092–3. doi: 10.1073/pnas.1715754114

25. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement system part I - molecular mechanisms of activation and regulation. *Front Immunol.* (2015) 6:262. doi: 10.3389/fimmu.2015.00262

26. Klose CSN, Artis D. Innate lymphoid cells control signaling circuits to regulate tissue-specific immunity. *Cell Res.* (2020) 30:475–91. doi: 10.1038/s41422-020-0323-8

27. Galli SJ, Gaudenzio N. Human mast cells as antigen-presenting cells: when is this role important in vivo? *J Allergy Clin Immunol.* (2018) 141:92–3. doi: 10.1016/j.jaci.2017.05.029

28. Dorrington MG, Fraser IDC. NF-kappaB signaling in macrophages: dynamics, crosstalk, and signal integration. *Front Immunol.* (2019) 10:705. doi: 10.3389/fimmu.2019.00705

29. Allie SR, Randall TD. Resident memory B cells. *Viral Immunol.* (2020). [Epub ahead of print]. doi: 10.1089/vim.2019.0141

30. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* (2007) 81:1–5. doi: 10.1189/jlb.0306164

31. Venereau E, Ceriotti C, Bianchi ME. DAMPs from cell death to new life. *Front Immunol.* (2015) 6:422. doi: 10.3389/fimmu.2015.00422

32. Hauser CJ, Otterbein LE. Danger signals from mitochondrial DAMPS in trauma and post-injury sepsis. *Eur J Trauma Emerg Surg.* (2018) 44:317–24. doi: 10.1007/s00068-018-0963-2

33. Roh JS, Sohn DH. Damage-associated molecular patterns in inflammatory diseases. *Immune Netw.* (2018) 18:e27. doi: 10.4110/in.2018.18.e27

34. Muire PJ, Schwacha MG, Wenke JC. Systemic T cell exhaustion dynamics is linked to early high mobility group box protein 1 (HMGB1) driven hyper-inflammation in a polytrauma rat model. *Cells.* (2021) 10:1646. doi: 10.3390/cells10071646

35. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm.* (2010) 2010:1–21. doi: 10.1155/2010/672395

36. Xiao W, Mindrinos MN, Seok J, Cuschieri J, Cuenca AG, Gao H, et al. A genomic storm in critically injured humans. *J Exp Med.* (2011) 208:2581–90. doi: 10.1084/jem.20111354

37. Gentile LF, Cuenca AG, Efron PA, Ang D, Bihorac A, Mckinley BA, et al. Persistent inflammation and immunosuppression: a common syndrome and new horizon for surgical intensive care. *J Trauma Acute Care Surg.* (2012) 72:1491–501. doi: 10.1097/TA.0b013e318256e000

38. Manson J, Cole E, De'ath HD, Vulliamy P, Meier U, Pennington D, et al. Early changes within the lymphocyte population are associated with the development of multiple organ dysfunction syndrome in trauma patients. *Crit Care.* (2016) 20:176. doi: 10.1186/s13054-016-1341-2

39. Thompson KB, Krispinsky LT, Stark RJ. Late immune consequences of combat trauma: a review of trauma-related immune dysfunction and potential therapies. *Mil Med Res.* (2019) 6:11. doi: 10.1186/s40779-019-0202-0

40. Sturm R, Xanthopoulos L, Heftrig D, Oppermann E, Vrdoljak T, Dunay IR, et al. Regulatory T cells modulate CD4 proliferation after severe trauma via IL-10. *J Clin Med.* (2020) 9:1052. doi: 10.3390/jcm9041052

41. Vazquez AC, Arriaga-Pizano L, Ferat-Osorio E. Cellular markers of immunosuppression in sepsis. *Arch Med Res.* (2021) 52:828–35. doi: 10.1016/j.arcmed.2021.10.001

42. Kimura F, Shimizu H, Yoshidome H, Ohtsuka M, Miyazaki M. Immunosuppression following surgical and traumatic injury. *Surg Today.* (2010) 40:793–808. doi: 10.1007/s00595-010-4323-z

43. Yang Y, Ye Y, Chen C, Kong C, Su X, Zhang X, et al. Acute traumatic brain injury induces CD4+ and CD8+ T cell functional impairment by upregulating the expression of PD-1 via the activated sympathetic nervous system. *Neuroimmunomodulation.* (2019) 26:43–57. doi: 10.1159/000495465

44. Ruan X, Darwiche SS, Cai C, Scott MJ, Pape HC, Billiar TR. Anti-HMGB1 monoclonal antibody ameliorates immunosuppression after peripheral tissue trauma: attenuated T-lymphocyte response and increased splenic CD11b (+) Gr-1 (+) myeloid-derived suppressor cells require HMGB1. *Mediators Inflamm.* (2015) 2015:458626. doi: 10.1155/2015/458626

45. Islam MN, Bradley BA, Ceredig R. Sterile post-traumatic immunosuppression. *Clin Transl Immunol.* (2016) 5:e77. doi: 10.1038/cti.2016.13

46. Campbell B, Budreau D, Williams-Perez S, Chakravarty S, Galet C, Mcgonagill P. Admission lymphopenia predicts infectious complications and

mortality in traumatic brain injury victims. *Shock.* (2022) 57:189–98. doi: 10.1097/SHK.0000000000001872

47. Wang J-W, Li J-P, Song Y-L, Zhao Q-H. Humoral and cellular immunity changed after traumatic brain injury in human patients. *Ann Clin Lab Sci.* (2017) 47:10–6.

48. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol.* (2010) 10:467–78. doi: 10.1038/nri2781

49. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* (2009) 9:162–74. doi: 10.1038/nri2506

50. Muller-Heck RM, Bosken B, Michiels I, Dudda M, Jager M, Flohe SB. Major surgical trauma impairs the function of natural killer cells but does not affect monocyte cytokine synthesis. *Life (Basel).* (2021) 12:13. doi: 10.3390/life12010013

51. Yang H, Wang H, Andersson U. Targeting inflammation driven by HMGB1. *Front Immunol.* (2020) 11:484. doi: 10.3389/fimmu.2020.00484

52. Kumar R, Herbert PE, Warrens AN. An introduction to death receptors in apoptosis. *Int J Surg.* (2005) 3:268–77. doi: 10.1016/j.ijsu.2005.05.002

53. Arnold R, Brenner D, Becker M, Frey CR, Krammer PH. How T lymphocytes switch between life and death. *Eur J Immunol.* (2006) 36:1654–8. doi: 10.1002/eji.200636197

54. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. *Nat Rev Immunol.* (2007) 7:532–42. doi: 10.1038/nri2115

55. Brenner D, Krammer PH, Arnold R. Concepts of activated T cell death. *Crit Rev Oncol Hematol.* (2008) 66:52–64. doi: 10.1016/j.critrevonc.2008.01.002

56. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. *Int J Cell Biol.* (2010) 2010:214074. doi: 10.1155/2010/214074

57. Roth S, Cao J, Singh V, Tiedt S, Hundeshagen G, Li T, et al. Post-injury immunosuppression and secondary infections are caused by an AIM2 inflammasome-driven signaling cascade. *Immunity.* (2021) 54:648–659.e8. doi: 10.1016/j.immuni.2021.02.004

58. Bock M, Bergmann CB, Jung S, Biberthaler P, Heimann L, Hanschen M. Platelets differentially modulate CD4(+) Treg activation via GPIIa/ IIIb-, fibrinogen-, and PAR4-dependent pathways. *Immunol Res.* (2022) 70:185–96. doi: 10.1007/s12026-021-09258-5

59. Abe R, Hirasawa H, Oda S, Sadahiro T, Nakamura M, Watanabe E, et al. Up-regulation of interleukin-10 mRNA expression in peripheral leukocytes predicts poor outcome and diminished human leukocyte antigen-DR expression on monocytes in septic patients. *J Surg Res.* (2008) 147:1–8. doi: 10.1016/j.jss.2007.07.009

60. Desborough JP. The stress response to trauma and surgery. *Br J Anaesth.* (2000) 85:109–17. doi: 10.1093/bja/85.1.109

61. Ballard-Croft C, Maass DL, Sikes P, White JM, Horton J. Activation of stress-responsive pathways by the sympathetic nervous system in burn trauma. *Shock.* (2002) 18:38–45. doi: 10.1097/00024382-200207000-00008

62. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol.* (2006) 6:318–28. doi: 10.1038/nri1810

63. Hotamisligil GS, Davis RJ. Cell signaling and stress responses. *Cold Spring Harb Perspect Biol.* (2016) 8:a006072. doi: 10.1101/cshperspect.a006072

64. Powell K, Shah K, Hao C, Wu YC, John A, Narayan RK, et al. Neuromodulation as a new avenue for resuscitation in hemorrhagic shock. *Bioelectron Med.* (2019) 5:17. doi: 10.1186/s42234-019-0033-z

65. Gans IM, Coffman JA. Glucocorticoid-mediated developmental programming of vertebrate stress responsivity. *Front Physiol.* (2021) 12:812195. doi: 10.3389/fphys.2021.812195

66. Prete A, Yan Q, Al-Tarrah K, Akturk HK, Prokop LJ, Alahdab F, et al. The cortisol stress response induced by surgery: a systematic review and meta-analysis. *Clin Endocrinol (Oxf).* (2018) 89:554–67. doi: 10.1111/cen.13820

67. Smith SM, Vale WW. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialog Clin Neurosci.* (2006) 8:383–95. doi: 10.31887/DCNS.2006.8.4/ssmith

68. Charkoudian N, Wallin BG. Sympathetic neural activity to the cardiovascular system: integrator of systemic physiology and interindividual characteristics. *Compr Physiol.* (2014) 4:825–50. doi: 10.1002/cphy.c130038

69. Burford NG, Webster NA, Cruz-Topete D. Hypothalamic-pituitary-adrenal axis modulation of glucocorticoids in the cardiovascular system. *Int J Mol Sci.* (2017) 18:2150. doi: 10.3390/ijms18102150

70. Barman SM. 2019 Ludwig lecture: rhythms in sympathetic nerve activity are a key to understanding neural control of the cardiovascular system. *Am J Physiol Regul Integr Comp Physiol.* (2020) 318:R191–205. doi: 10.1152/ajpregu.00298.2019

71. Pavlov VA, Tracey KJ. Neural regulators of innate immune responses and inflammation. *Cell Mol Life Sci.* (2004) 61:2322–31. doi: 10.1007/s00018-004-4102-3

72. Huston JM. The vagus nerve and the inflammatory reflex: wandering on a new treatment paradigm for systemic inflammation and sepsis. *Surg Infect (Larchmt).* (2012) 13:187–93. doi: 10.1089/sur.2012.126

73. Tracey KJ. The inflammatory reflex. *Nature.* (2020) 420:853–9. doi: 10.1038/nature01321

74. Sudo N. Microbiome, HPA axis and production of endocrine hormones in the gut. In: Lyte M, Cryan J editors. *Microbiology Endocrinology: The Microbiota-Gut Brain Axis in Health and Disease*. New York, NY: Springer (2014). doi: 10.1007/978-1-4939-0897-4_8

75. Mayer EA, Tillisch K, Gupta A. Gut/brain axis and the microbiota. *J Clin Invest.* (2015) 125:926–38. doi: 10.1172/JCI76304

76. Crile G. Nitrous oxide anaesthesia and a note on anoci-association, a new principle in operative surgery. *Surg Gynecol Obstet.* (1911) 13:170–3.

77. Montagne A, Toga AW, Zlokovic BV. Blood-brain barrier permeability and gadolinium: benefits and potential pitfalls in research. *JAMA Neurol.* (2016) 73:13–4. doi: 10.1001/jamaneurol.2015.2960

78. Turnbull AV, Rivier C. Regulation of the HPA axis by cytokines. *Brain Behav Immun.* (1995) 9:253–75. doi: 10.1006/brbi.1995.1026

79. Kadry H, Noorani B, Cucullo L. A blood-brain barrier overview on structure, function, impairment, and biomarkers of integrity. *Fluids Barriers CNS.* (2020) 17:69. doi: 10.1186/s12987-020-00230-3

80. Dobson GP, Morris JL, Biros E, Davenport LM, Letson HL. Major surgery leads to a proinflammatory phenotype: differential gene expression following a laparotomy. *Ann Med Surg (Lond).* (2021) 71:102970. doi: 10.1016/j.amsu.2021.102970

81. Osler W. *The Evolution of Modern Medicine*. New Haven, CT: Yale, University Press (1921). p. 243.

82. Dobson GP. Addressing the global burden of trauma in major surgery. *Front Surg.* (2015) 2:43. doi: 10.3389/fsurg.2015.00043

83. Suga H, Goto Y, Kawaguchi O, Hata K, Takasago T, Saeki TW, et al. Ventricular perspective on efficiency. In: Burkhoff D, Schaefer J, Schaffner K, Yue DT editors. *Myocardial Optimization and Efficiency, Evolutionary Aspects and Philosophy of Science Considerations*. New York, NY: Springer-Verlag (1993). p. 43–65.

84. London GM. Role of arterial wall properties in the pathogenesis of systolic hypertension. *Am J Hypertens.* (2005) 18(1 Pt 2):19S–22S. doi: 10.1016/j.amjhyper.2004.10.001

85. Kass DA. Ventricular arterial stiffening: integrating the pathophysiology. *Hypertension.* (2005) 46:185–93. doi: 10.1161/01.HYP.0000168053.34306.d4

86. Guarracino F, Baldassarri R, Pinsky MR. Ventriculo-arterial decoupling in acutely altered hemodynamic states. *Crit Care.* (2013) 17:213–20. doi: 10.1186/cc12522

87. Cholley B, Le Gall A. Ventriculo-arterial coupling: the comeback? *J Thorac Dis.* (2016) 8:2287–9. doi: 10.21037/jtd.2016.08.34

88. Dobson GP, Arsyad A, Letson HL. The adenosine hypothesis revisited: a possible role for arterial compliance and its implications to coronary perfusion. *Front Physiol.* (2017) 8:824. doi: 10.3389/fphys.2017.00824

89. Onorati F, Santini F, Dandale R, Ucci G, Pechlivanidis K, Menon T, et al. "Polarizing" microplegia improves cardiac cycle efficiency after CABG for unstable angina. *Int J Cardiol.* (2013) 167:2739–46. doi: 10.1016/j.ijcard.2012.06.099

90. Granfeldt A, Letson HL, Hyldebrandt JA, Wang ER, Salcedo PA, Nielson TK, et al. Small-volume 7.5% NaCl adenosine, lidocaine and Mg2+ has multiple benefits during hypotensive and blood resuscitation in the pig following severe blood loss: rat to pig translation. *Crit Care Med.* (2014) 42:e329–44. doi: 10.1097/CCM.0000000000000225

91. Ye Z, Coutinho T, Pellikka PA, Villarraga HR, Borlaug BA, Kullo IJ. Associations of alterations in pulsatile arterial load with left ventricular longitudinal strain. *Am J Hypertens.* (2015) 28:1325–31. doi: 10.1093/ajh/hpv039

92. Antonini-Canterin F, Poli S, Vriz O, Pavan D, Bello VD, Nicolosi GL. The ventricular-arterial coupling: from basic pathophysiology to clinical application in the echocardiography laboratory. *J Cardiovasc Echogr.* (2013) 23:91–5. doi: 10.4103/2211-4122.127408

93. Ky B, French B, May Khan A, Plappert T, Wang A, Chirinos JA, et al. Ventricular-arterial coupling, remodeling, and prognosis in chronic heart failure. *J Am Coll Cardiol.* (2013) 62:1165–72. doi: 10.1016/j.jacc.2013.03.085

94. Howard BM, Kornblith LZ, Christie SA, Conroy AS, Nelson MF, Campion EM, et al. Characterizing the gut microbiome in trauma: significant changes in microbial diversity occur early after severe injury. *Trauma Surg Acute Care Open.* (2017) 2:e000108. doi: 10.1136/tsaco-2017-000108

95. Wiel E, Vallet B, ten Cate H. The endothelium in intensive care. *Crit Care Clin.* (2005) 21:403–16. doi: 10.1016/j.ccc.2005.03.001

96. Bennett HS. Morphological aspects of extracellular polysaccharides. *J Histochem Cytochem.* (1963) 11:14–23. doi: 10.1177/11.1.14

97. Luft JH. The structure and properties of the cell surface coat. *Int Rev Cytol.* (1976) 45:291–382. doi: 10.1016/S0074-7696(08)60081-9

98. Aird WC. Spatial and temporal dynamics of the endothelium. *J Thromb Haemost.* (2005) 3:1392–406. doi: 10.1111/j.1538-7836.2005.01328.x

99. Johansson PI, Henriksen HH, Stensballe J, Gybel-Brask M, Cardenas JC, Baer LA, et al. Traumatic endotheliopathy: a prospective observational study of 424 severely injured patients. *Ann Surg.* (2017) 265:597–603. doi: 10.1097/SLA.0000000000001751

100. Gonzalez Rodriguez E, Ostrowski SR, Cardenas JC, Baer LA, Tomasek JS, Henriksen HH, et al. Syndecan-1: a quantitative marker for the endotheliopathy of trauma. *J Am Coll Surg.* (2017) 225:419–27. doi: 10.1016/j.jamcollsurg.2017.05.012

101. Halbgebauer R, Braun CK, Denk S, Mayer B, Cinelli P, Radermacher P, et al. Hemorrhagic shock drives glycocalyx, barrier and organ dysfunction early after polytrauma. *J Crit Care.* (2018) 44:229–37. doi: 10.1016/j.jcrc.2017.11.025

102. Richards JE, Samet RE, Grissom TE. Scratching the surface: endothelial damage in traumatic hemorrhagic shock. *Adv Anesth.* (2021) 39:35–51. doi: 10.1016/j.aan.2021.07.003

103. Tiruppathi C, Minshall RD, Paria BC, Vogel SM, Malik AB. Role of Ca2+ signaling in the regulation of endothelial permeability. *Vasc Pharm.* (2003) 39:173–85. doi: 10.1016/S1537-1891(03)00007-7

104. Reitsma S, Slaaf DW, Vink H, Van Zandvoort MA, Oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Arch.* (2007) 454:345–59. doi: 10.1007/s00424-007-0212-8

105. Chappell D, Westphal M, Jacob M. The impact of the glycocalyx on microcirculatory oxygen distribution in critical illness. *Curr Opin Anaesthesiol.* (2009) 22:155–62. doi: 10.1097/ACO.0b013e328328d1b6

106. Biddle C. Like a slippery fish, a little slime is a good thing: the glycocalyx revealed. *AANA J.* (2013) 81:473–80.

107. Aditianingsih D, George YWH. Guiding principles of fluid and volume therapy. *Best Pract Res Clin Anaesthesiol.* (2014) 28:249–60. doi: 10.1016/j.bpa.2014.07.002

108. Gall LS, Vulliamy P, Gillespie S, Jones TF, Pierre RSJ, Breukers SE, et al. The S100A10 pathway mediates an occult hyperfibrinolytic subtype in trauma patients. *Ann Surg.* (2019) 269:1184–91. doi: 10.1097/SLA.0000000000002733

109. Moore EE, Moore HB, Kornblith LZ, Neal MD, Hoffman M, Mutch NJ, et al. Trauma-induced coagulopathy. *Nat Rev Dis Primers.* (2021) 7:30. doi: 10.1038/s41572-021-00264-3

110. Krocker JD, Lee KH, Henriksen HH, Wang Y-WW, Schoof EM, Karvelsson ST, et al. Exploratory investigation of the plasma proteome associated with the endotheliopathy of trauma. *Int J Mol Sci.* (2022) 23:6213. doi: 10.3390/ijms23116213

111. Fu BM, Tarbell JM. Mechano-sensing and transduction by endothelial surface glycocalyx: composition, structure, and function. *Wiley Interdiscip Rev Syst Biol Med.* (2013) 5:381–90. doi: 10.1002/wsbm.1211

112. van Hinsbergh VW. Endothelium–role in regulation of coagulation and inflammation. *Semin Immunopathol.* (2012) 34:93–106. doi: 10.1007/s00281-011-0285-5

113. Schott U, Solomon C, Fries D, Bentzer P. The endothelial glycocalyx and its disruption, protection and regeneration: a narrative review. *Scand J Trauma Resusc Emerg Med.* (2016) 24:48. doi: 10.1186/s13049-016-0239-y

114. Hahn RG. Water content of the endothelial glycocalyx layer estimated by volume kinetic analysis. *Intensive Care Med Exp.* (2020) 8:29. doi: 10.1186/s40635-020-00317-z

115. Moore KH, Murphy HA, George EM. The glycocalyx: a central regulator of vascular function. *Am J Physiol Regul Integr Comp Physiol.* (2021) 320:R508–18. doi: 10.1152/ajpregu.00340.2020

116. Luft JH. Fine structures of capillary and endocapillary layer as revealed by ruthenium red. *Fed Proc.* (1966) 25:1773–83.

117. Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev.* (2004) 84:869–901. doi: 10.1152/physrev.00035.2003

118. Woodcock TE, Woodcock TM. Revised Starling equation and the glycocalyx model of transvascular fluid exchange: an improved paradigm for prescribing intravenous fluid therapy. *Br J Anaesth.* (2012) 108:384–94. doi: 10.1093/bja/aer515

119. Chappell D, Jacob M. Role of the glycocalyx in fluid management: small things matter. *Best Pract Res Clin Anaesthesiol.* (2014) 28:227–34. doi: 10.1016/j.bpa.2014.06.003

120. Brohi K, Cohen MJ, Ganter MT, Matthay MA, Mackersie RC, Pittet JF. Acute traumatic coagulopathy: initiated by hypoperfusion: modulated through the protein C pathway? *Ann Surg.* (2007) 245:812–8. doi: 10.1097/01.sla.0000256862.79374.31

121. Holcomb JB, Jenkins D, Rhee P, Johannigman J, Mahoney PF, Mehta S, et al. Damage control resuscitation: directly addressing the early coagulopathy of trauma. *J Trauma.* (2007) 62:307–10. doi: 10.1097/TA.0b013e3180324124

122. Moore HB, Moore EE, Gonzalez E, Chapman MP, Chin TL, Silliman CC, et al. Hyperfibrinolysis, physiologic fibrinolysis, and fibrinolysis shutdown: the spectrum of postinjury fibrinolysis and relevance to antifibrinolytic therapy. *J Trauma Acute Care Surg.* (2014) 77:811–7. doi: 10.1097/TA.0000000000000341

123. Dobson GP, Letson HL, Sharma R, Sheppard F, Cap AP. Mechanisms of early traumatic-induced coagulopathy (TIC): the clot thickens or not? *J Trauma Acute Care Surg.* (2015) 79:301–9. doi: 10.1097/TA.0000000000000729

124. D'elia RV, Harrison K, Oyston PC, Lukaszewski RA, Clark GC. Targeting the "cytokine storm" for therapeutic benefit. *Clin Vaccine Immunol.* (2013) 20:319–27. doi: 10.1128/CVI.00636-12

125. Corps KN, Roth TL, McGavern DB. Inflammation and neuroprotection in traumatic brain injury. *JAMA.* (2015) 72:355–62. doi: 10.1001/jamaneurol.2014.3558

126. Mira JC, Brakenridge SC, Moldawer LL, Moore FA. Persistent inflammation, immunosuppression and catabolism syndrome. *Crit Care Clin.* (2017) 33:245–58. doi: 10.1016/j.ccc.2016.12.001

127. Qasim Z, Butler FK, Holcomb JB, Kotora JG, Eastridge BJ, Brohi K, et al. Selective prehospital advanced resuscitative care – developing a strategy to prevent prehospital deaths from noncompressible torso hemorrhage. *Shock.* (2022) 57:7–14. doi: 10.1097/SHK.0000000000001816

128. Moore EE, Moore FA, Harken AH, Johnson JL, Ciesla D, Banerjee A. The two-event construct of postinjury multiple organ failure. *Shock.* (2005) 24(Suppl. 1):71–4. doi: 10.1097/01.shk.0000191336.01036.fe

129. Dewar DC, Butcher NE, King KL, Balogh ZJ. Post injury multiple organ failure. *Trauma.* (2011) 13:81–91. doi: 10.1177/1460408610386657

130. Zeng Y, Tarbell JM. The adaptive remodeling of endothelial glycocalyx in response to fluid shear stress. *PLoS One.* (2014) 9:e86249. doi: 10.1371/journal.pone.0086249

131. Hahn RG, Patel V, Dull RO. Human glycocalyx shedding: systematic review and critical appraisal. *Acta Anaesthesiol Scand.* (2021) 65:590–606. doi: 10.1111/aas.13797

132. Dobson GP. On being the right size: heart design, mitochondrial efficiency, and lifespan potential. *Clin Exp Pharm Physiol.* (2003) 30:590–7. doi: 10.1046/j.1440-1681.2003.03876.x

133. Kluge MA, Fetterman JL, Vita JA. Mitochondria and endothelial function. *Circ Res.* (2013) 112:1171–88. doi: 10.1161/CIRCRESAHA.111.300233

134. Bhatti JS, Bhatti GK, Reddy PH. Mitochondrial dysfunction and oxidative stress in metabolic disorders – a step towards mitochondria based therapeutic strategies. *Biochim Biophys Acta Mol Basis Dis.* (2017) 1863:1066–77. doi: 10.1016/j.bbadis.2016.11.010

135. Berry BJ, Trewin AJ, Amitrano AM, Kim M, Wojtovich AP. Use the protonmotive force: mitochondrial uncoupling and reactive oxygen species. *J Mol Biol.* (2018) 430:3873–91. doi: 10.1016/j.jmb.2018.03.025

136. Canyon SJ, Dobson GP. The effect of adenosine and lidocaine infusion on myocardial high energy phosphates and pH during regional ischemia in the rat model in vivo. *Can J Physiol Pharmacol.* (2006) 84:903–12. doi: 10.1139/y06-035

137. Argaud L, Gateau-Roesch O, Muntean D, Chalabreysse L, Loufouat J, Robert D, et al. Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. *J Mol Cell Cardiol.* (2005) 38:367–74. doi: 10.1016/j.yjmcc.2004.12.001

138. Baines CP. The mitochondrial permeability transition pore as a target of cardioprotective signaling. *Am J Physiol Heart Circ Physiol.* (2007) 293:H903–4. doi: 10.1152/ajpheart.00575.2007

139. West AP, Shadel GS, Ghosh S. Mitochondria in innate immune responses. *Nat Rev Immunol.* (2011) 11:389–402. doi: 10.1038/nri2975

140. Dehne N, Brüne B. Sensors, transmitters, and targets in mitochondrial oxygen shortage-a hypoxia-inducible factor relay story. *Antioxid Redox Signal.* (2014) 20:339–52. doi: 10.1089/ars.2012.4776

141. Kunkel GH, Chaturvedi P, Tyagi SC. Mitochondrial pathways to cardiac recovery: TFAM. *Heart Fail Rev.* (2016) 21:499–517. doi: 10.1007/s10741-016-9561-8

142. Cherry AD, Piantadosi CA. Regulation of mitochondrial biogenesis and its intersection with inflammatory responses. *Antioxid Redox Signal.* (2015) 22:965–76. doi: 10.1089/ars.2014.6200

143. Zhao Z, Wang M, Tian Y, Hilton T, Salsbery B, Zhou EZ, et al. Cardiolipin-mediated procoagulant activity of mitochondria contributes to traumatic brain injury–associated coagulopathy in mice. *Blood.* (2016) 127:2763–72. doi: 10.1182/blood-2015-12-688838

144. Cap AP, Hunt B. Acute traumatic coagulopathy. *Curr Opin Crit Care.* (2014) 20:638–45. doi: 10.1097/MCC.0000000000000158

145. Boudreau LH, Duchez AC, Cloutier N, Soulet D, Martin N, Bollinger J, et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood.* (2014) 124:2173–83. doi: 10.1182/blood-2014-05-573543

146. Riedel B, Rafat N, Browne K, Burbury K, Schier R. Perioperative implications of vascular endothelial dysfunction: current understanding of this critical sensor-effector organ. *Curr Anesthesiol Rep.* (2013) 3:151–61. doi: 10.1007/s40140-013-0024-7

147. Johansson PI, Stensballe J, Ostrowski SR. Shock induced endotheliopathy (SHINE) in acute critical illness - a unifying pathophysiologic mechanism. *Crit Care.* (2017) 21:25. doi: 10.1186/s13054-017-1605-5

148. Henriksen HH, Mcgarrity S, Sigurethardottir RS, Nemkov T, D'alessandro A, Palsson BO, et al. Metabolic systems analysis of shock-induced endotheliopathy (SHINE) in trauma: a new research paradigm. *Ann Surg.* (2020) 272:1140–8. doi: 10.1097/SLA.0000000000003307

149. Hart DA. Human heterogeneity and survival of the species: how did it arise and being sustained?—the conundrum facing researchers. *J Biomed Sci Eng.* (2021) 14:212–21. doi: 10.4236/jbise.2021.145018

150. Tsukamoto T, Pepe HC. Animal models for trauma research: what are the options? *Shock.* (2009) 31:3–10. doi: 10.1097/SHK.0b013e31817fdabf

151. Dobson GP, Letson HL, Biros E, Morris JL. Specific pathogen-free (SPF) animal status as a variable in biomedical research: have we come full circle? *EBioMedicine (Lancet).* (2019) 41:42–3. doi: 10.1016/j.ebiom.2019.02.038

152. Letson HL, Morris JL, Biros E, Dobson GP. Conventional and specific-pathogen free rats respond differently to anesthesia and surgical trauma. *Sci Rep.* (2019) 9:9399. doi: 10.1038/s41598-019-45871-z

153. Dobson GP, Morris J, Biros E, Letson HL. Specific pathogen-free animals for civilian and military trauma: a cautionary note in the translation of new drug therapies. *Shock.* (2020) 54:232–6. doi: 10.1097/SHK.0000000000001495

154. Foster HL. Housing of disease-free vertebrates. *Ann N Y Acad Sci.* (1959) 78:80–8. doi: 10.1111/j.1749-6632.1959.tb53096.x

155. Masopust D, Sivula CP, Jameson SC. Of mice, dirty mice and men: using mice to understand human immunology. *J Immunol.* (2017) 199:383–8. doi: 10.4049/jimmunol.1700453

156. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature.* (2016) 532:512–6. doi: 10.1038/nature17655

157. Schubert KA, Boerema AS, Vaanholt LM, De Boer SF, Strijkstra AM, Daan S. Daily torpor in mice: high foraging costs trigger energy-saving hypothermia. *Biol Lett.* (2010) 6:132–5. doi: 10.1098/rsbl.2009.0569

158. Dobson GP. The August Krogh principle: seeking unity in diversity. *Shock.* (2014) 42:480. doi: 10.1097/SHK.0000000000000229

159. Bouma HR, Carey HV, Kroese GM. Hibernation: the immune system at rest? *J Leukocyte Biol.* (2010) 88:619–24. doi: 10.1189/jlb.0310174

160. Franconi F, Campesi I, Colombo D, Antonini P. Sex-gender variable: methodological recommendations for increasing scientific value of clinical studies. *Cells.* (2019) 8:476. doi: 10.3390/cells8050476

161. Gölz C, Kirchhoff FP, Westerhorstmann J, Schmidt M, Hirnet T, Rune GM, et al. Sex hormones modulate pathogenic processes in experimental traumatic brain injury. *J Neurochem.* (2019). [Epub ahead of print]. doi: 10.1111/jnc.14678

162. Gupte R, Brooks WM, Vukas RR, Pierce JD, Harris JL. Sex differences in traumatic brain injury: what we know and what we should know. *J Neurotrauma.* (2019). [Epub ahead of print]. doi: 10.1089/neu.2018.6171

163. Choudhry MA, Bland KI, Chaudry IH. Trauma and immune response–effect of gender differences. *Injury.* (2007) 38:1382–91. doi: 10.1016/j.injury.2007.09.027

164. Chaudry IH, Bland KI. Cellular mechanisms of injury after major trauma. *Br J Surg.* (2009) 96:1097–8. doi: 10.1002/bjs.6697

165. Shoemaker WC, Beez M. Pathophysiology, monitoring, and therapy of shock with organ failure. *Appl Cardiopul Pathophysiol.* (2010) 14:5–15.

166. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev.* (2018) 281:8–27. doi: 10.1111/imr.12621

167. Mehta P, Mcauley DF, Brown M, Sanchez E, Tattersall RS, Mansen JJ. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet.* (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0

168. Moore TJ, Zhang H, Anderson G, Alexander GC. Estimated costs of pivotal trials for novel therapeutic agents approved by the US food and drug administration, 2015-2016. *JAMA Intern Med.* (2018) 178:1451–7. doi: 10.1001/jamainternmed.2018.3931

169. Moore FD. The growth of surgical biology. *Ann Surg.* (1953) 138:807–22. doi: 10.1097/00000658-195311000-00023

170. Letson HL, Granfeldt A, Jensen TH, Mattson TH, Dobson GP. ALM supports a high flow, hypotensive, vasodilatory state with improved O2 delivery and cerebral protection in a pig model of non-compressible hemorrhage. *J Surg Res.* (2020) 253:127–38. doi: 10.1016/j.jss.2020.03.048

171. Letson HL, Dobson GP. 3.0% NaCl adenosine, lidocaine, Mg2+ (ALM) bolus and 4 hours 'drip' infusion reduces non-compressible hemorrhage by 60% in a rat model. *J Trauma Acute Care Surg.* (2017) 82:1063–72. doi: 10.1097/TA.0000000000001454

172. Letson HL, Dobson GP. Adenosine, lidocaine and Mg2+ (ALM) fluid therapy attenuates systemic inflammation, platelet dysfunction and coagulopathy after non-compressible truncal hemorrhage. *PLos One.* (2017) 12:e0188144. doi: 10.1371/journal.pone.0188144

173. Letson HL, Dobson GP. Adenosine, lidocaine and Mg2+ (ALM) resuscitation fluid protects against experimental traumatic brain injury. *J Trauma Acute Care Surg.* (2018) 84:908–16. doi: 10.1097/TA.0000000000001874

174. Letson HL, Morris JL, Biros E, Dobson GP. ALM fluid therapy leads to 72 hr survival after hemorrhagic shock: a model for studying differential gene expression and extending biological time. *J Trauma Acute Care Surg.* (2019) 87:606–13. doi: 10.1097/TA.0000000000002397

175. Griffin MJ, Letson HL, Dobson GP. Adenosine, lidocaine and Mg2+ (ALM) induces a reversible hypotensive state, reduces lung edema and prevents coagulopathy in the rat model of polymicrobial sepsis. *J Trauma Acute Care Surg.* (2014) 77:471–8. doi: 10.1097/TA.0000000000000361

176. Griffin MJ, Letson HL, Dobson GP. Small-volume adenosine, lidocaine and Mg2+ (ALM) 4 hour infusion leads to 88% survival after 6 days of experimental sepsis in the rat without antibiotics. *Clin Vaccine Immunol.* (2016) 23:863–72. doi: 10.1128/CVI.00390-16

177. Granfeldt A, Letson HL, Dobson GP, Shi W, Vinten-Johansen J, Tonnesen E. Cardioprotective and anti-inflammatory effects of treatment with adenosine, lidocaine and Mg2+ in a porcine model of endotoxemia. *Circulation.* (2013) 18:682–70.

178. Letson HL, Biros E, Morris JL, Dobson GP. ALM fluid therapy shifts sympathetic hyperactivity to parasympathetic dominance in the rat model of non-compressible hemorrhagic shock. *Shock.* (2022) 57:264–73. doi: 10.1097/SHK.0000000000001886

179. Letson HL, Dobson GP. Differential contributions of platelets and fibrinogen to early coagulopathy in a rat model of hemorrhagic shock. *Thromb Res.* (2016) 141:58–61. doi: 10.1016/j.thromres.2016.03.007

180. Torres Filho IP, Torres LN, Salgado C, Dubick MA. Novel adjunct drugs reverse endothelial glycocalyx damage after hemorrhagic shock in rats. *Shock.* (2017) 48:583–9. doi: 10.1097/SHK.0000000000000895

181. Seyhan AA. Lost in translation: the valley of death across preclinical and clinical divide – identification of problems and overcoming obstacles. *Transl Med Commun.* (2019) 4:18. doi: 10.1186/s41231-019-0050-7

182. Downing NS, Shah ND, Aminawung JA, Pease AM, Zeitoun JD, Krumholz HM, et al. Postmarket safety events among novel therapeutics approved by the US food and drug administration between 2001 and 2010. *JAMA.* (2017) 317:1854–63. doi: 10.1001/jama.2017.5150

183. Huston JM, Tracey KJ. The pulse of inflammation: heart rate variability, the cholinergic anti-inflammatory pathway and implications for therapy. *J Intern Med.* (2011) 269:45–53. doi: 10.1111/j.1365-2796.2010.02321.x

184. Matteoli G, Boeckxstaens GE. The vagal innervation of the gut and immune homeostasis. *Gut.* (2013) 62:1214–22. doi: 10.1136/gutjnl-2012-302550

185. Dobson GP. Addressing the global burden of sepsis: importance of a systems-based approach. *Crit Care Med.* (2014) 42:e797–8. doi: 10.1097/CCM.0000000000000595

186. Chappell D, Jacob M, Hofmann-Kiefer K, Bruegger D, Rehm M, Conzen P, et al. Hydrocortisone preserves the vascular barrier by protecting the endothelial glycocalyx. *Anesthesiology.* (2007) 107:776–84. doi: 10.1097/01.anes.0000286984.39328.96

187. Pries AR, Secomb TW, Gaehtgens P. The endothelial surface layer. *Pflügers Arch.* (2000) 440:653–66. doi: 10.1007/s004240000307

188. Pillinger NL, Kam PCA. Endothelial glycocalyx: basic science and clinical implications. *Anaesth Intensive Care.* (2017) 45:3. doi: 10.1177/0310057X1704500305

189. Vink H, Duling BR. Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries. *Circ Res.* (1996) 79:581–9. doi: 10.1161/01.RES.79.3.581

190. Squire JM, Chew M, Nneji G, Neal C, Barry J, Michel C. Quasi-periodic substructure in the microvessel endothelial glycocalyx: a possible explanation for molecular filtering? *J Struct Biol.* (2001) 136:239–55. doi: 10.1006/jsbi.2002.4441

191. Weinbaum S, Zhang X, Han Y, Vink H, Cowin SC. Mechanotransduction and flow across the endothelial glycocalyx. *Proc Natl Acad Sci USA.* (2003) 100:7988–95. doi: 10.1073/pnas.1332808100

192. Arkill KP, Knupp C, Michel CC, Neal CR, Qvortrup K, Rostgaard J, et al. Similar endothelial glycocalyx structures in microvessels from a range of mammalian tissues: evidence for a common filtering mechanism? *Biophys J.* (2011) 101:1046–56. doi: 10.1016/j.bpj.2011.07.036

193. Arkill KP. A reinterpretation of evidence for the endothelial glycocalyx filtration structure. *Front Cell Dev Biol.* (2021) 9:734661. doi: 10.3389/fcell.2021.734661

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Effect of scalp nerve block with ropivacaine on postoperative pain in pediatric patients undergoing craniotomy: A randomized controlled trial

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Background: Scalp nerve block (SNB) is widely used for postoperative pain control, intraoperative hemodynamic control, and opioid-sparing in adult craniotomies. However, there are few studies of SNB in pediatric patients undergoing craniotomy. In the present study, we aimed to investigate the effect of SNB on postoperative pain, intraoperative hemodynamic stability, and narcotic consumption in pediatric craniotomy under general anesthesia.

Methods: This trial is a single-center, prospective, randomized, and double-blind study. A total of 50 children aged between 2 and 12 years who are undergoing elective brain tumor surgery will be randomly allocated in a 1:1 ratio to receive either 0.2% ropivacaine for SNB (group SNB, intervention group, $n = 25$) or the same volume of saline (group Ctrl, control group, $n = 25$). The primary outcome was to assess the score of postoperative pain intensity at time 1, 4, 8, 12, 24, and 48 h postoperatively using the FLACC score method. Secondary outcomes were to record intraoperative hemodynamic variables (MAP and HR) during skull-pin fixation, skin incision and end of skin closure, intraoperative total consumption of remifentanil and propofol, postoperative opioid consumption, and the incidence of postoperative nausea and vomiting.

Results: Fifty patients were analyzed ($n = 25$ in SNB group; $n = 25$ in control group). Compared to the control group, postoperative pain intensity was significantly relieved in the SNB group up to 8 h post-operatively. In addition, SNB provided good intraoperative hemodynamic stability, reduced intraoperative overall propofol and remifentanil consumption rate, and postoperative fentanyl consumption compared to the control group. However, the incidence of postoperative nausea and vomiting was not different between SNB and the control group.

Conclusions: In pediatric craniotomies, SNB with 0.2% ropivacaine provides adequate postoperative pain control and good intraoperative hemodynamic stability during noxious events compared to the control group.

Clinical trial registration: Chinese Clinical Trial Registry [No: ChiCTR2100050594], Prospective registration.

KEYWORDS

scalp nerve block, postoperative pain, pediatric craniotomy, hemodynamic stability, ropivacaine

Introduction

For a long time, postoperative pain has not received sufficient attention in pediatric craniotomy patients (1, 2). On the one hand, young children, particularly infants, cannot properly describe their pain and it is sometimes difficult to distinguish painful or emotional responses in young children, so the pain after surgery is often inappropriately considered associated with emotional responses (3–5). On the other hand, many neurosurgeons fear that the use of opioids may interfere with neurologic examination (6). Moreover, opioid-induced side effects, such as nausea, vomiting, and especially respiratory depression may lead to disastrous results (7, 8). Therefore, prevention and treatment of postoperative pain in pediatric craniotomy patients is still a challenging clinical problem.

Inadequate postoperative pain control in children following craniotomy may cause severe consequences such as agitation, intracranial hypertension, and postoperative hemorrhage, which may increase morbidity and mortality (9, 10). Given the side effects of opioids, it is necessary to minimize reliance on opioid analgesia in craniotomy patients. Multimodal analgesia which combines low doses of systemic analgesics with local anesthetics for scalp infiltration or regional scalp nerve block has been proposed to prevent postoperative pain in adult craniotomy patients (11–13). However, the optimal postoperative analgesic management for pediatric craniotomy patients remains elusive.

SNB has been widely used as the principal anesthetic in awake craniotomies or served as an adjuvant method to general anesthesia in adult supratentorial craniotomies (13, 14). SNB can attenuate postoperative pain, decrease opioid and narcotic agent consumption and prevent intraoperative hemodynamic responses to noxious stimulation (12, 15). However, over the past decade, the studies of SNB mostly focused on adult craniotomy patients, and there are few studies of SNB in pediatric patients undergoing craniotomy.

The purpose of our study was to determine if SNB with ropivacaine reduce postoperative pain score in pediatric patients undergoing craniotomy. Our primary hypothesis was that SNB before surgery would improve postoperative pain control, and the secondary hypothesis was that SNB would provide intraoperative hemodynamic stability, and reduce perioperative opioid and narcotic agent consumption.

Materials and methods

Participants

This prospective, randomized, placebo-controlled, double-blind study was approved by the Institutional Ethical Committee of Xinhua Hospital, Medical School, Shanghai Jiaotong University, and was registered in the Chinese Clinical Trial Registry (registration number: ChiCTR2100050594). Written informed consent was obtained prior to study enrollment. Pediatric patients aged 2–12 years presenting for elective supratentorial craniotomies will be recruited from Xinhua Hospital, Medical School, Shanghai Jiaotong University from July 2020 to October 2021.

Patient recruitment and assignment to groups

Sixty pediatric patients aged 2–12 years with American Society of Anesthesiologists (ASA) physical status I or II scheduled to undergo elective supratentorial brain tumor surgery and to receive general anesthesia were prospectively screened for possible inclusion. The exclusion criteria included: (1) Pediatric patients aged>12 or <2 years; (2) Children with mental disorders; (3) Children whose authorized surrogates are unwilling to participate in the study; (4) Children with severe diseases or cardiac insufficiency; (5) Emergency craniotomies; (6) Children with severe kidney or liver diseases; (7) Children with severe coagulation disorders; (8) Children who cannot be weaned from endotracheal intubation following surgery; (9) Children with a history of allergy to opioids or other anesthetics; (10) Children with a history of analgesic substance abuse.

Randomization

After meeting the eligibility criteria and signing the informed consent to participate in the study, Patients were randomized in a 1:1 ratio into two groups using computer-generated randomized numbers. Groups differed according to the performance of a SNB with either 0.2% ropivacaine (Group SNB, intervention group) or the same volume of saline (Group Ctrl, control group). The SNB was performed by the attending

anesthesiologists who were blinded to the agents which has been prepared by a nurse non-involved in the study in identical syringes. Patients, children's guardians, anesthesiologists, and neurosurgeons were blind to group assignment. The outcome assessors who were blinded to randomization and did not participate in anesthetic management and data recording or analysis. All of them received the use of evaluation scale training and recorded pain scores and complications postoperatively.

Anesthesia and analgesia

All patients received standardized anesthetic monitoring including non-invasive blood pressure (BP), heart rate (HR), pulse oximetry saturation (SpO_2), invasive arterial pressure, end-tidal carbon dioxide partial pressure ($P_{ET}CO_2$), and anesthesia gas monitoring. General anesthesia was induced with intravenous atropine (0.01 mg/kg), midazolam (0.1 mg/kg), propofol (2–3 mg/kg), fentanyl (1–2 μg/kg), and cisatracurium (0.2 mg/kg). In addition, dexamethasone (0.2 mg/kg to a maximum dose of 5 mg) was given after anesthesia induction. Following anesthesia induction, continuous invasive blood pressure monitoring was established through a radial arterial catheterization, and a jugular vein catheter was inserted. Mechanical ventilation was performed in all patients (50% air in oxygen) to maintain an O_2 saturation of >98% and an end-expiratory CO_2 of 30–35 mmHg.

Anesthesia was maintained with 0.5 MAC (minimum alveolar concentration) sevoflurane at an inhalational concentration of 1–1.5% and an intravenous infusion with propofol 3–6 mg/kg/h and remifentanil 0.05–0.25 μg/kg/min. Cisatracurium was administered intraoperatively as needed and was reversed at the end of the surgery with neostigmine (0.04 mg/kg) and atropine (0.015 mg/kg). Mean arterial blood pressure and heart rate were maintained within 20% of baseline measures. Remifentanil was adjusted by steps of 0.05–0.1 μg/kg/min if intraoperative MAP or HR over or below 20% of baseline values. If the adjustment failed, propofol was adjusted by steps of 0.5–1 mg/kg/h until stabilization within the 20% range. Baseline values were defined as 3-min averaged values immediately before the performance of SNB. Fentanyl (1 μg/kg) was given 20 min before the end of surgery. Additionally, all patients received tropisetron hydrochloride (0.2 mg/kg to a maximum dose of 5 mg) as an emesis prophylaxis 30 min before the end of surgery. No other intraoperative adjuvant analgesics were given.

After surgery, mechanical ventilation was discontinued, and the patient was ready for tracheal extubation when consciousness and sufficient spontaneous breathing recovered and hemodynamics was stable. All the patients were equipped with nurse-controlled intravenous analgesia (NCIA) device containing a fentanyl solution (15 μg/kg fentanyl, 0.2 mg/kg dexamethasone to a maximum dose of 5 mg and 0.2 mg/kg tropisetron hydrochloride to a maximum dose of 5 mg, the total volume was diluted to 100 ml with 0.9% normal saline). The NCIA device was connected to the IV line before the end of surgery (parameters: background dose-rate: 2 ml/h; boluses: 2 ml, 15 min refractory time). If the score of postoperative pain <3, the Acute Pain Services who were blinded to study treatment allocation will consider discontinuing the NCIA. The care physicians or nurses had been informed of the adequate NCIA use the day before surgery and blinded to randomization.

Regional scalp block

Bilateral scalp nerve blocks were performed by the anesthesiologist before skull-pin fixation and after induction of general anesthesia. The block was performed by the attending anesthesiologists who were blinded to the agents. The anesthetic solution was prepared by a nurse, who was not participating in patient anesthetic management and data recording or analysis, according to the computer-generated randomization list. For SNB group patients, the syringe was containing 20 mL of 0.2% ropivacaine. For control group patients, the syringe was containing 20 mL of 0.9% normal saline.

Bilateral scalp nerve blocks were done at several points over the scalp. (1) Bilateral supraorbital nerve (1–2 ml); (2) Bilateral supratrochlear nerves (1–2 ml); (3) Bilateral zygomatic temporal nerve (1–2 ml); (4) Bilateral auriculotemporal nerve (1–2 ml); (5) Bilateral greater occipital nerve (2–3 ml); (6) Bilateral lesser occipital nerve (2–3 ml).

Outcomes

The primary measured outcome was the score of postoperative pain which was assessed at time 1, 4, 8, 12, 24, and 48 h postoperatively. In our study, postoperative pain was evaluated by the Faces, Legs, Activity, Cry, and Consolability Scale (FLACC, 0–10 scores) following pediatric surgery (16). Secondary outcomes included: the intraoperative hemodynamic variables (MAP and HR) during baseline, skull-pin fixation, skin incision, and end of skin closure; The overall consumption rate of propofol (mg/kg/h) and remifentanil (μg/kg/min); The total amount of fentanyl consumption and the total number of compressions of NCIA device within 48 h postoperatively; The incidence of postoperative nausea and vomiting (PONV) and complications both from local anesthetic and the nerve block were also assessed.

Sample size calculation

The sample size was calculated based on data available in published studies and our clinical experience at the study centers

using the G* Power software (version 3.1.9.2, Franz Faul, Kiel University, Germany) (9, 10). According to previous studies, the incidence of moderate postoperative pain in children is ∼50% in pediatric craniotomy patients (10). Assuming a two-sided α value of 0.05 and a β value of 0.2, we estimated that 21 patients in each group would be required to detect a 1.8-point difference in the FLACC score. Considering a 20% dropout rate, 27 patients were recruited in each group. Therefore, the total sample size was 54 patients in this study.

Statistical analyses

SPSS version 22.0 (International Business Machines Inc.) was used for data analysis. All analyses were conducted using the modified intention-to-treat principle. All data are either presented as median (IQR) or mean (SD), or as frequency and percentage (%), respectively. Categorical variables (sex, ASA physical status) were presented as frequencies and percentages. The chi-square test was used for comparing proportions, the Shapiro–Wilk test was used to test the normal distribution of continuous variables. Student's *t*-test was used to compare normally distributed outcome variables between the two randomized groups. MAP and HR were compared using 2-way mixed-design analysis of variance (ANOVA) and Tukey's multiple comparisons test for *post-hoc* comparisons. FLACC score was compared using the Mann-Whitney U test for non-parametric variables (17, 18). Two-tailed analyses were conducted, and a $p \leq 0.05$ was considered statistically significant.

Results

Demographic characteristics

As in Figure 1, a total of 60 patients who underwent major craniotomy were enrolled in the study, of whom 54 patients were randomized into group control and group SNB, with 27 patients in the group control and 27 patients in the group SNB (Figure 1). Among them, two patients in the group control and two patients in the group SNB were excluded from analysis for delaying extubation postoperatively, the outcomes in those patients could not be assessed. Ultimately, 50 patients were completed in this study. There were no significant differences between group control and group SNB in demographic characteristics of patients and operative variables, including sex, age, weight, ASA status, the duration of operation and anesthesia, recovery time from anesthesia (Table 1, $P > 0.05$).

Primary outcome

Pain intensity was evaluated at 1, 4, 8, 12, 24, and 48 h after surgery. FLACC pain scores were significantly decreased in the SNB group compared to the control group at postoperative 1, 4, and 8 h (Table 2, $P < 0.05$). The pain intensity gradually decreased after postoperative 8 h. However, there was no significant difference at postoperative 12, 24, and 48 h between the two groups (Table 2, $P > 0.05$).

Secondary outcomes

The MAP and HR were significantly higher in group control than in group SNB at the time of skull-pin fixation and skin incision (Figure 2, $P < 0.05$). However, there was no significant difference at the time of end of skin closure between two groups (Figure 2, $P > 0.05$).

The overall intraoperative consumption rate of propofol and remifentanil was significantly higher in group control than in group SNB (Figure 3, $P < 0.001$). The total amount of medicine used in the postoperative analgesia pump was calculated. The total amount of fentanyl consumption and the total number of compressions of NCIA were significantly higher in group control than in group SNB during postoperative 48 h (Figure 4, $P < 0.001$).

There was no statistical difference in the incidence of PONV between the two groups (Table 3, $P > 0.05$). In our study, no adverse effects such as post-operative scalp infection, hematoma, or local anesthetic toxicity were observed in patients during the study period (Table 3, $P > 0.05$).

Discussion

In the present study, we found that bilateral SNB with ropivacaine improved postoperative pain control for up to 8 h compared to the control group in pediatric craniotomy patients. Furthermore, bilateral SNB provided good intraoperative hemodynamic stability and reduced propofol and opioid consumption compared to the control group.

Regional anesthesia is mainly used to provide postoperative analgesia. Preemptive analgesia with local anesthetics is an effective method of postoperative pain control (13). The theory was that preemptive analgesia prior to surgery can prevent central sensitization caused by noxious stimuli and inflammation (19, 20). Previous studies demonstrated that preoperative SNB has beneficial to postoperative pain in adult craniotomy patients (12, 13). In our study, we found that SNB with 0.2% ropivacaine provided preferable analgesia which relieved postoperative pain for up to 8 h postoperatively. The previous studies showed the persistent analgesic time of SNB on postoperative pain was 4–48 h in adult craniotomy patients (12, 15). The reasons for the different duration of SNB on postoperative pain may be related to the type of local anesthetic, the dose of local anesthetic, and the compound application of epinephrine. In the present study, we did not add epinephrine for SNB. In light of the mean duration of operation was over

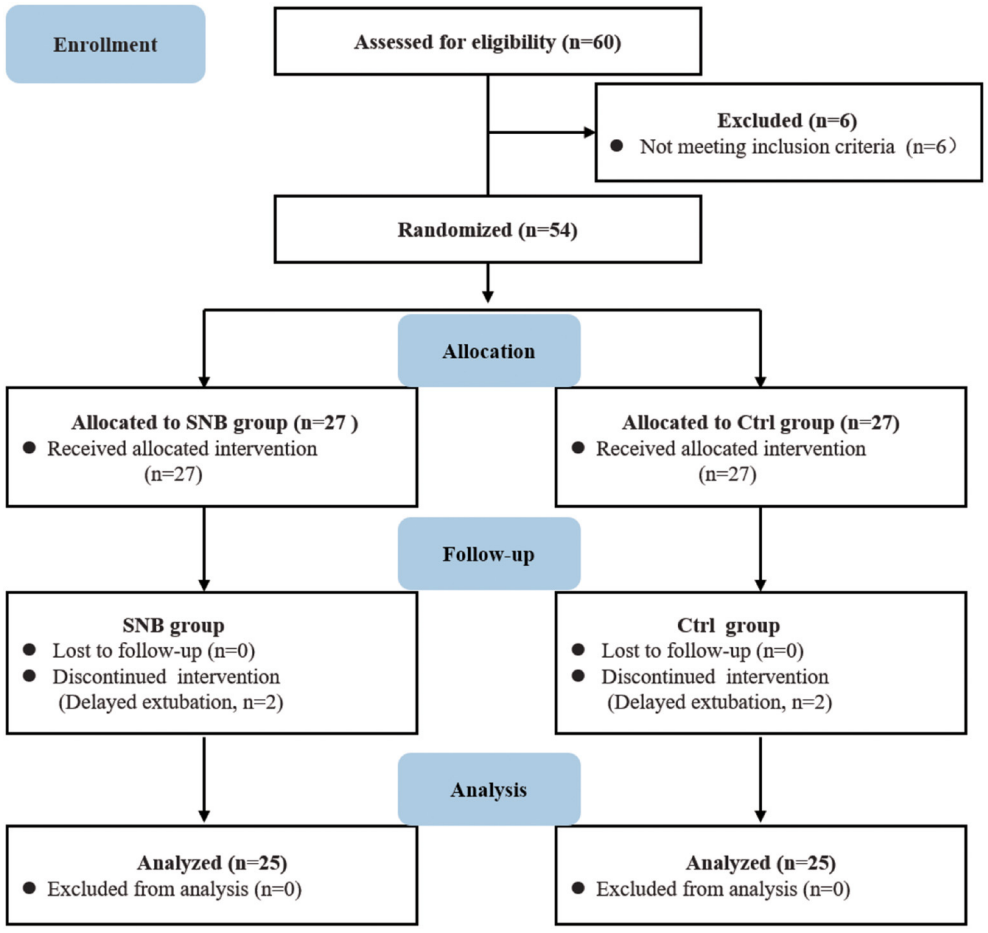

FIGURE 1
CONSORT diagram showing patient recruitment and follow-up. group Ctrl, control group; group SNB, scalp nerve block group.

TABLE 1 Demographic characteristics of group Ctrl and group SNB.

Characteristics	Ctrl ($n = 25$)	SNB ($n = 25$)	*P*-value
Sex (female/male)	9/16	9/16	1.00
Age (year)	6.36 ± 2.99	6.72 ± 3.42	0.69
Weight (kg)	26.39 ± 11.42	26.68 ± 12.84	0.93
ASA physical status [1/2, *n* (% 1)]	10/15 (40.0)	11/14 (44.0)	1.00
Duration of operation (h)	4.83 ± 1.22	4.43 ± 1.37	0.28
Duration of anesthesia (h)	5.43 ± 0.79	5.12 ± 1.27	0.35
Time for recovery from anesthesia (min)	12.20 ± 3.51	11.92 ± 4.09	0.79

Data are presented as mean ± SD or percentage (%) as indicated. ASA, American Society of Anesthesiologists; group Ctrl, control group; group SNB, scalp nerve block group.

TABLE 2 Pain scores across postoperative time points.

FLACC scores (h)	Ctrl ($n = 25$)	SNB ($n = 25$)	*P*-value
1	2 (2–3)	1 (0–1.5)	<0.001
4	4 (4–5)	2 (2–3)	<0.001
8	5 (4–6)	3 (2.5–4.5)	<0.01
12	4 (3–5)	3 (2–4.5)	>0.05
24	3 (2–5)	3 (1–4)	>0.05
48	2 (0.5–3)	2 (0–3)	>0.05

Data are presented as median (IQR). group Ctrl, control group; group SNB, scalp nerve block group; FLACC, face, legs, activity, cry, and consolability scale.

4 h in scalp nerve block group, the persistent analgesic time of SNB on postoperative analgesia was up to 8 h. Therefore, it is supposed to be at least 12 h to return the sensitivity of the scalp nerve, which was far longer than the duration of action of ropivacaine (about 3 h). This long-lasting effect on postoperative analgesia might be due to preemptive analgesia.

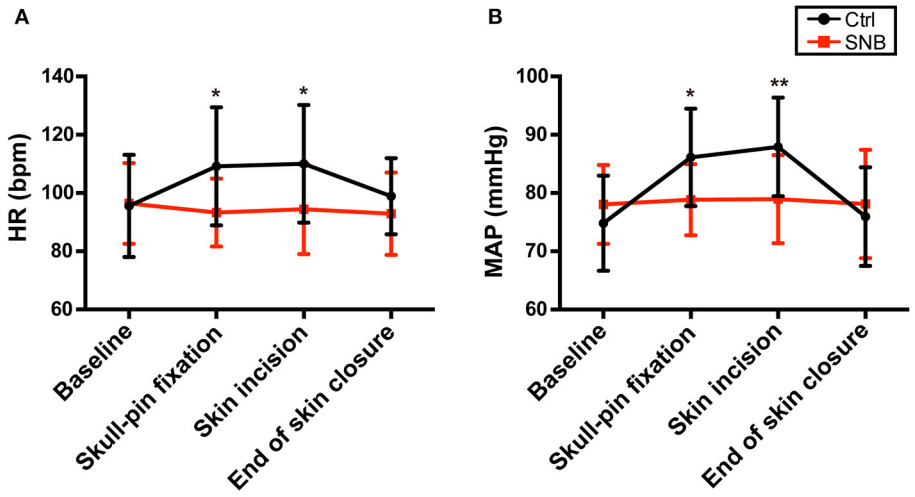

FIGURE 2
The intraoperative hemodynamic variables (HR and MAP) in group Ctrl and group SNB. Changes in HR **(A)** and MAP **(B)** during baseline, skull-pin fixation, skin incision, and end of skin closure in group Ctrl and group SNB. MAP, mean arterial blood pressure; HR, heart rate. Data are presented as mean ± SD. *$P < 0.05$; **$P < 0.01$. group Ctrl, control group; group SNB, scalp nerve block group.

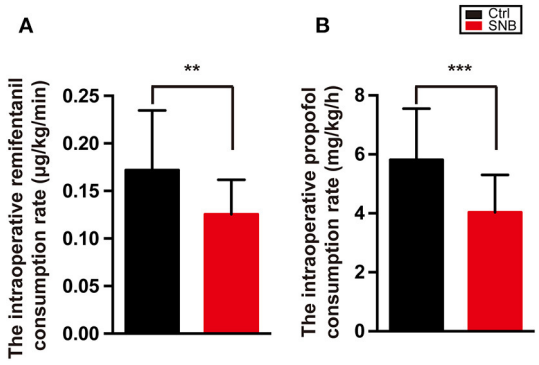

FIGURE 3
The intraoperative total remifentanil **(A)** and propofol **(B)** consumption rate in group Ctrl and group SNB. Data are presented as mean ± SD. **$P < 0.01$; ***$P < 0.001$. group Ctrl, control group; group SNB, scalp nerve block group.

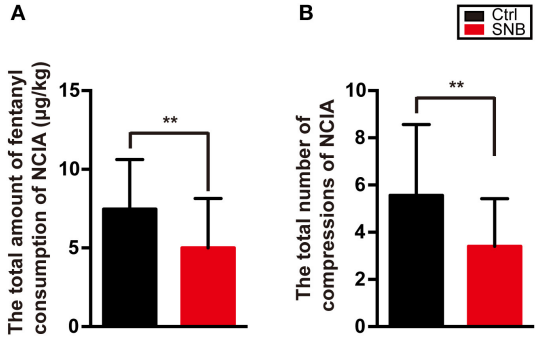

FIGURE 4
The total amount of fentanyl consumption **(A)** and the total number of compressions **(B)** of NCIA in group Ctrl and in group SNB within postoperative 48 h. Data are presented as mean ± SD. **$P < 0.01$. NCIA, nurse-controlled intravenous analgesia; group Ctrl, control group; group SNB, scalp nerve block group.

Hemodynamic stabilization is important to neurosurgery patients both in the intraoperative and postoperative periods. The elevation of blood pressure may cause an abrupt increase of intracranial pressure and favor bleeding in injured parenchyma with fragile hemostasis (21, 22). In the absence of regional anesthesia, deep anesthesia is usually used to control hemodynamic variations in response to noxious stimulation, including an increase in opioid and/or hypnotic anesthetic agent concentrations (23–25). However, too deep anesthesia may cause deleterious consequences such as hypotension, bradycardia, and increase postoperative morbidity and mortality (26, 27). In our study, we found that the hemodynamics were stable during dramatic noxious stimulation in the SNB group compared to the control group. Moreover, SNB decreased the intraoperative propofol and remifentanil total consumption, consistent with the previous studies in adult craniotomy patients (12, 15).

Another advantage of combining SNB with general anesthesia in pediatric craniotomy patients has the potential for decreasing intraoperative general anesthetic requirements.

TABLE 3 The incidence of PONV, local anesthetic toxicity, and SNB complications.

Complications	Ctrl ($n = 25$)	SNB ($n = 25$)	*P*-value
Postoperative nausea and vomiting within 48 h	10/25	11/25	1.00
Local anesthetic toxicity	0/25	0/25	1.00
Post-operative scalp infection or hematoma	0/25	0/25	1.00

Data are presented as the total number of patients (n). PONV, postoperative nausea and vomiting; group Ctrl, control group; group SNB, scalp nerve block group.

Pediatric neurosurgery usually takes a long time, so it requires a lot of general anesthetics in the absence of regional anesthesia (9, 10). However, the neurotoxicity of general anesthetics have been well-documented in some animal models and clinical trials (28–30). The dose of general anesthetics is an important factor in neurotoxicity in the developing brain (31). Therefore, it is necessary to pay attention to the neuron death of general anesthetics exposure in pediatric craniotomy patients. In the present study, combining SNB with general anesthesia significantly decreased intraoperative general anesthetic consumption. An eventual beneficial effect of SNB on neurological prognosis should be the object of a specifically designed study.

Postoperative nausea and vomiting (PONV) are the common and distressing symptoms after craniotomy. PONV may not only generate lower patient satisfaction but cause deleterious consequences such as intracranial hypertension and postoperative intracranial hemorrhage (32). In light of opioids being a risk factor for PONV, the use of regional anesthesia and non-opioid analgesics has been proposed to decrease PONV (33, 34). In our study, opioid consumption was decreased both in the intraoperative and postoperative periods. However, the incidence of PONV had no significant difference between the two groups within 48 h postoperatively. Although we used dexamethasone and tropisetron hydrochloride intraoperative for PONV prophylaxis, the incidence of PONV was up to 42% (10 patients in the control group and 11 patients in the SNB group) within the first 48 h after surgery. The high incidence of PONV in our study might be related to the duration of surgery, use of volatile anesthetic agents, brain tumor size, and postoperative intracranial pressure.

Ropivacaine is widely used in pediatric regional anesthesia for its minimal cardiotoxicity and neurotoxicity (35). Additionally, ropivacaine has a relatively short onset time and long effect duration as compared to bupivacaine and lidocaine, respectively (36). It has become one of the most popular local anesthetics for pediatric regional blocks (36, 37). In our study, we used 0.2% ropivacaine for SNB and the total dosage of ropivacaine did not exceed 2 mg/kg. The concentration was demonstrated safety in pediatrics which the concentration of ropivacaine was 0.1–0.375% and the total dosage was should not exceed 3 mg/kg for caudal blocks (37, 38).

Our study has several limitations. First, we only examined the effect of 0.2% ropivacaine on postoperative pain and did not examine the effect of other concentrations of ropivacaine on postoperative pain. Second, we did not examine the plasma concentration of ropivacaine in our patients to rule out the risk of toxicity with our SNB technique. Because the scalp is rich in blood vessels with rapid local anesthetic uptake, local anesthetic injection may predispose to local anesthetic toxicity (39). However, we did not find any local anesthetics toxicity of 0.2% ropivacaine for SNB in our study. Third, epinephrine was recommended in well-vascularized areas to maximize block duration and minimize acute rises in anesthetic plasma concentration (40). In the present study, we did not supplement with epinephrine during SNB. It is possible to prolong the postoperative analgesia time by adding epinephrine. Fourth, postoperative pain was assessed by the FLACC in our study, which was commonly used for the evaluation of postoperative pain aged 1–18 years for hospitalized children (16). Although children older than 7 years of age can use NRS or VAS for self-assessment, some children were apathetic after neurosurgery, so the reliability may decline through self-assessment methods such as NRS and VAS in children after neurosurgery.

SNB has been proposed as an adjuvant to general anesthesia for postoperative pain control, intraoperative hemodynamic control, and opioid-sparing in adult craniotomy. However, the SNB technique is not commonly used in pediatric craniotomy patients so far. In the present study, we demonstrated that SNB combined with general anesthesia significantly improved postoperative pain control, intraoperative hemodynamic control, and reduced opioid consumption in pediatric craniotomy patients compared to the control group. It was no adverse effects with 0.2% ropivacaine for SNB pediatric craniotomy patients. Therefore, the SNB is a safety technique in pediatric craniotomy patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Ethical Committee of Xinhua

Hospital, Medical School, Shanghai Jiao Tong University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

LN designed, planned, performed the experiments, analyzed the data, and contributed to writing the manuscript. YP designed, planned, performed the experiments, and analyzed the data. DX designed the experiments and wrote the manuscript. ML designed the experiments. LJ oversaw the research phases. QZ assisted in completing the experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Kulikov A, Tere V, Sergi PG, Bilotta F. Prevention and treatment of postoperative pain in pediatric patients undergone craniotomy: systematic review of clinical evidence. *Clin Neurol Neurosurg.* (2021) 205:106627. doi: 10.1016/j.clineuro.2021.106627

2. Shay JE, Kattail D, Morad A, Yaster M. The postoperative management of pain from intracranial surgery in pediatric neurosurgical patients. *Paediatr Anaesth.* (2014) 24:724–33. doi: 10.1111/pan.12444

3. Ferland CE, Vega E, Ingelmo PM. Acute pain management in children: challenges and recent improvements. *Curr Opin Anaesthesiol.* (2018) 31:327–32. doi: 10.1097/ACO.0000000000000579

4. Vittinghoff M, Lonnqvist PA, Mossetti V, Heschl S, Simic D, Colovic V, et al. Postoperative pain management in children: guidance from the pain committee of the European society for paediatric anaesthesiology (espa pain management ladder initiative). *Paediatr Anaesth.* (2018) 28:493–506. doi: 10.1111/pan.13373

5. Zontag D, Honigman L, Kuperman P, Treister R. Effects of pain-reporting education program on children's pain reports-results from a randomized controlled post-operative pediatric pain trial. *Front Pediatr.* (2021) 9:672324. doi: 10.3389/fped.2021.672324

6. Dunn LK, Naik BI, Nemergut EC, Durieux ME. Post-craniotomy pain management: beyond opioids. *Curr Neurol Neurosci Rep.* (2016) 16:93. doi: 10.1007/s11910-016-0693-y

7. Colvin LA, Bull F, Hales TG. Perioperative opioid analgesia-when is enough too much? a review of opioid-induced tolerance and hyperalgesia. *Lancet.* (2019) 393:1558–68. doi: 10.1016/S0140-6736(19)30430-1

8. Algera MH, Kamp J, van der Schrier R, van Velzen M, Niesters M, Aarts L, et al. Opioid-induced respiratory depression in humans: a review of pharmacokinetic-pharmacodynamic modelling of reversal. *Br J Anaesth.* (2019) 122:e168–79. doi: 10.1016/j.bja.2018.12.023

9. Xing F, An LX, Xue FS, Zhao CM, Bai YF. Postoperative analgesia for pediatric craniotomy patients: a randomized controlled trial. *BMC Anesthesiol.* (2019) 19:53. doi: 10.1186/s12871-019-0722-x

10. Zhao C, Zhang N, Shrestha N, Liu H, Ge M, Luo F. Dexamethasone as a ropivacaine adjuvant to pre-emptive incision-site infiltration analgesia in pediatric craniotomy patients: a prospective, multicenter, randomized, double-blind, controlled trial. *Paediatr Anaesth.* (2021) 31:665–75. doi: 10.1111/pan.14178

11. Ban VS, Bhoja R, McDonagh DL. Multimodal analgesia for craniotomy. *Curr Opin Anaesthesiol.* (2019) 32:592–9. doi: 10.1097/ACO.0000000000000766

12. Carella M, Tran G, Bonhomme VL, Franssen C. Influence of levobupivacaine regional scalp block on hemodynamic stability, intra- and postoperative opioid consumption in supratentorial craniotomies: a randomized controlled trial. *Anesth Analg.* (2021) 132:500–11. doi: 10.1213/ANE.0000000000005230

13. Guilfoyle MR, Helmy A, Duane D, Hutchinson PJA. Regional scalp block for postcraniotomy analgesia: a systematic review and meta-analysis. *Anesth Analg.* (2013) 116:1093–102. doi: 10.1213/ANE.0b013e3182863c22

14. Zetlaoui PJ, Gauthier E, Benhamou D. Ultrasound-guided scalp nerve blocks for neurosurgery: a narrative review. *Anaesth Crit Care Pain Med.* (2020) 39:876–82. doi: 10.1016/j.accpm.2020.06.019

15. Yang Y, Ou M, Zhou H, Tan L, Hu Y, Li Y, et al. Effect of scalp nerve block with ropivacaine on postoperative pain in patients undergoing craniotomy: a randomized, double blinded study. *Sci Rep.* (2020) 10:2529. doi: 10.1038/s41598-020-59370-z

16. Crellin DJ, Harrison D, Santamaria N, Babl FE. Systematic review of the face, legs, activity, cry and consolability scale for assessing pain in infants and children: is it reliable, valid, and feasible for use? *Pain.* (2015) 156:2132–51. doi: 10.1097/j.pain.0000000000000305

17. Oksuz G, Arslan M, Urfalioglu A, Guler AG, Teksen S, Bilal B, et al. Comparison of quadratus lumborum block and caudal block for postoperative analgesia in pediatric patients undergoing inguinal hernia repair and orchiopexy surgeries: a randomized controlled trial. *Reg Anesth Pain Med.* (2020) 45:187–91. doi: 10.1136/rapm-2019-101027

18. Alseoudy MM, Abdelbaser I. Ultrasound-guided retrolaminar block versus ilioinguinal nerve block for postoperative analgesia in children undergoing inguinal herniotomy: a randomized controlled trial. *J Clin Anesth.* (2021) 74:110421. doi: 10.1016/j.jclinane.2021.110421

19. Van Backer JT, Jordan MR, Leahy DT, Moore JS, Callas P, Dominick T, et al. Preemptive analgesia decreases pain following anorectal surgery: a prospective, randomized, double-blinded, placebo-controlled trial. *Dis Colon Rectum.* (2018) 61:824–9. doi: 10.1097/DCR.0000000000001069

20. Dahl JB, Kehlet H. Preventive analgesia. *Curr Opin Anaesthesiol.* (2011) 24:331–8. doi: 10.1097/ACO.0b013e328345afd9

21. Shapiro HM, Wyte SR, Harris AB, Galindo A. Acute. intraoperative intracranial hypertension in neurosurgical patients: mechanical and pharmacologic factors *Anesthesiology.* (1972) 37:399–405. doi: 10.1097/00000542-197210000-00008

22. Hayashi M, Kobayashi H, Kawano H, Handa Y, Hirose S. Treatment of systemic hypertension and intracranial hypertension in cases of brain hemorrhage. *Stroke.* (1988) 19:314–21. doi: 10.1161/01.STR.19.3.314

23. Pardey Bracho GF, Pereira de Souza Neto E, Grousson S, Mottolese C, Dailler F. Opioid consumption after levobupivacaine scalp nerve block for craniosynostosis surgery. *Acta Anaesthesiol Taiwan.* (2014) 52:64–9. doi: 10.1016/j.aat.2014.05.006

24. Brown EN, Pavone KJ, Naranjo M. Multimodal general anesthesia: theory and practice. *Anesth Analg.* (2018) 127:1246–58. doi: 10.1213/ANE.0000000000003668

25. Egan TD. Are opioids indispensable for general anaesthesia? *Br J Anaesth.* (2019) 122:e127–35. doi: 10.1016/j.bja.2019.02.018

26. Sieber F, Neufeld KJ, Gottschalk A, Bigelow GE, Oh ES, Rosenberg PB, et al. Depth of sedation as an interventional target to reduce postoperative delirium: mortality and functional outcomes of the strategy to reduce the incidence of postoperative delirium in elderly patients randomised clinical trial. *Br J Anaesth.* (2019) 122:480–9. doi: 10.1016/j.bja.2018.12.021

27. Kertai MD, White WD, Gan TJ. Cumulative duration of "triple low" state of low blood pressure, low bispectral index, and low minimum alveolar concentration of volatile anesthesia is not associated with increased mortality. *Anesthesiology.* (2014) 121:18–28. doi: 10.1097/ALN.0000000000000281

28. Walkden GJ, Gill H, Davies NM, Peters AE, Wright I, Pickering AE. Early childhood general anesthesia and neurodevelopmental outcomes in the avon longitudinal study of parents and children birth cohort. *Anesthesiology.* (2020) 133:1007–20. doi: 10.1097/ALN.0000000000003522

29. Glatz P, Sandin RH, Pedersen NL, Bonamy AK, Eriksson LI, Granath F. Association of anesthesia and surgery during childhood with long-term academic performance. *JAMA Pediatr.* (2017) 171:e163470. doi: 10.1001/jamapediatrics.2016.3470

30. Xu D, Wang B, Xu B, Yin C, Ning L, Li X, et al. Midazolam exposure impedes oligodendrocyte development via the translocator protein and impairs myelination in larval zebrafish. *Mol Neurobiol.* (2022) 59:93–106. doi: 10.1007/s12035-021-02559-8

31. Vutskits L, Xie Z. Lasting impact of general anaesthesia on the brain: mechanisms and relevance. *Nat Rev Neurosci.* (2016) 17:705–17. doi: 10.1038/nrn.2016.128

32. Uribe AA, Stoicea N, Echeverria-Villalobos M, Todeschini AB, Esparza Gutierrez A, Folea AR, et al. Postoperative nausea and vomiting after craniotomy: an evidence-based review of general considerations, risk factors, and management. *J Neurosurg Anesthesiol.* (2021) 33:212–20. doi: 10.1097/ANA.0000000000000667

33. Kovac AL. Postoperative nausea and vomiting in pediatric patients. *Paediatr Drugs.* (2021) 23:11–37. doi: 10.1007/s40272-020-00424-0

34. Roberts GW, Bekker TB, Carlsen HH, Moffatt CH, Slattery PJ, McClure AF. Postoperative nausea and vomiting are strongly influenced by postoperative opioid use in a dose-related manner. *Anesth Analg.* (2005) 101:1343–8. doi: 10.1213/01.ANE.0000180204.64588.EC

35. Dobereiner EF, Cox RG, Ewen A, Lardner DR. Evidence-based clinical update: which local anesthetic drug for pediatric caudal block provides optimal efficacy with the fewest side effects? *Can J Anaesth.* (2010) 57:1102–10. doi: 10.1007/s12630-010-9386-1

36. Johr M. Regional anaesthesia in neonates, infants and children: an educational review. *Eur J Anaesthesiol.* (2015) 32:289–97. doi: 10.1097/EJA.0000000000000239

37. Suresh S, Ecoffey C, Bosenberg A, Lonnqvist PA, de Oliveira GS Jr, de Leon Casasola O, et al. The European society of regional anaesthesia and pain therapy/american society of regional anesthesia and pain medicine recommendations on local anesthetics and adjuvants dosage in pediatric regional anesthesia. *Reg Anesth Pain Med.* (2018) 43:211–6. doi: 10.1097/AAP.0000000000000702

38. Morton NS. Ropivacaine in children. *Br J Anaesth.* (2000) 85:344–6. doi: 10.1093/bja/85.3.344

39. Colley PS, Heavner JE. Blood levels of bupivacaine after injection into the scalp with and without epinephrine. *Anesthesiology.* (1981) 54:81–4. doi: 10.1097/00000542-198101000-00018

40. Tschopp C, Tramer MR, Schneider A, Zaarour M, Elia N. Benefit and harm of adding epinephrine to a local anesthetic for neuraxial and locoregional anesthesia: a meta-analysis of randomized controlled trials with trial sequential analyses. *Anesth Analg.* (2018) 127:228–39. doi: 10.1213/ANE.0000000000003417

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Longitudinal assessment of the inflammatory response: The next step in personalized medicine after severe trauma

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Infections in trauma patients are an increasing and substantial cause of morbidity, contributing to a mortality rate of 5–8% after trauma. With increased early survival rates, up to 30–50% of multitrauma patients develop an infectious complication. Trauma leads to a complex inflammatory cascade, in which neutrophils play a key role. Understanding the functions and characteristics of these cells is important for the understanding of their involvement in the development of infectious complications. Recently, analysis of neutrophil phenotype and function as complex biomarkers, has become accessible for point-of-care decision making after trauma. There is an intriguing relation between the neutrophil functional phenotype on admission, and the clinical course (e.g., infectious complications) of trauma patients. Potential neutrophil based cellular diagnostics include subsets based on neutrophil receptor expression, responsiveness of neutrophils to formyl-peptides and FcγRI (CD64) expression representing the infectious state of a patient. It is now possible to recognize patients at risk for infectious complications when presented at the trauma bay. These patients display increased numbers of neutrophil subsets, decreased responsiveness to fMLF and/or increased CD64 expression. The next step is to measure these biomarkers over time in trauma patients at risk for infectious complications, to guide decision making regarding timing and extent of surgery and administration of (preventive) antibiotics.

KEYWORDS

trauma, inflammatory, neutrophil, infection, longitudinal

Outline

Trauma leads to a complex inflammatory cascade that can cause an acquired immunodeficiency, which makes patients prone to develop infectious complications (1). Neutrophils play a key role in these processes (2). Distinct neutrophil subtypes with different functions exist, and these cells participate in the development and/or

recovery of infectious complications after trauma (3–5). Recently, analysis of neutrophil phenotype and function as complex biomarkers has become accessible for point-of-care decision-making in the management of trauma patients (6). The aim of this narrative review is to discuss the potential use of longitudinal assessment of the neutrophil compartment by point-of-care flow cytometry. The goal of these sequential measurements over time is to guide clinical decision-making regarding end goals of resuscitation, indication and timing of immune protective surgery in a damage control setting as well as definitive surgery and the administration of (preventive) antibiotics.

The immunological response to trauma is initiated at the moment of injury. At this point, two inflammatory responses are induced in parallel. The systemic pro-inflammatory response to tissue damage makes patients susceptible to multi organ dysfunction syndrome (MODS), while the simultaneous immune paralysis makes patients susceptible to late infectious complications (7). Additional trauma caused by (operative) interventions can worsen this inflammatory response, thereby, increasing the risk of inflammatory and infectious complications (1). From a clinical perspective, these processes can complicate the course of trauma patients by early pro-inflammatory complications during the first 4 days after trauma and by late-onset (>5 days) sepsis, which is suggested to have a relation with the magnitude of the inflammatory response in the beginning (8–11).

This relation between extent of the inflammatory response and the activation of the immune system can be measured by multiple neutrophil biomarkers for both phenotype and function (12). The next step is to measure neutrophil biomarkers at different time points after trauma to guide decision making regarding timing and burden (i.e., approach and extent) of surgery and administration of (preventive) antibiotics.

Clinical relevance

Injury is the leading cause of death in young individuals (13). Due to advances in the organization of trauma care, hemorrhage control, surgical approaches and resuscitation, overall mortality after trauma has declined up to 75% in recent decades (14–17). With increased early survival rates, infections are the most common complications affecting trauma patients (18, 19). Thus, infections are an increasing and substantial cause of morbidity, contributing to a mortality rate of 5–8% after trauma (17, 20). A large number of trauma patients are able to survive the first critical phase after trauma, which creates new challenges in terms of diagnostics and treatment in longitudinal management of trauma patients.

Injury induces an immediate innate immune response, leading to a complex inflammatory cascade that induces both immune activation and a refractory immune state in parallel (21). Neutrophils are the first immune cells responding to tissue damage and invading pathogens and they are known to play a key role in regulating the inflammatory response after trauma (2). Recently, analysis of neutrophil phenotype and function (e.g., neutrophil size, neutrophil subsets, neutrophil activation markers, responsiveness of these markers to bacterial stimuli) has become accessible to such extent that they can be used for point-of-care (POC) decision making after trauma (6).

Until now, studies mainly focused on the assessment of the neutrophil functional phenotype on admission at the trauma bay. In this setting, a relation between neutrophil phenotype on admission and the clinical course of trauma patients (e.g., inflammatory, and infectious complications) was demonstrated (8–11). However, the course of the inflammatory response is dynamic in time and is driven by both patient characteristics, injury type and severity, timing and burden (i.e., extent) of (operative) interventions. Further determination of these changes over time will increasingly facilitate personalized care of injured patients. As it is now possible to measure the neutrophil functional phenotype at any time point after trauma in a fast and non-labor intensive manner (12), the longitudinal course of the inflammatory response and the impact of additional interventions can be measured.

Inflammatory responses after trauma

The innate immune response is initiated by injury-related tissue damage, coined as "the first hit" (22). The amplitude of this response is thought to be determined by the severity and type of injury (6, 9, 11, 23). The response is characterized by two responses developing in parallel. Firstly, a **pro-inflammatory response** is mediated by a systemic inflammatory response to tissue damage, in reaction to damage-associated molecular patterns (DAMPs) and microbe-associated molecular patterns (MAMPs) (24). A dysregulated pro-inflammatory response can increase susceptibility to (multi) organ failure such as ARDS or MODS (9). Neutrophils might also help to resolve tissue damage by the release DNA structures that are formed like a web, namely neutrophil extracellular traps (NETs). NETs could contribute to preventing dissemination of (larger) pathogens (25). However, when neutrophils produce too many NETs as a reaction to trauma, these toxic structures further amplify the pro-inflammatory response, contributing to an imbalanced immune reaction (26). Secondly, an **anti-inflammatory response** is mediated by a refractory immune state, which leads to immune paralysis and susceptibility to infectious complications e.g., sepsis. Recently, it became clear that although these responses appear clinically distinct, the underlying immunological mechanisms, in terms of the appearance of neutrophil subsets and pro-inflammatory

cytokines e.g., IL-6 and IL-10, start simultaneously and immediately after the injury (8, 27–30).

Additional trauma caused by surgical intervention, generally referred to as "a second hit," can contribute to a further dysregulation of the immune response, thereby increasing the risk of inflammatory/infectious complications (31). Over the past decade surgical procedures were shortened to minimize the effect of such a second hit. This physiological and immune protective surgery is becoming more and more recognized as important in the early phases of treatment after trauma. Over time, different strategies have been practiced for treating multitrauma patients that are considered too unstable for early total care. The concept of damage control surgery (DCS) has come up since the 1990s, focusing on improving survival by rapidly controlling hemorrhage and contamination by a brief initial operation. Only when a patient becomes hemodynamically stable, definitive surgery is performed (22). Nowadays, a strategy that focusses on early fixation of bones, combined with a continual reassessment of the reaction and response to injury and surgery, has gained popularity. When physiology (e.g., in terms of physiological or immunological response) deteriorates, fixation should be delayed until the patient stabilizes (32). This strategy is coined early appropriate care.

Over the past decades, due to advanced hemorrhage control (damage control surgery and damage control orthopedics) and (damage control) resuscitation, a decrease in mortality and a significant lowering of the incidence of inflammatory complications (e.g., ARDS, MODS) was observed (33–35). This had consequences for studies before 2010 and after 2012, during which the inflammatory marker interleukin-6 (IL-6) was measured (11, 36, 37). In the earlier period 2006–2010 higher injury severity scores (ISS) and higher concentrations of IL-6 were found to correlate with inflammatory complications. Interestingly, this correlation between IL-6 and ISS was lost in studies published after 2012, where a lower incidence of inflammatory complications was observed (see **Table 1**) (11, 36–38). Thus, despite a pronounced initial inflammatory response, the systemic inflammation was lower leading to a decrease in inflammatory complications most likely due to ongoing better management of trauma care (39).

In addition, in a cohort study with thirty-three patients with acute burn patients, a clear dose response relation was found between the degree of skin injury and the percentage of banded neutrophils. Furthermore, patients with additional thermal inhalation injuries were characterized by a decreased responsiveness of fNLF of neutrophils in peripheral blood. This indicates that burn injuries cause a mild activation of systemic cellular inflammation, and that additional thermal inhalation injury induces refractory systemic neutrophils, possibly leading to an increased susceptibility to infectious complications. These results are not published.

Currently, the mortality after trauma is increasingly caused by neurotrauma (33, 40, 41). This illustrates that the pro-inflammatory response, that used to be causing hyper-inflammatory systemic conditions, seems more under control (33). This leads now to an anti-inflammatory imbalance with new clinical challenges, such as recognition and treatment of late (>5 days) infections mainly resulting in increased morbidity. A dysregulation of the immune response, in which neutrophils play a pivotal role, predisposes patients to these infectious complications (11). Understanding the functions and characteristics of neutrophils is important to understand their involvement in the development of infectious complications.

Potential neutrophil biomarkers for treatment of trauma patients

The immediate immune response directly after trauma is regulated by many humoral and cellular mediators including leukocytes, coagulation, systemic cytokine release and complement cascades (42). Neutrophils are the most abundant innate effector cells and belong to a final common pathway causing tissue damage upon hyper activation of these cells (2). Therefore, research over the past decades has focused on identification of complex activation of neutrophils to identify trauma patients with high risk on adverse outcome. An overview of the investigated markers is described below.

Cell size and granularity of neutrophils

White blood cell count (WBC) and differentiation into leukocyte subsets have been extensively investigated in trauma patients. A marked increase of WBC and especially neutrophilic granulocytes has been noted after trauma, but this increase in cell numbers *per se* was found to be of no prognostic value (43, 44). In addition, WBC morphological parameters have been assessed to identify patients at risk. An increase in neutrophil cell size was observed in non-survivors after severe trauma, implying an increased cell size to be a predictor for mortality (45). Thereafter, in another cohort, a rise in neutrophil cell size preceded the clinical manifestation of organ dysfunction

TABLE 1 Incidence of SIRS, sepsis and ARDS were tested using Fisher's exact test.

	Early period	Late period	*P*-value
Number of patients (n)	19	15	
Study period	2006–2010	2012–2016	
SIRS	14	14	0.18
Sepsis	4	1	0.34
ARDS/MODS	8	0	0.003*

Significant value in indicated with *. SIRS, systemic inflammatory response syndrome; ARDS, acute respiratory distress syndrome; MODS, multiple organ dysfunction syndrome.

in every patient with a complication (46). Unfortunately, it remains to be determined which precise mechanisms underlie the increase in neutrophil cell size.

A partial explanation for an increased cell-size could be the influx of immature neutrophils in the peripheral blood (47, 48). It is known that immature neutrophils appear in the bloodstream as a result of the inflammatory reaction immediately after trauma; generally referred to as a left shift (49, 50). These young neutrophils with a larger cell size are known to have a C shaped ("band") nucleus instead of a lobulated nucleus. The neutrophil left shifts have proven their diagnostic value in patients with a bacterial infection (51). However, routine analyzers are not able to recognize a left shift of banded neutrophils in trauma patients, while they are able to do so in infectious patient groups (52). So, the question remains if banded neutrophils in trauma patients are different in function and phenotype from banded neutrophils in patients suffering from an infection. Further research has focused on phenotyping neutrophils based on receptor expression (CD16/CD62L), as this is a more accurate method to identify different phenotypes of neutrophils in trauma patients (6).

Subsets of neutrophils

Under homeostatic conditions, only a homogeneous population of mature neutrophils identified by $CD16^{bright}/CD62L^{bright}$ cells circulate in the peripheral blood. However, during acute inflammation, neutrophils in the peripheral blood can be divided into different subsets based on the expression of specific surface proteins (CD16/FcγRIII and CD62L/L-selectin) (3). Immediately after trauma, large numbers of immature neutrophils with a banded shaped nucleus ($CD16^{dim}/CD62L^{bright}$) enter the circulation and after several days also hypersegmented neutrophils ($CD16^{bright}/CD62L^{dim}$) can be observed (**Figure 1**) (50, 53). Interestingly, these neutrophil subsets based on the expression of CD16 and CD62L have different functions (3, 54). Landmark studies in healthy controls, who received lipopolysaccharide (LPS) inducing systemic inflammation, showed that banded (immature) neutrophils display a higher killing capacity than segmented neutrophils and that hypersegmented neutrophils are associated with almost immediate bacterial outgrowth, due to impaired intracellular killing after adequate phagocytosis (3). In addition, polytrauma patients who developed infectious complications had significantly higher percentage $CD16^{dim}/CD62L^{bright}$ (banded) neutrophils in the blood immediately after trauma compared with polytrauma patients who did not develop infectious complications (6).

Neutrophil left shifts (influx of immature $CD16^{dim}$ (banded) neutrophils into the blood) are observed immediately after trauma but quickly disappear after approximately 1 day (5). This well-known phenomenon is explained by a massive mobilization of neutrophils from the bone marrow (50). This can lead to a subsequent imbalance of the neutrophil compartment and the bone marrow might be unable to fully compensate in time for the loss of these well-functioning neutrophils that can live up to 5 days under homeostatic conditions (55, 56). This phenomenon might be the driving force for the clinical observation that the trauma patient becomes highly sensitive to infectious complications after 5–6 days post trauma (**Figure 1**) (57).

On the other hand, $CD62L^{dim}$ (hypersegmented) neutrophils appear in the peripheral blood several days (>3 days) after trauma with a peak after 10 days (5). These $CD62L^{dim}$ neutrophils were shown to have impaired killing capacity and a reduced acidification capacity, but they have immunoregulatory characteristics that could be important for keeping the balance in the immune response (54). The presence of high numbers of $CD62L^{dim}$ neutrophils during the days to weeks following severe trauma might play a significant role in the risk of infectious complications in trauma patients but this concept needs more experimental support. It is, however, known that the first systemic infectious complications that have consequences for the clinical course of the patient arise at the end of the first week after trauma (58). This coincides with the rising counts of $CD62L^{low}$ cells during the first week after trauma, so it is tempting to hypothesize that high numbers of $CD62L^{low}$ neutrophils can lead to an increased risk of infectious complications (**Figure 1**) (10).

Trauma patients with high numbers of banded neutrophils directly after trauma and high numbers of hypersegmented neutrophils after 10 days in the peripheral blood seem also at risk for infectious complications (**Figure 1**). In these patients, longitudinal measurement of the neutrophil compartment and the amount of $CD16^{dim}$ and $CD62L^{dim}$ neutrophils could guide the clinician regarding decisions on the timing and extent (burden) of surgery. A patient that is not at risk could benefit from early total care, while a patient displaying extensive neutrophil subsets could benefit from damage control immune protective surgery.

Responsiveness to inflammatory mediators (e.g., formyl-peptides/fMLF)

In addition to the assessment of neutrophil subsets by receptor expression, the functionality of neutrophils can be investigated in terms of responsiveness of neutrophil to formyl peptides (e.g., fMLF). fMLF activates neutrophils by interactions with their formyl peptide receptors (FPR) (59–63). A decreased responsiveness of neutrophils to fMLF at initial presentation was demonstrated in trauma patients who later developed septic shock (10). In addition, low responsiveness of neutrophils to fMLF immediately after trauma preceded by almost a week the development of septic complications after > 5 days (11). Thus,

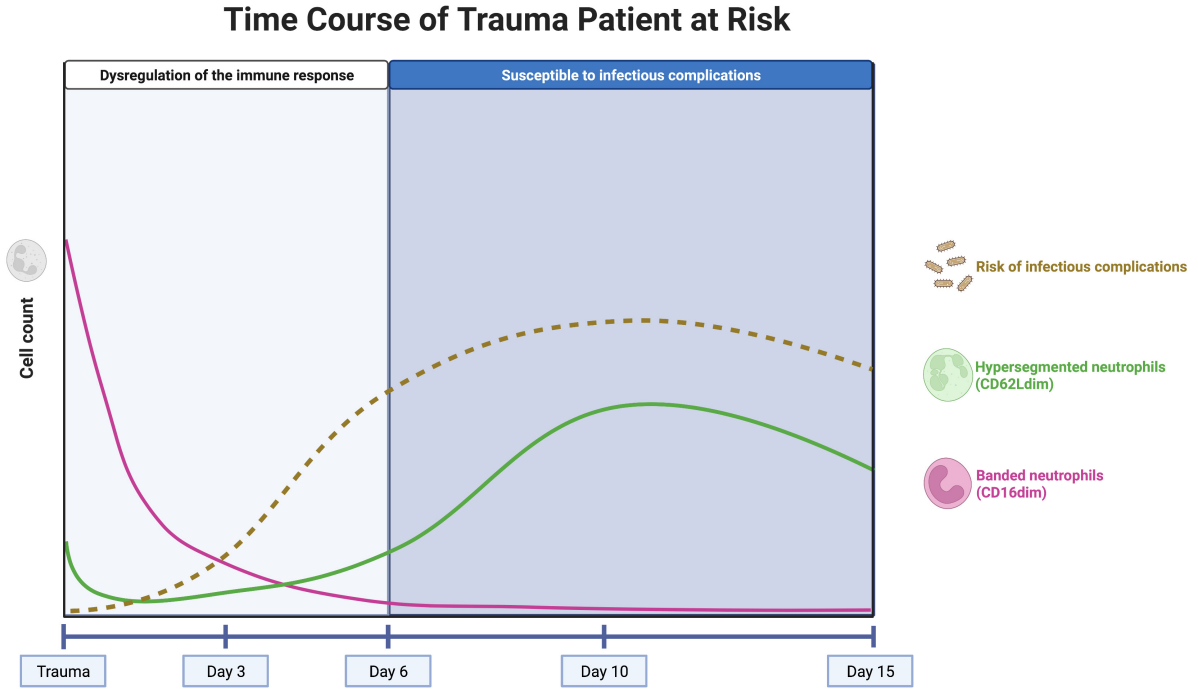

FIGURE 1
Concept of the neutrophil subset response over time in a trauma patient at risk for infectious complications. The pink line represents the course of well-functioning banded ($CD16^{dim}$) neutrophils, entering the circulation immediately after trauma. The influx of these cells stops after approximately 1 day and the neutrophils have an average lifetime of 5 days. The green line represents the course of hypersegmented ($CD62L^{dim}$) neutrophils, with impaired killing capacity. These cells appear after several days, with a peak after 10 days. The brown, dashed line represents the risk of infectious complications over time. The risk increases after 3–5 days, when there are no banded neutrophils left in the circulation. After 10 days, the peak of hypersegmented neutrophils coincides with the first systemic infectious complications. Created with BioRender.com.

responsiveness to formyl-peptides appears to be a sensitive marker as it is involved in an essential part of the immune system that is activated during the pathogenesis of sepsis and septic shock. Modulation of this responsiveness can aid the clinician in identifying patients at risk. No longitudinal data has been published yet about the responsiveness of neutrophils in trauma patients. These patients might also benefit from longitudinal monitoring to guide decision making.

Expression of FCγRI (CD64) on neutrophils

Specific expression of neutrophil receptors could be used as an extra tool to measure markers associated with specific components of the immune response. Neutrophil CD64 is a receptor that is upregulated on neutrophils within 1–6 h after administration of pro-inflammatory cytokines or bacterial cell wall components such as LPS (64). In the absence of a pro-inflammatory stimulus, CD64 levels on neutrophils start to decrease after 48 h and are return to baseline within 7 days (65). Thus, neutrophil CD64 is a relatively specific marker for bacterial infections (66). This property of CD64 has been utilized as a diagnostic marker of infection particularly for sepsis in intensive care patients (67). This could potentially also be a marker to aid the decision to early antibiotics in patients at risk for bacterial infections. Longitudinal measurement of CD64 expression in patients admitted at the hospital could aid the clinician with early diagnosis and follow up of bacterial infections (64, 68–71).

Burden of surgery: Timing + intensity

There are different strategies for treating severely injured trauma patients. Damage control surgery (DCS) is characterized by a brief initial operation to stabilize the patient, followed by several short interventions (**Figure 2**) (22). This approach of physiological and immune protective surgery is commonly chosen when a patient is too unstable to survive definitive fixation in the acute moment (1). The alternative to damage control surgery is early total care (ETC) with definitive fixation within the first 24–36 h (**Figure 2**) (32). Recently, there has been a trend toward early appropriate care (EAC), which means providing a plan of action based on continuous assessment

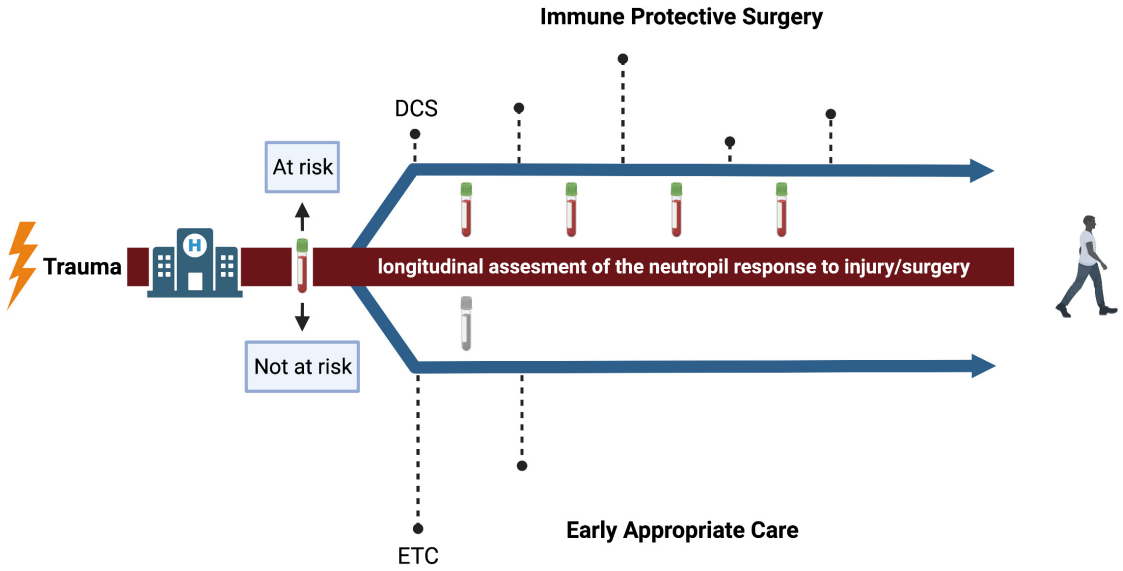

FIGURE 2
Conceptual representation of surgical strategies after trauma. The dashed lines represent the timing of surgical interventions and the height of the dashed lines represent the intensity. The upper arrow represents a proposed strategy for patients that, after flow cytometric measurement of the neutrophil compartment, are expected to be at risk for infectious complications. The impact of surgery is minimized and adjusted over time to reassessment of the neutrophil response. The lower arrow represents a proposed strategy for patients that are expected to be not at risk. Here, the approach of early appropriate care is suggested. DCS, damage control surgery; ETC, early total care. Created with BioRender.com.

and reaction to the response to injury and surgery (31, 72). It is based on fixation of the fractured bones at an early stage, unless physiology deteriorates and definitive fixation should be postponed (32). However, the challenge is to correctly assess the response to injury and surgery to select the patients that benefit from delayed definitive fixation of bones.

Currently, there is no adequate method to estimate the physiological status of an injured patient over time. As shown in **Figure 1**, the trauma patient at risk of infectious complications is characterized by the presence of prominent neutrophil subsets in the peripheral blood directly after trauma (6). The change in the occurrence of these subsets over time might be associated with the risk of developing infectious complications (10). The impact of extensive surgery on the amount of neutrophil subsets in the blood and neutrophil responsiveness, has not yet been described. The next essential step is to investigate if the imbalance in the immune response further deteriorates, in terms of e.g., influx of banded/hypersegmented neutrophils of a further decreased responsiveness to fMLF, due to (extensive) surgery. Possibly, this could possibly aid decision making regarding timing and extent of surgery. Assessment of neutrophil subsets and responsiveness before and after surgery in patients that are deemed "at risk" directly after trauma is likely to give information about the impact of surgery and might give the opportunity to compare different surgical strategies. Thus, longitudinal assessment of changes in the neutrophil compartment might be the promising next step in personalized medicine in trauma patients, because it quantifies the inflammatory response caused by the first hit and subsequent hits.

Antibiotic interventions

Infectious complications in trauma patients are treated with antibiotics. However, not all antibiotics may be useful at every moment after trauma. Patients with increased $CD62L^{dim}$ subsets and decreased fMLF responses, could suffer from invading bacteria that reside inside the neutrophils for up to 5 days after trauma (3, 55, 73, 74). *Staphylococcus aureus (S. aureus)* is a common causative pathogen of infections after trauma and it is known that it has the ability to survive and proliferate inside neutrophils, as in a Trojan horse (3, 75). Treatment with antibiotics that could kill bacteria that are contained inside the neutrophil would protect vulnerable patients against severe infections that typically occur >5 days after trauma. The antibiotics quinolones, rifamycins and sulfamethoxazole-trimethoprim seem effective against *S. Aureus* inside a human neutrophil (74). Moreover, the infectious state of a patient should be monitored by measuring the neutrophil receptor CD64. In patients with a suspected infection, high levels of CD64 are of diagnostic value (66). Moreover, when the infection is diagnosed and antibiotics are administered, sequential measurement of CD64 gives early information on the effect of the antibiotics (76). If the expression of CD64 remains high, the bacteria could be resistant, or the antibiotic is not able to reach

the bacteria. Later, a low expression of CD64 after cessation of antibiotic treatment, confirms a successfully treated infection. Thus, sequentially analyzing neutrophil subsets, estimating the killing capacity and monitoring the infectious status by CD64 on neutrophils may individualize both indication, timing and type of antibiotic used in severely injured trauma patients.

Conclusion

The extent of the inflammatory response after trauma can be measured with an automatic point-of-care flow cytometry approach. It is known that there is a relation between the neutrophil functional phenotype on admission, and the clinical course (e.g., infectious complications) of the trauma patient. Potential neutrophil associated biomarkers include determination of subsets based on neutrophil receptor expression, responsiveness of neutrophils to formyl-peptides (fMLF) and CD64 expression representing the infectious state of a patient. By this technology it is likely possible to identify patients at risk for infectious complications when presented at the trauma bay. These patients display large extents of banded neutrophils directly after trauma and high numbers of hypersegmented neutrophils after 10 days, decreased responsiveness to fMLF and/or increased CD64 expression. The next step is to measure these biomarkers over time to guide decision making regarding timing and extent of surgery to achieve early appropriate care and determine the administration of (preventive) antibiotics.

Author contributions

EF wrote the first draft of the manuscript. LK, LL, NV, KW, and FH critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Lord JM, Midwinter MJ, Chen YF, Belli A, Brohi K, Kovacs EJ, et al. The systemic immune response to trauma: an overview of pathophysiology and treatment. *Lancet.* (2014) 384:1455–65. doi: 10.1016/S0140-6736(14)60687-5

2. Kubes P. The enigmatic neutrophil: what we do not know. *Cell Tissue Res.* (2018) 371:399–406. doi: 10.1007/s00441-018-2790-5

3. Leliefeld PHC, Pillay J, Vrisekoop N, Heeres M, Tak T, Kox M, et al. Differential antibacterial control by neutrophil subsets. *Blood Adv.* (2018) 2:1344–55. doi: 10.1182/bloodadvances.2017015578

4. Pillay J, Ramakers BP, Kamp VM, Loi ALT, Lam SW, Hietbrink F, et al. Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia. *J Leukoc Biol.* (2010) 88:211–20. doi: 10.1189/jlb.1209793

5. Bongers SH, Chen N, van Grinsven E, van Staveren S, Hassani M, Spijkerman R, et al. Kinetics of neutrophil subsets in acute, subacute, and chronic inflammation. *Front Immunol.* (2021) 12:674079. doi: 10.3389/fimmu.2021.674079

6. Spijkerman R, Hesselink L, Bongers S, van Wessem KJP, Vrisekoop N, Hietbrink F, et al. Point-of-care analysis of neutrophil phenotypes: a first step toward immuno-based precision medicine in the trauma ICU. *Crit Care Explor.* (2020) 2:e0158. doi: 10.1097/cce.0000000000000158

7. Hesselink L, Spijkerman R, Van Wessem KJP, Koenderman L, Leenen LPH, Huber-Lang M, et al. Neutrophil heterogeneity and its role in infectious complications after severe trauma. *World J Emerg Surg.* (2019) 14:1–11. doi: 10.1186/s13017-019-0244-3

8. Hietbrink F, Oudijk EJ, Braams R, Koenderman L, Leenen L. Aberrant regulation of polymorphonuclear phagocyte responsiveness in multitrauma patients. *Shock.* (2006) 26:558–64. doi: 10.1097/01.shk.0000233196.40989.78

9. Hietbrink F, Koenderman L, Althuizen M, Leenen LPH. Modulation of the innate immune response after trauma visualised by a change in functional PMN phenotype. *Injury.* (2009) 40:851–5. doi: 10.1016/j.injury.2008.11.002

10. Hietbrink F, Koenderman L, Althuizen M, Pillay J, Kamp V, Leenen LPH. Kinetics of the innate immune response after trauma: implications for the development of late onset sepsis. *Shock.* (2013) 40:21–7. doi: 10.1097/SHK.0b013e318295a40a

11. Groeneveld KM, Koenderman L, Warren BL, Jol S, Leenen LPH, Hietbrink F. Early decreased neutrophil responsiveness is related to late onset sepsis in multitrauma patients: an international cohort study. *PLoS One.* (2017) 12:e0180145. doi: 10.1371/journal.pone.0180145

12. Spijkerman R, Hesselink L, Hellebrekers P, Vrisekoop N, Hietbrink F, Leenen LPH, et al. Automated flow cytometry enables high performance point-of-care analysis of leukocyte phenotypes. *J Immunol Methods.* (2019) 474:112646. doi: 10.1016/j.jim.2019.112646

13. CDC. *Top Ten Leading Causes of Death in the U.S. for Ages 1-44 from 1981-2020.* Atlanta, GA: CDC (2020).

14. Murray CJL, Lopez AD. Global mortality, disability, and the contribution of risk factors: global burden of disease study. *Lancet.* (1997) 349:1436–42. doi: 10.1016/S0140-6736(96)07495-8

15. GBD 2017 DALYS and Hale Collaborators. The global burden of disease. *Lancet.* (2018) 392:1859–922.

16. World Health Organization. *The Injury Chart Book*. Geneva: World Health Organization (2002).

17. Haagsma JA, Graetz N, Bolliger I, Naghavi M, Higashi H, Mullany EC, et al. The global burden of injury: incidence, mortality, disability-adjusted life years and

time trends from the global burden of disease study 2013. *Inj Prev.* (2016) 22:3–18. doi: 10.1136/injuryprev-2015-041616

18. Komori A, Iriyama H, Kainoh T, Aoki M, Naito T, Abe T. The impact of infection complications after trauma differs according to trauma severity. *Sci Rep.* (2021) 11:1–8. doi: 10.1038/s41598-021-93314-5

19. Eguia E, Bunn C, Kulshrestha S, Markossian T, Durazo-Arvizu R, Baker MS, et al. Trends, cost, and mortality from sepsis after trauma in the United States: an evaluation of the national inpatient sample of hospitalizations, 2012–2016. *Crit Care Med.* (2020) 48:1296–303. doi: 10.1097/ccm.0000000000004451

20. Wafaisade A, Lefering R, Bouillon B, Sakka SG, Thamm OC, Paffrath T, et al. Epidemiology and risk factors of sepsis after multiple trauma: an analysis of 29,829 patients from the trauma registry of the German society for trauma surgery. *Crit Care Med.* (2011) 39:621–8. doi: 10.1097/CCM.0b013e318206d3df

21. Hietbrink F, Koenderman L, Rijkers GT, Leenen LPH. Trauma: the role of the innate immune system. *World J Emerg Surg.* (2006) 1:1–11. doi: 10.1186/1749-7922-1-15

22. Rotondo MF, Schwab CW, McGonigal MD, Phillips GR, Fruchterman TM, Kauder DR, et al. 'Damage control': an approach for improved survival in exsanguinating penetrating abdominal injury. *J Trauma Inj Infect Crit Care.* (1993) 35:375–83. doi: 10.1097/00005373-199309000-00008

23. Visser T, Pillay J, Pickkers P, Leenen LPH, Koenderman L. Homology in systemic neutrophil response induced by human experimental endotoxemia and by trauma. *Shock.* (2012) 37:145–51. doi: 10.1097/SHK.0b013e31823f14a4

24. Hazeldine J, Hampson P, Lord JM. The impact of trauma on neutrophil function. *Injury.* (2014) 45:1824–33. doi: 10.1016/j.injury.2014.06.021

25. Hidalgo, A, Libby P, Soehnlein O, Aramburu I, Papayannopoulos V, Silvestre-Roig C. Neutrophil extracellular traps.: from physiology to pathology. *Cardiovasc Res.* (2021) cvab329. doi: 10.1093/cvr/cvab329 [Epub ahead of print].

26. Wang J. Neutrophils in tissue injury and repair. *Cell Tissue Res.* (2018) 371:531–9. doi: 10.1007/s00441-017-2785-7

27. Visser T, Pillay J, Koenderman L, Leenen LPH. Postinjury immune monitoring: can multiple organ failure be predicted? *Curr Opin Crit Care.* (2008) 14:666–72. doi: 10.1097/MCC.0B013E3283196522

28. Xiao W, Mindrinos MN, Seok J, Cuschieri J, Cuenca AG, Gao H, et al. A genomic storm in critically injured humans. *J Exp Med.* (2011) 208:2581–90. doi: 10.1084/JEM.20111354

29. Hazeldine J, Naumann DN, Toman E, Davies D, Bishop JRB, Su Z, et al. Prehospital immune responses and development of multiple organ dysfunction syndrome following traumatic injury: a prospective cohort study. *PLoS Med.* (2017) 14:1–29. doi: 10.1371/journal.pmed.1002338

30. Timmermans K, Kox M, Vaneker M, van den Berg M, John A, van Laarhoven A, et al. Plasma levels of danger-associated molecular patterns are associated with immune suppression in trauma patients. *Intensive Care Med.* (2016) 42:551–61. doi: 10.1007/S00134-015-4205-3

31. Pape HC, Giannoudis P, Krettek C. The timing of fracture treatment in polytrauma patients: relevance of damage control orthopedic surgery. *Am J Surg.* (2002) 183:622–9. doi: 10.1016/S0002-9610(02)00865-6

32. Berwin JT, Pearce O, Harries L, Kelly M. Managing polytrauma patients. *Injury.* (2020) 51:2091–6. doi: 10.1016/j.injury.2020.07.051

33. Van Wessem KJP, Leenen LPH. Reduction in mortality rates of postinjury multiple organ dysfunction syndrome: a shifting paradigm? A prospective population-based cohort study. *Shock.* (2018) 49:33–8. doi: 10.1097/SHK.0000000000000938

34. van Wessem KJP, Leenen LPH. Incidence of acute respiratory distress syndrome and associated mortality in a polytrauma population. *Trauma Surg Acute Care Open.* (2018) 3:e000232. doi: 10.1136/tsaco-2018-000232

35. Sauaia A, Moore EE, Johnson JL, Chin TL, Banerjee A, Sperry JL, et al. Temporal trends of postinjury multiple-organ failure: still resource intensive, morbid, and lethal. *J Trauma Acute Care Surg.* (2014) 76:582–93. doi: 10.1097/TA.0000000000000147

36. Hietbrink F, Koenderman L, Leenen LPH. Intramedullary nailing of the femur and the systemic activation of monocytes and neutrophils. *World J Emerg Surg.* (2011) 6:1–6. doi: 10.1186/1749-7922-6-34

37. Hietbrink F, Koenderman L, Van Wessem KJP, Leenen LPH. The Impact of intramedullary nailing of tibia fractures on the innate immune system. *Shock.* (2015) 44:209–14. doi: 10.1097/SHK.0000000000000405

38. Heeres M, Visser T, van Wessem KJP, Koenderman AHL, Strengers PFW, Koenderman L, et al. The effect of C1-esterase inhibitor on systemic inflammation in trauma patients with a femur fracture – the CAESAR study: study protocol for a randomized controlled trial. *Trials.* (2011) 12:223. doi: 10.1186/1745-6215-12-223

39. van Wessem, KJP, Leenen LPH, Hietbrink F. Physiology dictated treatment after severe trauma: timing is everything. *Eur J Trauma Emerg Surg.* (2022) doi: 10.1007/s00068-022-01916-z [Epub ahead of print].

40. Evans JA, Van Wessem KJP, McDougall D, Lee KA, Lyons T, Balogh ZJ. Epidemiology of traumatic deaths: comprehensive population-based assessment. *World J Surg.* (2010) 34:158–63. doi: 10.1007/S00268-009-0266-1

41. Pfeifer R, Tarkin IS, Rocos B, Pape HC. Patterns of mortality and causes of death in polytrauma patients–has anything changed? *Injury.* (2009) 40:907–11. doi: 10.1016/J.INJURY.2009.05.006

42. Huber-Lang M, Lambris JD, Ward PA. Innate immune responses to trauma review-article. *Nat Immunol.* (2018) 19:327–41. doi: 10.1038/s41590-018-0064-8

43. Chang DC, Cornwell EE, Phillips J, Paradise J, Campbell K. Early leukocytosis in trauma patients: what difference does it make? *Curr Surg.* (2003) 60:632–5. doi: 10.1016/j.cursur.2003.07.011

44. Lam SW, Leenen LPH, Van Solinge WW, Hietbrink F, Huisman A. Evaluation of hematological parameters on admission for the prediction of 7-day in-hospital mortality in a large trauma cohort. *Clin Chem Lab Med.* (2011) 49:493–9. doi: 10.1515/CCLM.2011.069

45. Lam SW, Leenen LPH, Van Solinge WW, Hietbrink F, Huisman A. Comparison between the prognostic value of the white blood cell differential count and morphological parameters of neutrophils and lymphocytes in severely injured patients for 7-day in-hospital mortality. *Biomarkers.* (2012) 17:642–7. doi: 10.3109/1354750X.2012.712161

46. Hesselink L, Heeres M, Paraschiakos F, Ten Berg M, Huisman A, Hoefer IE, et al. A rise in neutrophil cell size precedes organ dysfunction after trauma. *Shock.* (2019) 51:439–46. doi: 10.1097/SHK.0000000000001200

47. Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. *J Trauma.* (1995) 39:411–7. doi: 10.1097/00005373-199509000-00003

48. van Hout GPJ, van Solinge WW, Gijsberts CM, Teuben MPJ, Leliefeld PHC, Heeres M, et al. Elevated mean neutrophil volume represents altered neutrophil composition and reflects damage after myocardial infarction. *Basic Res Cardiol.* (2015) 110:58. doi: 10.1007/S00395-015-0513-6

49. Köller M, Wick M, Muhr G. Decreased leukotriene release from neutrophils after severe trauma: role of immature cells. *Inflammation.* (2001) 25:53–9. doi: 10.1023/A:1007027712387

50. Pillay J, Kamp VM, Van Hoffen E, Visser T, Tak T, Lammers JW, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest.* (2012) 122:327–36. doi: 10.1172/JCI57990

51. Chung HN, Jong WC, Lee J. Delta neutrophil index in automated immature granulocyte counts for assessing disease severity of patients with sepsis. *Ann Clin Lab Sci.* (2008) 38:241–6.

52. Hellebrekers P, Hesselink L, Huisman A, ten Berg M, Koenderman L, Leenen LPH, et al. Recognizing the mobilization of neutrophils with banded nuclei early after trauma. *Int J Lab Hematol.* (2020) 42:e224–7. doi: 10.1111/ijlh.13272

53. Hampson P, Dinsdale RJ, Wearn CM, Bamford AL, Bishop JRB, Hazeldine J, et al. Neutrophil dysfunction, immature granulocytes, and cell-free DNA are early biomarkers of sepsis in burn-injured patients: a prospective observational cohort study. *Ann Surg.* (2017) 265:1241–9. doi: 10.1097/SLA.0000000000001807

54. Tak T, Wijten P, Heeres M, Pickkers P, Scholten A, Heck AJR, et al. Human CD62Ldim neutrophils identified as a separate subset by proteome profiling and in vivo pulse-chase labeling. *Blood.* (2017) 129:3476–85. doi: 10.1182/blood-2016-07-727669

55. Pillay J, Den Braber I, Vrisekoop N, Kwast LM, De Boer RJ, Borghans JAM, et al. In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood.* (2010) 116:625–7. doi: 10.1182/blood-2010-01-259028

56. Overbeeke C, Tak T, Koenderman L. The journey of neutropoiesis : how complex landscapes in bone marrow guide continuous neutrophil lineage determination. *Blood.* (2021) 39:2285–93. doi: 10.1182/blood.2021012835

57. Livingston DH, Anjaria D, Wu J, Hauser CJ, Chang V, Deitch EA, et al. Bone marrow failure following severe injury in humans. *Ann Surg.* (2003) 238:748–53. doi: 10.1097/01.sla.0000094441.38807.09

58. Cole E, Davenport R, Willett K, Brohi K. The burden of infection in severely injured trauma patients and the relationship with admission shock severity. *J Trauma Acute Care Surg.* (2014) 76:730–5. doi: 10.1097/TA.0b013e31829fdbd7

59. Carp H. Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils. *J Exp Med.* (1982) 155:264–75. doi: 10.1084/JEM.155.1.264

60. Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, et al. Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. *J Biol Chem*. (1984) 259:5430–9. doi: 10.1016/S0021-9258(18)91029-X

61. Chatham WW, Turkiewicz A, Blackburn WD. Determinants of neutrophil HOCl generation: ligand-dependent responses and the role of surface adhesion. *J Leukoc Biol*. (1994) 56:654–60. doi: 10.1002/JLB.56.5.654

62. Ye RD, Boulay F, Ji MW, Dahlgren C, Gerard C, Parmentier M, et al. International union of basic and clinical pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol Rev*. (2009) 61:119–61. doi: 10.1124/PR.109.001578

63. Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev*. (2007) 219:88–102. doi: 10.1111/J.1600-065X.2007.00550.X

64. Li S, Huang X, Chen Z, Zhong H, Peng Q, Deng Y, et al. Neutrophil CD64 expression as a biomarker in the early diagnosis of bacterial infection: a meta-analysis. *Int J Infect Dis*. (2013) 17:e902–13. doi: 10.1016/j.ijid.2012.07.017

65. Schiff DE, Rae J, Martin TR, Davis BH, Curnutte JT. Increased phagocyte FcγRI expression and improved Fcγ-receptor- mediated phagocytosis after in vivo recombinant human interferon-γ treatment of normal human subjects. *Blood*. (1997) 90:3187–94. doi: 10.1182/blood.v90.8.3187

66. Fjaertoft G, Håkansson LD, Pauksens K, Sisask G, Venge P. Neutrophil CD64 (FcgammaRI) expression is a specific marker of bacterial infection: a study on the kinetics and the impact of major surgery. *Scand J Infect Dis*. (2007) 39:525–35. doi: 10.1080/00365540601113693

67. Mahmoodpoor A, Paknezhad S, Shadvar K, Hamishehkar H, Movassaghpour AA, Sanaie S, et al. Flow cytometry of CD64, HLA-DR, CD25, and TLRs for diagnosis and prognosis of sepsis in critically Ill Patients admitted to the intensive care unit: a review article. *Anesthesiol pain Med*. (2018) 8:e83128. doi: 10.5812/AAPM.83128

68. Mahmoodpoor A, Movassaghpour A, Talebi M, Shadvar K, Soleimanpour H. Value of flow cytometry (HLA-DR, CD14, CD25, CD13, CD64) in prediction of prognosis in critically ill septic patients admitted to ICU: a pilot study. *J Clin Anesth*. (2020) 61:109646. doi: 10.1016/J.JCLINANE.2019.109646

69. Cong S, Ma T, Di X, Tian C, Zhao M, Wang K. Diagnostic value of neutrophil CD64, procalcitonin, and interleukin-6 in sepsis: a meta-analysis. *BMC Infect Dis*. (2021) 21:384. doi: 10.1186/s12879-021-06064-0

70. Thiriet C, Mahjoub K, Courte G, Labroca P, Cravoisy A, Lemarie J, et al. Automated measurement of neutrophil CD64 expression for diagnosing sepsis in critically ill patients. *Minerva Anestesiol*. (2019) 85:943–50. doi: 10.23736/S0375-9393.19.13420-7

71. Ye Z, Zou H, Liu S, Mei C, Chang X, Hu Z, et al. Diagnostic performance of neutrophil CD64 index in patients with sepsis in the intensive care unit. *J Int Med Res*. (2019) 47:4304–11. doi: 10.1177/0300060519860677

72. Pfeifer R, Pape HC. Diagnostik und versorgungsstrategien beim polytraumatisierten patienten. *Der Chir*. (2016) 87:165–75. doi: 10.1007/s00104-015-0139-0

73. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. (2013) 13:159–75. doi: 10.1038/nri3399

74. Bongers S, Hellebrekers P, Leenen LPH, Koenderman L, Hietbrink F. Intracellular penetration and effects of antibiotics on staphylococcus aureus inside human neutrophils: a comprehensive review. *Antibiotics*. (2019) 8:1–22. doi: 10.3390/antibiotics8020054

75. Fraunholz M, Sinha B. Intracellular *Staphylococcus aureus*: live-in and let die. *Front Cell Infect Microbiol*. (2012) 2:43. doi: 10.3389/fcimb.2012.00043

76. Jämsä J, Ala-Kokko T, Huotari V, Ohtonen P, Savolainen ER, Syrjälä H. Neutrophil CD64, C-reactive protein, and procalcitonin in the identification of sepsis in the ICU – post-test probabilities. *J Crit Care*. (2018) 43:139–42. doi: 10.1016/J.JCRC.2017.08.038

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Role of prothrombin complex concentrate in the severe trauma patient

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Introduction

Traumatic hemorrhagic shock causes an estimated annual loss of more than 75 million years of life globally, and mostly affects young people (1). It is a time-dependent process, in which prehospital and emergency interventions must be sufficient to prevent the lethal triad of hypothermia, acidosis, and coagulopathy (2), and to avoid trauma-induced coagulopathy (TIC). Both are key determinants of trauma-associated mortality, so early control is essential to improve prognosis and reduce this outcome (3). During hemorrhagic shock, special attention needs to be paid to hyperfibrinolysis and deficiency of coagulation factors. The latter may be deficient for various reasons, such as loss due to hemorrhage, dilution due to vigorous fluid resuscitation, or the activation of pathophysiological mechanisms in TIC that mediate dysfunction (1, 4, 5).

From a therapeutic point of view, the administration of fresh frozen plasma (FFP) has conventionally been the only option to compensate TIC. However, this strategy is associated with a number of limitations such as fluid overload, variability in the quantity and quality of coagulation factors administered, as well as the need for compatibility with the patient's blood group and thawing prior to infusion. The use of FFP has also been associated with an increased risk of respiratory distress and multiple organ failure (6).

Prothrombin complex concentrate (PCC) could be considered an option within the emergent management of severe trauma patients, given its advantages such as lower infusion volume, immediate bioavailability, and compatibility with all blood groups. Additionally, a correct assistance of these patients requires a time-dependent management, thus, PCC can be administered immediately which makes it highly favorable. It is important to note that the composition of individual coagulation factors and the content of anticoagulants, especially heparin, as well as the indications vary among PCC from different manufacturers (7, 8). Therefore, their function and indications also differ.

Use of prothrombin complex in the undercoagulated patient

In recent years, the management of undercoagulated patients has improved with the introduction of new direct-acting oral anticoagulants, such as thrombin and factor

X inhibitors. Nonetheless, the incorporation of these drugs into the standard therapeutic arsenal of this population presents a challenge for urgent reversal of coagulation in the undercoagulated severe trauma patient (9). Although these direct-acting drugs have antidotes, not all are marketed in Spain, despite being authorized by the Spanish Agency for Medicines and Medical Devices (AEMPS). Accordingly, in these cases, the therapeutic alternatives involve waiting for the half-life of the drug, dialyzing the patient, if feasible, or administering high doses of PCC (25–50 IU/kg) together with tranexamic acid (9, 10). Studies have identified the reversal capacity of this strategy, reporting a good safety profile, and indeed the authors of one such paper recommend performing a comparison between PCC and andexanet alfa (antidote against thrombin inhibitors) to establish the best alternative in each case (7).

However, the approved indications for PCC are limited to antagonizing the effect of vitamin K-dependent coagulation factor inhibitors and treating congenital coagulation factor deficiency. The latest guidelines for severe polytrauma patients recommend the direct application of PCC in the emergent reversal of patients anticoagulated with vitamin K antagonists, based on the patient's weight and international normalized ratio (INR) (11). Furthermore, observational studies in traumatic brain injuries have found that PCC, as in spontaneous intracerebral hemorrhage, rapidly normalizes the INR, limiting the intracerebral injury and improving the prognosis of these patients (12).

Application of PCC in the urgent management of the polytrauma patient

Although some experimental studies report rapid recovery of hemostasis with the use of PCC, its application in the urgent management of the polytrauma patient with TIC is underdeveloped (13). This may be due in part to concerns over the risk of using a drug that increases the availability of thrombin and, therefore, the patient's thromboembolic risk. However, evidence regarding the reversal of coagulation in anticoagulated patients shows that the risk—estimated to be around 1.8%—is relatively low (14).

It is important to note that the pharmacological management of TIC should not focus solely on the urgent infusion of coagulation factors in the form of PCC. The possibility of administering both fibrinogen and tranexamic acid must first be evaluated (3, 4, 10). Moreover, the accumulation of thrombin that cannot interact with fibrinogen will not result in the fibrin necessary to generate the clot; furthermore, if hyperfibrinolysis occurs, the TIC will not be reversed. In fact, the isolated use of PCC in trauma does not allow optimization of viscoelastic test parameters unless it is combined with the administration of fibrinogen (15). Specifically, the parameter that should guide our actions when deciding to administer PCC is extrinsic thromboelastometry [EXTEM CT (clotting time)], provided that fibrinogen values have been restored (10). In this regard, it should be noted that the use of PCC has been reported to reduce the use of blood products compared to FFP (16). However, in this study, the control group was a historical cohort in which the baseline blood fibrinogen concentration was not considered, possibly generating bias. Other studies suggest that one benefit of PCC is that fewer transfusions are required after its use; even so, the authors are cautious about the use of PCC as first-line therapy in the management of severe polytrauma patients (17).

When should we use prothrombin complex to reverse TIC?

Various factors should be considered when deciding when to use PCC to reverse TIC: (i) Is this a massive bleed? (ii) Is there compromised space or organ-specific involvement (e.g., central nervous system, pharyngeal hematoma, intraocular hematoma, etc.)? (iii) Is there persistent major bleeding? (iv) Is urgent surgery or surgery with high bleeding risk needed? and (v) Are no other time-dependent options available (as happens in situations of war or catastrophes, etc.,)? Finally, the criterion of the healthcare team should carry a specific weight when determining the use of PCC. Given that early initiation of all medical-surgical interventions is essential for the successful management of TIC, we found studies that have evaluated the role of the normalization of hemostasis during the prehospital care of patients by emergency teams, some of which were successful and others somewhat contradictory (18, 19).

It is important to note that in order to have objective parameters on which to base the management of TIC in emergencies, the use of PCC must always be guided, either by conventional tests, such as INR, or by the support of viscoelastic tests (in case of EXTEM CT >80 s) (10, 16). In this regard, the algorithm presented herein (Figure 1) may be very useful for decision-making in these patients. The logistics and specific circumstances of each emergency unit must be taken into consideration, so that this algorithm can be adapted to the local protocol.

Conclusion

Limited therapeutic strategies are available today in the management of TIC, and those that are, are based on the

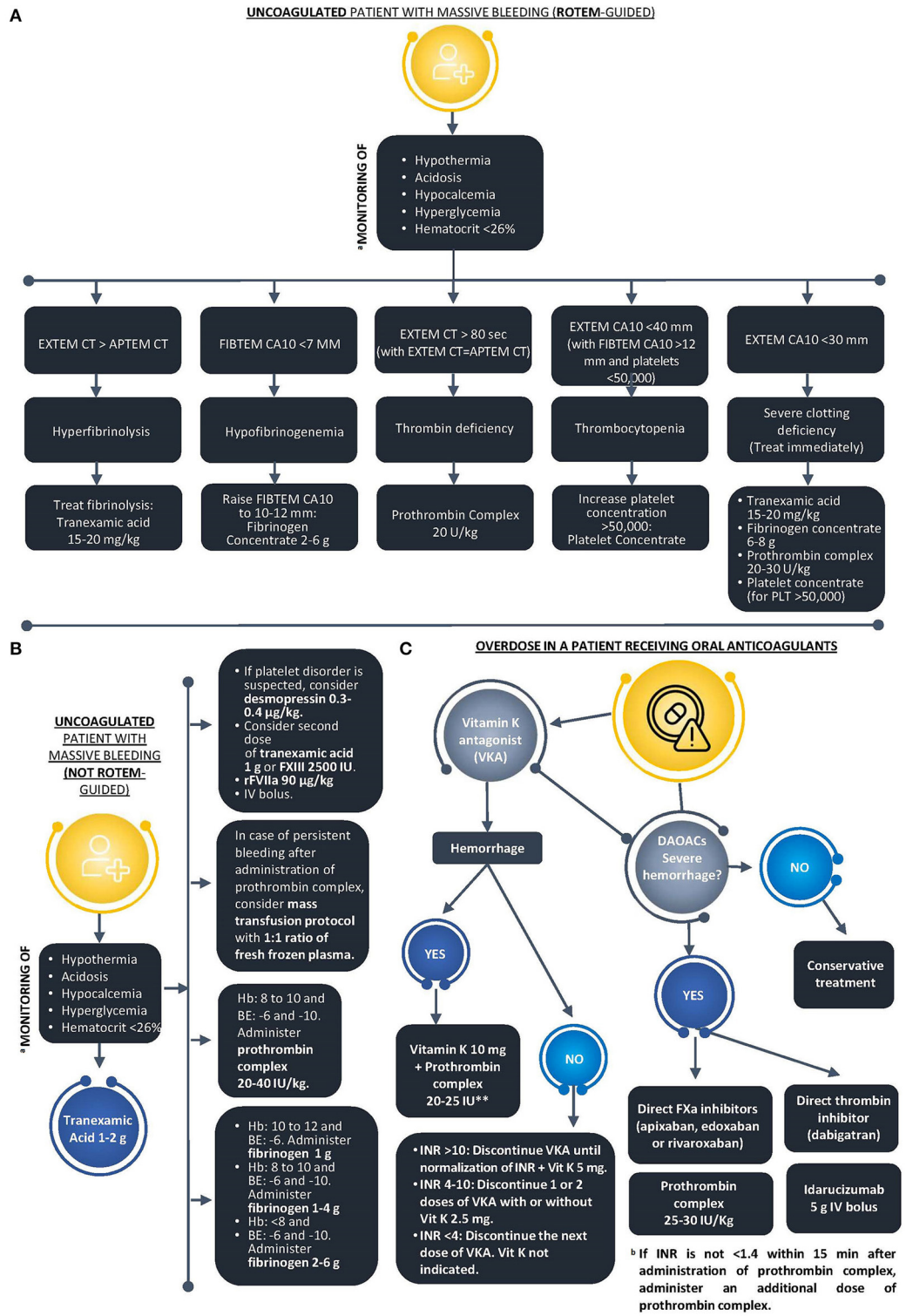

FIGURE 1
Coagulation support scheme for massive bleeding in two different scenarios: **(A)** Uncoagulated patient with massive bleeding (ROTEM-guided). **(B)** Uncoagulated patient with massive bleeding (not ROTEM-guided). **(C)** Overdose in a patient receiving oral anticoagulants. [a]The presence of the lethal triad is not a prerequisite to activate the algorithm; however, we stress the need to look for those signs during the approach of severe polytrauma patients. [b]Fixed doses 1000 UI. APTEM, aprotinin thromboelastometry; APTT, activated partial thromboplastin time; CT, clotting time; EXTEM, extrinsic thromboelastometry; FIBTEM, fibrinogen thromboelastometry; Hb, hemoglobin; HEPTEM, heparinase-modified thromboelastometry; INR, international normalized ratio; INTEM, intrinsic thromboelastometry; MCF, maximum clot firmness; ML, maximum lysis; PCC, prothrombin complex concentrate; PCF, platelet contractile force; PT, prothrombin time; ROTEM, rotational thromboelastometry.

correction of coagulopathy triggers and associated processes, and the replacement of coagulation factors. PCC is a useful therapeutic option with a proven cost-effectiveness balance in different emergency departments in Spain that should be analyzed in greater detail in the near future (10). However, more data from clinical trials are needed if we are to offer our severe trauma patients safe and personalized interventions. We must not forget that while increasing thrombin production is a fundamental process in the management of patients in traumatic hemorrhagic shock, fibrinolysis must be simultaneously controlled and platelet function and clot formation must be monitored. Otherwise, management will not be well balanced and the results may not be as expected, impacting on TIC and the outcome of the severe trauma patient.

Author contributions

JE-G and MQ-D made a substantial contribution to the concept of the article. JE-G drafted the preliminary version of the article. Both authors read and approved the final version of the article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Cannon JW. Hemorrhagic shock. *N Engl J Med.* (2018) 378:370–9. doi: 10.1056/NEJMra1705649

2. Quintana-Díaz M, Garay-Fernández M, Ariza-Cadena F. Advancing in the understanding of coagulopathy during hemorrhagic shock: from the triad to the deadly pentad. *Col J Anesthesiol.* (2022) 50. doi: 10.5554/22562087.e1038. Available online at: https://www.revcolanest.com.co/index.php/rca/article/view/1038

3. Diaz M, Alonso D, de Lorenzo y Mateos A. Coagulation and hemorrhage in the critically-ill Pattern, diagnostic tests and etiology. *Med Intensiva.* (2003) 27:605–14. doi: 10.1016/S0210-5691(03)79973-2

4. Egea-Guerrero J, Freire-Aragón M, Serrano-Lázaro A, Quintana-Díaz M. Resuscitative goals and new strategies in severe trauma patient resuscitation. *Med Intensiva.* (2014) 38:502–12. doi: 10.1016/j.medine.2014.06.001

5. Egea-Guerrero JJ, Ballesteros M, Quintana-Díaz M. Tranexamic acid in patients with multiple injuries: good, elegant, and cheap? *Emergencias.* (2019) 31:281–2.

6. Watson GA, Sperry JL, Rosengart MR, Minei JP, Harbrecht BG, Moore EE, et al. Fresh frozen plasma is independently associated with a higher risk of multiple organ failure and acute respiratory distress syndrome. *J Trauma Acute Care Surg.* (2009) 67:221–30. doi: 10.1097/TA.0b013e3181ad5957

7. van den Brink DP, Wirtz MR, Neto AS, Schöchl H, Viersen V, Binnekade J, et al. Effectiveness of prothrombin complex concentrate for the treatment of bleeding: a systematic review and meta-analysis. *J Thromb Haemost.* (2020) 18:2457–67. doi: 10.1111/jth.14991

8. Ghadimi K, Levy JH, Welsby IJ. Prothrombin complex concentrates for bleeding in the perioperative setting. *Anesth Analg.* (2016) 122:1287–300. doi: 10.1213/ANE.0000000000001188

9. Rossaint R, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernández-Mondéjar E, et al. The European guideline on management of major bleeding and coagulopathy following trauma. *Crit Care.* (2016) 20:1–55. doi: 10.1186/s13054-016-1265-x

10. Steiner T, Poli S, Griebe M, Hüsing J, Hajda J, Freiberger A, et al. Fresh frozen plasma versus prothrombin complex concentrate in patients with intracranial haemorrhage related to vitamin K antagonists (INCH): a randomised trial. *Lancet Neurol.* (2016) 15:566–73. doi: 10.1016/S1474-4422(16)00110-1

11. Spahn DR, Bouillon B, Cerny V, Duranteau J, Filipescu D, Hunt BJ, et al. The European guideline on management of major bleeding and coagulopathy following trauma: fifth edition. *Crit Care.* (2019) 23:98. doi: 10.1186/s13054-019-2347-3

12. Bavalia R, Abdoellakhan R, Brinkman HJM, Brekelmans MP, Hamulyák EN, Zuurveld M, et al. Emergencies on direct oral anticoagulants: management, outcomes, and laboratory effects of prothrombin complex concentrate. *Res Pract Thromb Haemost.* (2020) 4:569–81. doi: 10.1002/rth2.12336

13. Dickneite G. Prothrombin complex concentrate versus recombinant factor VIIa for reversal of coumarin anticoagulation. *Thromb Res.* (2007) 119:643–51. doi: 10.1016/j.thromres.2006.05.011

14. Chowdary P, Tang A, Watson D, Besser M, Collins P, Creagh MD, et al. Retrospective review of a prothrombin complex concentrate (Beriplex P/N) for the management of perioperative bleeding unrelated to oral anticoagulation. *Clin Appl Thromb Hemost.* (2018) 24:1159–69. doi: 10.1177/1076029617753537

15. Ponschab M, Voelckel W, Pavelka M, Schlimp CJ, Schöchl H. Effect of coagulation factor concentrate administration on ROTEM® parameters in major trauma. *Scand J Trauma Resusc Emerg Med.* (2015) 23:1–7. doi: 10.1186/s13049-015-0165-4

16. Schöchl H, Nienaber U, Maegele M, Hochleitner G, Primavesi F, Steitz B, et al. Transfusion in trauma: thromboelastometry-guided coagulation factor concentrate-based therapy versus standard fresh frozen plasma-based therapy. *Crit Care.* (2011) 15:1–9. doi: 10.1186/cc10078

17. Ferreira J, DeLosSantos M. The clinical use of prothrombin complex concentrate. *J Emerg Med.* (2013) 44:1201–10. doi: 10.1016/j.jemermed.2012.12.022

18. Schöchl H, Schlimp CJ, Maegele M. Tranexamic acid, fibrinogen concentrate, and prothrombin complex concentrate: data to support prehospital use? *Shock.* (2014) 41:44–6. doi: 10.1097/SHK.0000000000000093

19. Palomo-López N, Quintana-Díaz M, Egea-Guerrero JJ. Early fresh frozen plasma for hemorrhagic shock: are we sure? *Emergencias.* (2019) 31:353–4.

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Neutrophil phenotypes implicated in the pathophysiology of post-traumatic sepsis

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Background: The disruption of immune homeostasis after trauma is a major cause of post-traumatic organ dysfunction and/or sepsis. Recently, a variety of neutrophil phenotypes with distinct functions have been identified and suggested as involved in various clinical conditions. The association between neutrophil phenotypes and post-traumatic immunodeficiency has also been reported, yet the specific neutrophil phenotypes and their functional significance in post-traumatic sepsis have not been fully clarified. Therefore, we sought to investigate neutrophil phenotypic changes in a murine model, as these may hold prognostic value in post-traumatic sepsis.

Materials and methods: Third-degree burns affecting 25% of the body surface area were used to establish trauma model, and sepsis was induced 24 h later through cecal ligation and puncture (CLP). The Burn/CLP post-traumatic sepsis model and the Sham/CLP control model were established to assess the immunological status after trauma. Histopathological evaluation was performed on the spleen, liver, kidneys, and lung tissues. Immunological evaluation included the assessment of neutrophil markers using mass cytometry as well as cytokine measurements in serum and ascitic fluid through multiplex analysis using LUMINEX®.

Results: The Burn/CLP group had a lower survival rate than the Sham/CLP group. Histopathological examination revealed an impaired immune response and more advanced organ damage in the Burn/CLP group. Furthermore, the Burn/CLP group exhibited higher levels of transforming growth factor-beta 1 in the blood and generally lower levels of cytokines than the Sham/CLP group. CD11b, which is involved in neutrophil adhesion and migration, was highly expressed on neutrophils in the Burn/CLP group. The expression of CD172a, which is related to the inhibition of phagocytosis, was also upregulated on neutrophils in the Burn/CLP group. The expression of sialic acid-binding Ig-like lectin F and CD68 also differed between the two groups.

Conclusion: Different neutrophil phenotypes were observed between Burn/CLP and Sham/CLP groups, suggesting that neutrophils are implicated in the immune imbalance following trauma. However, further studies are needed to prove the causal relationships between neutrophil phenotypes and outcomes, including survival rate and organ dysfunction.

KEYWORDS

neutrophil phenotype, post-traumatic sepsis, innate immune system, CD11b, SIRPα, Siglec-F, CD68

Introduction

For over 50 years, trauma has been one of the leading causes of death worldwide, especially among working-age populations (1, 2). Although many patients die due to macrovascular injuries, severe brain injury, or hemorrhage in the hyperacute or acute phase after trauma, survival rates have increased owing to the improved management of trauma patients through transfusion therapy and surgical hemostasis techniques, among other medical advances (3–5).

Despite surviving the acute phase of trauma, a number of patients lose their lives within days to weeks after injury (6, 7). Subacute death in trauma patients is associated with the disruption of immune homeostasis (8). Once trauma occurs, the innate immune system drives a pro-inflammatory response, and an anti-inflammatory response mediated by the adaptive immune system then arises in response to an excessive initial pro-inflammatory signaling (8). An imbalance between pro- and anti-inflammatory responses can lead to the development of systemic inflammatory response syndrome (SIRS) or compensatory anti-inflammatory response syndrome (CARS), resulting in organ damage and/or sepsis (7, 8). However, the treatment of post-traumatic conditions caused by an immune imbalance mainly consists of supportive care, and no curative treatment has yet been established.

The innate immune response eliminates invading pathogens at the initial stage of infection, mainly through the activity of neutrophil and monocyte lineages (9, 10). In recent years, the existence of neutrophil phenotypes with distinct functions has been recognized (11–14). In particular, anti-inflammatory neutrophils have been suggested as involved in the development of CARS and subsequent secondary infections (9, 14). Polymorphonuclear myeloid-derived suppressor cells, which are produced by a variety of infections, including bacterial, and known to act as immunosuppressive agents in sepsis, have also attracted attention in recent years (15). Moreover, neutrophils polarize in the same manner as pro-inflammatory and anti-inflammatory macrophages, which are activated by Th1 and Th2 type cytokines, respectively (16). In the field of cancer, it has been shown that inhibition of transforming growth factor-beta 1 (TGF-β1) signaling, which is implicated in cell death and differentiation, increases the number of pro-inflammatory neutrophils (16). A previous study showed that the balance between pro- and anti-inflammatory neutrophils may be involved in the ventricular remodeling process during myocardial inflammation after infarction (17). Further, toll-like receptor (TLR) 4 deficiency in the central nervous system protected the brain after stroke by increasing the percentage of anti-inflammatory neutrophils (18).

Immunodeficiency after trauma has been studied from various perspectives (6–8, 19–24), including through a focus on neutrophils (25–27). However, the involvement of neutrophils in the pathogenesis of post-traumatic sepsis remains elusive. To investigate this issue, we conducted a study with two features. First, we employed the burn injury model as the experimental trauma model in this study. This model causes tissue damage with decreased circulating blood and has been used in many post-traumatic immunological studies (6, 7, 20–24). By using this model, we can avoid traumatic brain injury, which leads to specific immune responses (28, 29). Moreover, unlike other trauma models, the third-degree burn model is favorable from the perspective that there is no pain after the injury. Second, we employed mass cytometry (cytometry by time-of-flight: CyTOF) to reveal a wide range of functional changes in neutrophils and to investigate neutrophil phenotypes, which may influence the prognosis of post-traumatic sepsis patients.

Materials and methods

Ethics statement

All animal protocols performed in this study were approved by the Hokkaido University Animal Experiment Regulations and the Institutional Ethical Review Board at Hokkaido University (Approval number: 19-0167).

Mice

Outbred male Institute of Cancer Research (ICR) mice were purchased from the Sankyo Labo Service Corporation,

Inc. (Tokyo, Japan). Mouse housing was set up to maintain the appropriate temperature and humidity, with automatic light/dark switching every 12 h. The mice were supplied with standard chow (Nosan Corporation, Yokohama, Japan) and water *ad libitum* for the entire study. The mice were acclimated to the environment for at least 72 h before use in the experiments.

Trauma model

A burn trauma model was established as previously described (20, 22–24, 30). The mice were anesthetized *via* intraperitoneal administration of ketamine/xylazine (125/20 mg/kg), and buprenorphine (0.05 mg/kg) was subcutaneously administered for analgesia before treatment. After shaving of the dorsal hair, an anesthetized mouse was placed and immobilized in a plastic mold so as to expose 25% of the body surface area. The exposed dorsum was immersed in 90°C hot water for 9 s to induce burn, and in 24°C water for 9 s for the sham model. A subcutaneous injection of 1 ml of 0.9% pyrogen-free saline was administered immediately after the resuscitation procedure. A full-thickness and well-demarcated anesthetic third-degree burn injury was induced. No post-injury analgesics were used because the burns were applied to a painless depth. The mortality rate of model mice established using this protocol was below 5%, as previously reported (6, 7, 20, 23, 24) and observed in our laboratory.

Sepsis model

A cecal ligation and puncture (CLP) sepsis model was established as previously described (31–33). The mice were anesthetized in the same manner as for the burn protocol, and their abdominal fur was shaved. A 1 cm skin and peritoneal incision was made vertically in the midline of the abdomen, and the cecum was gently exposed after resection of the adipose tissue on both sides (34). Ligation was performed at 50% of the cecum, with two punctures with a 21-gauge needle 5 mm from the blind end. The method yielded a survival rate equivalent to the sepsis survival rate in humans (65–80%) (35–37). The cecum was returned to the abdominal cavity, whereafter the peritoneum was sutured with five stitches and the skin with three stitches using a 4-0 nylon thread. Resuscitation was performed *via* subcutaneous injection of 1 ml 0.9% pyrogen-free saline after CLP. The CLP procedure was performed on a hot plate (Heater Mat KN-475, Natsume Seisakusho, Co., Ltd., Tokyo, Japan) and maintained at approximately 38°C. To prevent surgical site infection, post-operative mice were kept in the supine position until the anesthetic effect wore off and they regained consciousness. To prevent post-operative hypothermia, the cage was placed on a hot plate until the mice woke up from anesthesia (38). Antibiotic treatment began 2 h after the CLP procedure, with a subcutaneous injection of imipenem/cilastatin (25 mg/kg) repeated every 12 h for the first 3 days (for a total of six injections) (39).

Schedule of experiments

Two murine models of post-traumatic sepsis, the Burn/CLP and Sham/CLP models, were established. The first day of trauma infliction was set as day 0, and CLP was performed 24 h after injury (day 1). Fifteen mice from each group were followed up until day 15 (14 days after CLP) to determine survival rates. Five mice from each group were used for histopathological, immunological, and serological evaluation. For histopathology only, five mice with neither trauma nor sepsis were used as controls in addition to the Burn/CLP and Sham/CLP models. Samples for histopathological, immunological, and serological examination were collected 24 h after CLP.

Histopathological evaluation

Twenty-four h after CLP, the mice were anesthetized and euthanized through the intraperitoneal administration of ketamine/xylazine (250/40 mg/kg) at twice the dose used in trauma and sepsis models, and were then placed in a supine position. The liver, kidney, spleen, and lung tissues were excised from five mice per group for histological evaluation. The organs were fixed in 10% formalin neutral buffer solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 2 weeks, following the fixation method in our other study, and the slides were prepared by Morphotechnology Co., Ltd. (Sapporo, Japan). Hematoxylin and eosin (H&E) staining was performed.

Cell preparation

Ascitic fluid and blood samples were collected from five mice per group. The mice were anesthetized and euthanized as done for histopathological evaluation and were then placed in a supine position. The abdominal skin was newly incised avoiding the CLP wound, the exposed peritoneum was then punctured, and 5 ml of 0.9% pyrogen-free saline was injected into the peritoneal cavity. The abdomen of each mouse was rubbed gently to ensure that the injected saline was evenly mixed. A 5 mm peritoneal incision was then made, through which as much ascitic fluid as possible (3–4 ml) was collected using a 2.5 ml syringe. Ascitic fluid was collected in a centrifuge tube pre-filled with 0.5 ml of 169 mM EDTA (EDTA tripotassium salt dihydrate 0.075 g/ml of water). Terminal blood collection was

subsequently conducted on mice maintained under anesthesia by puncturing the cardiac fossa with a 1 ml syringe pre-filled with 0.1 ml of 169 mM EDTA.

Cells in the samples were prepared in culture medium (C5) containing RPMI 1640, 5% heat-inactivated FCS, 1 mM glutamine, 10 mM HEPES, 2 mM non-essential amino acids, penicillin/streptomycin/fungizone, and 2.5×10^{-5} M 2-mercaptoethanol. All of the above-listed products included in the C5 medium were purchased from Life Technologies Corporation (Carlsbad, CA, USA). Blood samples were washed and centrifuged repeatedly with C5 after removal of red blood cells (RBCs) using ammonium chloride-based mouse RBC lysis buffer (JL-buffer), which was developed by the Lederer Lab at Harvard Medical School (20). Ascitic fluid samples were also washed and centrifuged repeatedly using C5. The pellet containing cells at the bottom of the centrifuge tube was collected with 0.5 ml of cell freezing medium CryoStor® (Biolife Solutions, Inc., Bothell, WA, USA) and stored in a tube. To avoid rapid cooling, the tubes filled with cells in the CryoStor® were set in Mr. Frosty® (Thermo Fisher Scientific, Waltham, MA, USA), which had been pre-chilled in a refrigerator at 4°C. The tubes with cells in Mr. Frosty were first placed in a refrigerator at 4°C for 20 min and then kept in a −80°C freezer overnight (12–24 h). The tubes with cells were taken from Mr. Frosty and immediately stored at −196°C in a liquid nitrogen tank until mass cytometry was performed.

Mass cytometry

Cytometry by time-of-flight was performed for immunological evaluation. All CyTOF staining procedures were performed at room temperature. Cells were first stained with a cisplatin viability staining reagent (Fluidigm Sciences, South San Francisco, CA, USA) for 5 min and washed *via* centrifugation. Fc-blocking agents were added to the cells for 10 min before adding the CyTOF antibody staining cocktail. Cells were stained for 30 min and washed with CyTOF staining buffer (calcium/magnesium-free PBS, 0.05% sodium azide, and 0.2% bovine serum albumin). For fixation and permeabilization of the cells, we used Maxpar® Fix I Buffer/Maxpar 10X Barcode Perm Buffer (Fluidigm Sciences), which was incubated with a palladium-based barcode reagent for 30 min. The samples were pooled into a single tube after washing off the barcode reagent. The Maxpar Nuclear Antigen Staining Buffer Set (Fluidigm Sciences) was used to fix and permeabilize the cells, which were then incubated with an intracellular antibody cocktail. After addition of the intracellular antibody cocktail for 30 min, the cells were washed and fixed with 1.6% paraformaldehyde. The cells and iridium intercalator solution (Max-Par Intercalator-Ir 500 mM; Fluidigm Sciences) were added together for 20 min, followed by a final wash. Cells were incubated with MilliQ-filtered distilled water (EMD Millipore, Billerica, MA, USA) at a concentration of 1×10^6 cells/ml containing EQ calibration beads (EQ Four Element Calibration Beads; Fluidigm Sciences). The cells were analyzed using a Helios mass cytometer (Fluidigm Sciences) on pooled single samples using the Normalizer and Single Cell Debarker software developed at the Nolan Lab (Stanford, Palo Alto, CA, USA). Normalization and deconvolution were also performed. The CyTOF staining panels used in this study are listed in **Supplementary Tables 1, 2**. OMIQ (Omiq, Inc., Santa Clara, CA, USA) was used for mass cytometric analysis. In OMIQ, we used automated optimized parameters for T-distributed stochastic neighbor embedding (opt-SNE) for the dimension reduction algorithm and FlowSOM for the clustering and visualization algorithms.

Serological evaluation

Cytokines in the serum and ascitic fluid were evaluated by multiplex analysis using Luminex® 100/200™. Five mice from each group were used for evaluation. Blood and ascitic fluid were collected 24 h after CLP in the same manner as the samples for mass cytometry. The obtained blood samples were centrifuged at 700 × *g* for 20 min at 25°C, and the ascitic fluid samples were centrifuged at 200 × *g* for 5 min at 25°C. The supernatants were collected and stored in a freezer at −20°C. The samples were subjected to multiplex analysis by Luminex® 100/200™ using the MILLIPLEX® MAP kit (Merck Millipore Corporation, Darmstadt, Germany).

Statistical analysis

Statistical analyses and calculations were performed using GraphPad Prism for Windows version 9.3.1 (Graph Pad Software, San Diego, CA, USA). Survival curves were estimated *via* the Kaplan–Meier method, and differences in survival were evaluated using the log-rank test. Numerical data from the OMIQ were exported and compared among groups using the two-sided non-parametric Mann–Whitney *U* test to assess significance. Statistical significance was set at $p < 0.05$.

Results

Survival outcome after cecal ligation and puncture

The 14-day survival rate after CLP was compared between the Burn/CLP and Sham/CLP groups, using 15 mice per group. A significant reduction in survival was observed in the Burn/CLP group (13.3%) when compared to the Sham/CLP group (66.7%) (log-rank, $p = 0.006$) (**Figure 1**).

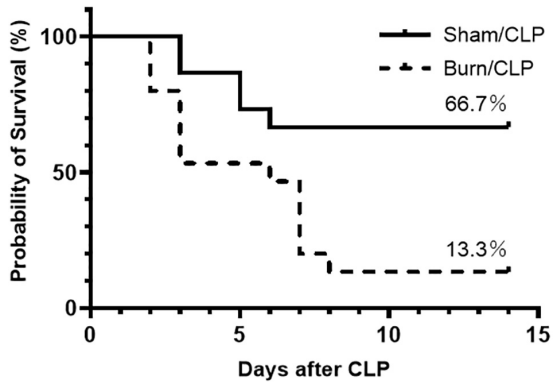

FIGURE 1
Kaplan–Meier survival curve for 14-day survival after CLP. CLP, cecal ligation and puncture.

Histopathological findings of major organs

We performed histopathological analysis of the spleen, liver, kidney, and lung tissues using H&E staining, which did not show staining defects caused by overfixation (Figure 2). Considerable numbers of infiltrating macrophages were observed in the spleens of the Sham/CLP group compared to a few in the Burn/CLP and control groups. The extent of macrophage infiltration into the spleen, scored using the scoring system (Supplementary Figure 1) for control, Sham/CLP, and Burn/CLP mice are shown in Supplementary Figures 2–4. The Sham/CLP group exhibited dilation of the sinusoid, while livers of the Burn/CLP group exhibited hepatocyte damage around the central vein, with dilation of the sinusoid. In the kidneys, acute tubular necrosis was observed in three of the five cases from the Burn/CLP group, while none was observed in the control and the Sham/CLP groups. Congestion was more noticeable in the lungs of the Burn/CLP group than the Sham/CLP group.

Cytokine levels in serum and ascitic fluid

The cytokine levels in serum and ascitic fluid are presented in heatmaps (Figure 3). Cytokine levels, including those of interferon-gamma (IFNγ), were generally lower in the Burn/CLP group than in the Sham/CLP group, except for TGF-β1. Serum TGFβ-1 levels were elevated in the Burn/CLP group (Figure 4).

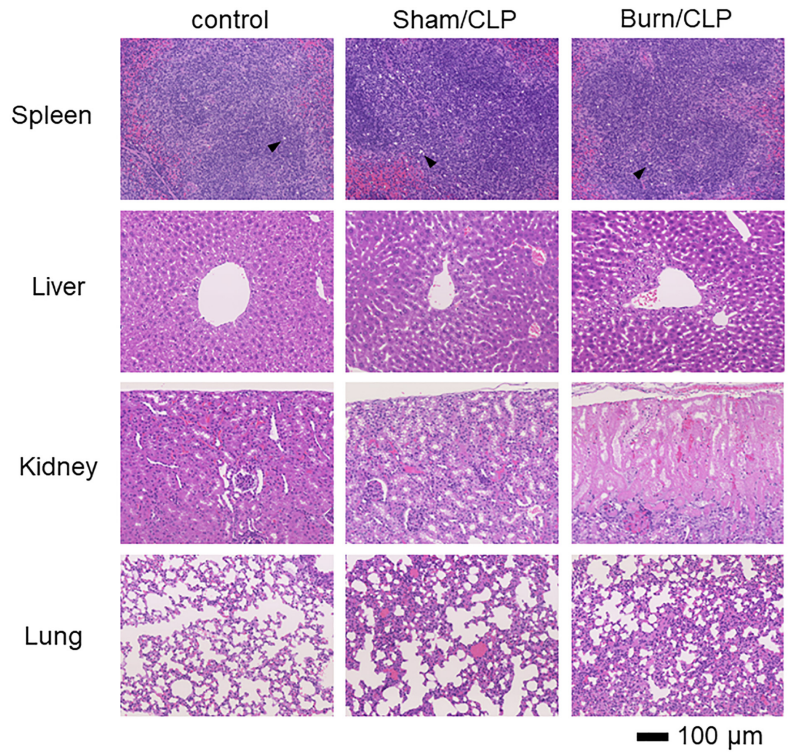

FIGURE 2
Histopathological findings in the Burn/CLP group, Sham/CLP group, and control cases. 10× (Objective lens) spleen, liver, kidney, and lung tissues. CLP, cecal ligation and puncture.

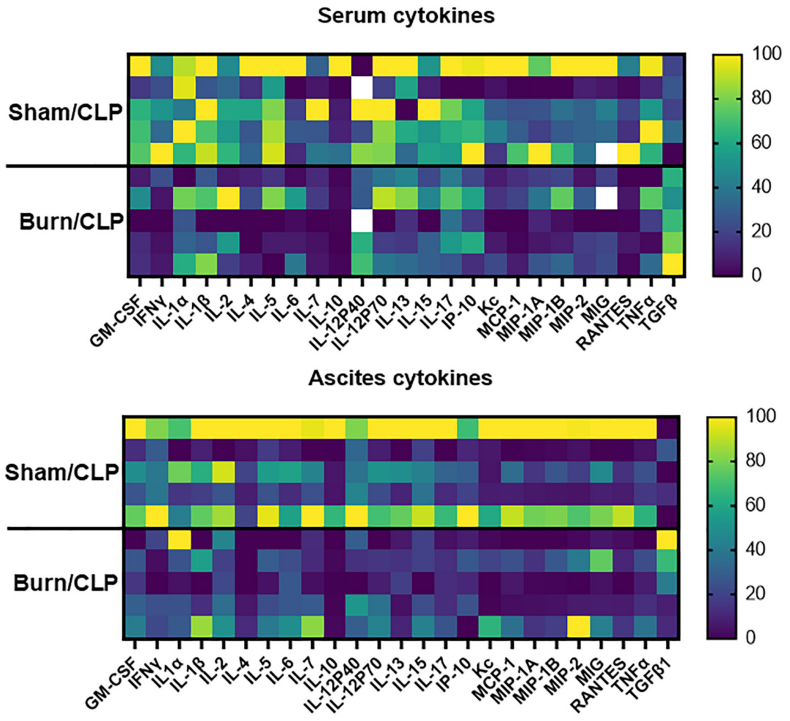

FIGURE 3
Heatmaps of the cytokine levels in serum and ascitic fluid. CLP, cecal ligation and puncture; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon-gamma; IL, interleukin; IP, interferon gamma-induced protein; KC, keratinocyte-derived chemokines; MCP, monocyte chemotactic protein; MIG, monokine induced by interferon-gamma; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; TNF, tumor necrosis factor; TGF-β1, transforming growth factor-beta 1.

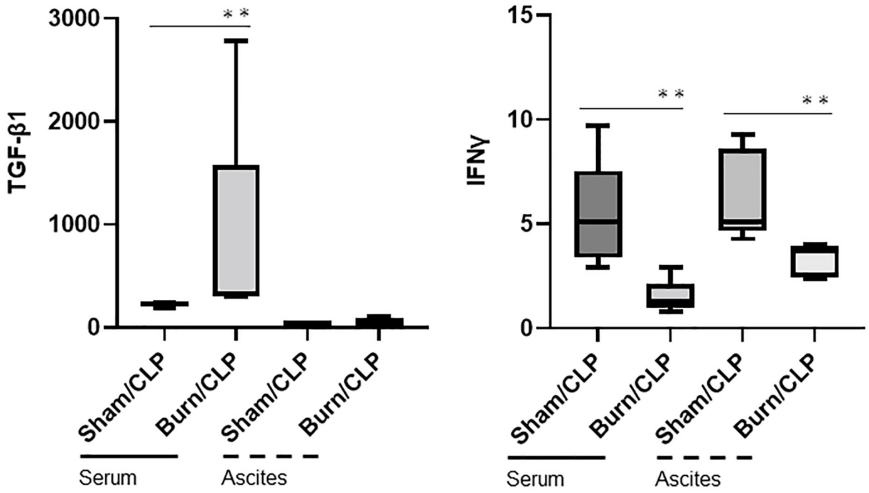

FIGURE 4
Transforming growth factor-beta 1 and IFNγ levels in serum and ascitic fluid. CLP, cecal ligation and puncture; TGF-β1, transforming growth factor-beta 1; IFN-γ, interferon gamma. $^{**}p < 0.01$.

Cell phenotyping *via* mass cytometry

The number of events in both groups was adjusted *via* the subsampling method in OMIQ analysis so as to ensure that event numbers are comparable. Lymphocyte antigen 6 G (Ly6G)-positive cells were identified as neutrophils, and CD68-positive cells were identified as monocytes or macrophages through dimension reduction maps created by opt-SNE

(Figure 5A). The files were concatenated per group for each blood and ascitic fluid sample, filtered based on CD45, and the contour plots of dimension reduction maps were compared (Figure 5B). CD45-positive cells were differentially abundant between the two groups both in blood and ascitic fluid, with a relative decrease of neutrophil islands in Burn/CLP group.

Neutrophil marker expression

Marker expression levels are presented *via* heatmaps in Supplementary Figures 5, 6. We examined whether there were any apparent differences in the scatter plot of dimension reduction maps, which were concatenated per group, with the z-channel set to several markers. The expression of CD11b on neutrophils in ascitic fluid was significantly higher in the Burn/CLP group than in the Sham/CLP group. CD11b expression on neutrophils in the blood also tended to be higher in the Burn/CLP group (Figure 6A). CD172a (signal-regulatory protein alpha: SIRPα) appeared as differentially expressed on neutrophils between the two groups, being significantly higher on neutrophils in blood samples of the Burn/CLP group, with a similar trend observed for ascitic fluid samples (Figure 6B). Sialic acid-binding lg-like lectin F (Siglec-F), which is used as an eosinophil surface marker, was partly expressed on the neutrophil island in ascitic fluid samples. The expression of Siglec-F on neutrophils in ascitic fluid exhibited significant differences between the two groups (Figure 6C). Eosinophils, which are generally known to express high levels of Siglec-F, were identified (Figure 7A), and we generated metaclusters including eosinophils (metacluster I: MC-I in Figure 7B), Siglec-F positive neutrophils (MC-F and MC-T in Figure 7B), and Siglec-F negative neutrophils (MC-C in Figure 7B). Siglec-F expression in MC-I, MC-F, MC-T, and MC-C were compared in histograms (Figure 7C). Siglec-F expression in MC-F and MC-T was similar and lower than that in MC-I, i.e., eosinophils. Siglec-F expression in MC-C was even lower than in MC-F and MC-T. In addition, histograms of Ly6G expression in MC-I, MC-F, MC-T, and MC-C (Figure 7D) are shown to reveal that MC-I lacked Ly6G expression while its expression was similar in MC-F, MC-T, and MC-C. Since Siglec-F expression on neutrophils is found mainly in inflammatory tissues and not in peripheral blood, Siglec-F was not included in the blood panel in this study due to conflicts with other markers. We also examined the expression of the above-mentioned markers on monocytes and macrophages, obtaining similar results (Supplementary Figure 7).

CD68-high neutrophils in the Burn/cecal ligation and puncture group

The contour plots of the two groups were overlaid to assess differences. While notable differences were not recorded for the blood samples, areas without overlap in the neutrophil island were noted for the ascitic fluid samples (Figure 8A, surrounded by the dashed line). The clustering algorithm FlowSOM was used to create metaclusters and examine the non-overlapping area in greater detail. The scatter plot of the dimension reduction map with metaclusters was created.

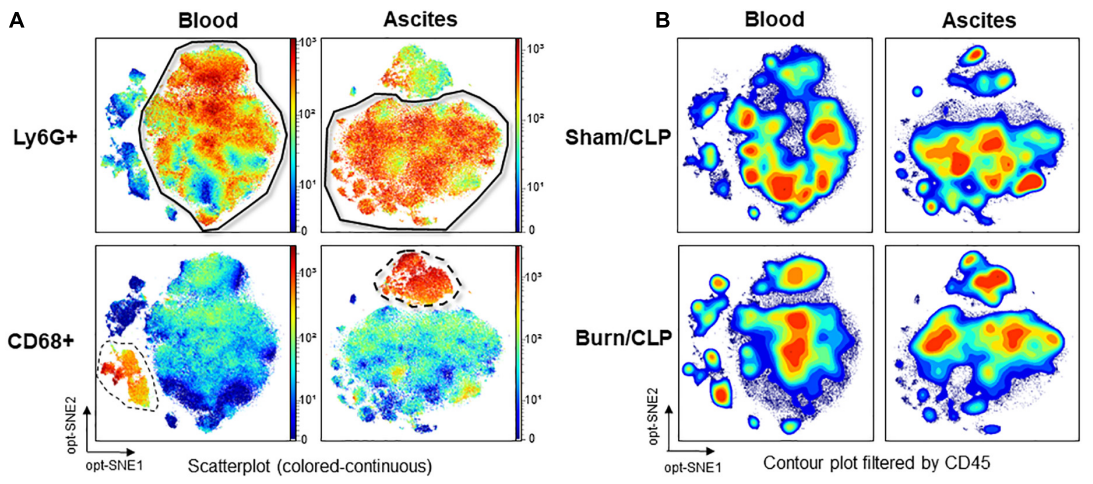

FIGURE 5
Identification of neutrophils and monocytes/macrophages on the dimension reduction map and CD45 expression. **(A)** Identification of neutrophil islands based on Ly6G and monocyte/macrophage islands based on CD68 in the scatterplot (colored-continuous) of the dimension reduction map, all files concatenated, with the z-channel set to Ly6G or CD68. **(B)** The expression of CD45 in contour plot, with the files concatenated by groups. CD, cluster of differentiation; CLP, cecal ligation and puncture; Ly6G, lymphocyte antigen 6 G; opt-SNE, optimized parameters for T-distributed stochastic neighbor embedding.

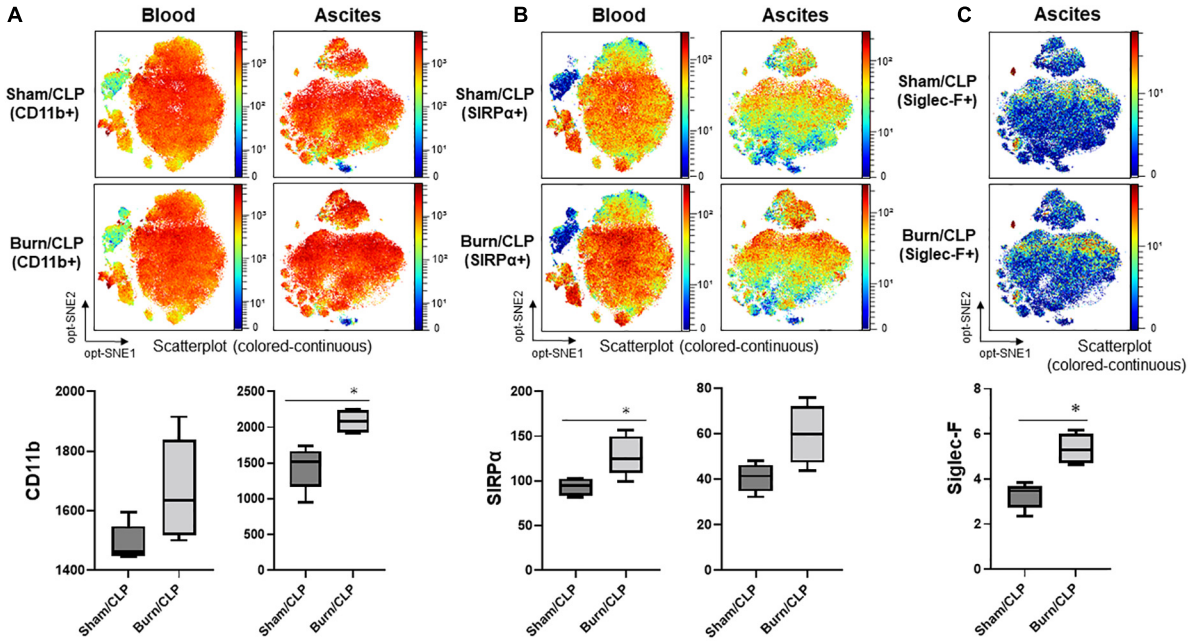

FIGURE 6
The expression of neutrophil markers. **(A)** The expression of CD11b. **(B)** The expression of SIRPα. **(C)** The expression of Siglec-F. CD, cluster of differentiation; CLP, cecal ligation and puncture; opt-SNE, optimized parameters for T-distributed stochastic neighbor embedding; Siglec-F, sialic acid-binding lg-like lectin F; SIRPα, signal-regulatory protein alpha. *$p < 0.05$.

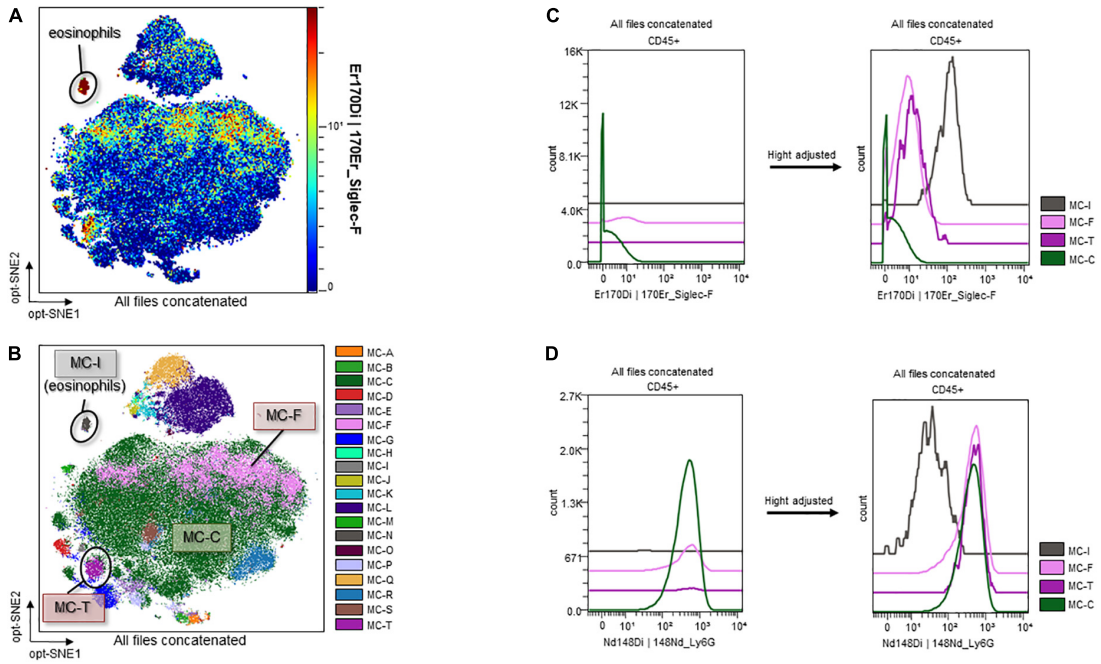

FIGURE 7
Sialic acid-binding lg-like lectin F and Ly6G expression on eosinophils and Siglec-F-positive/negative neutrophils. **(A)** Identification of eosinophils. **(B)** Metaclusters re-created with emphasis on Siglec-F expression. MC-I: eosinophils; MC-F and MC-T: Siglec-F-positive neutrophils; MC-C: Siglec-F-negative neutrophils. **(C)** Histograms of Siglec-F expression. **(D)** Histograms of Ly6G expression. CD, cluster of differentiation; Ly6G, lymphocyte antigen 6 G; MC, metacluster; opt-SNE, optimized parameters for T-distributed stochastic neighbor embedding; Siglec-F, sialic acid-binding lg-like lectin F.

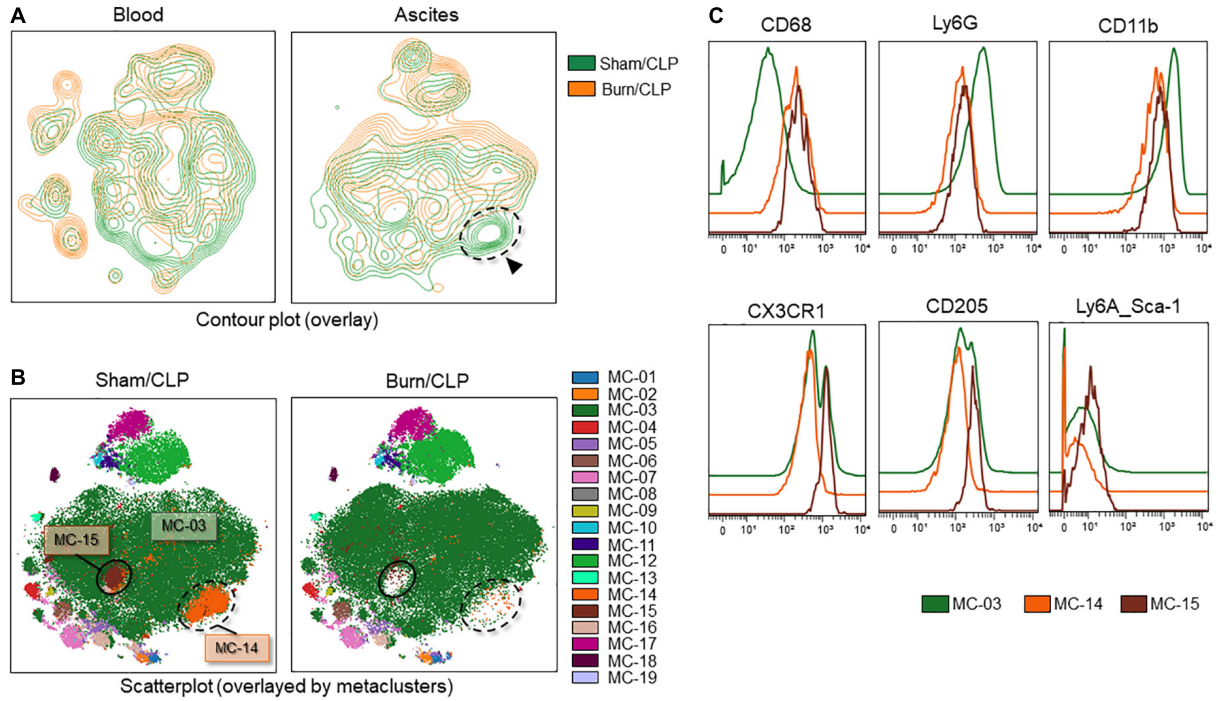

FIGURE 8
Deficiency of CD68-high neutrophils in the Burn/CLP group. **(A)** Comparison between the Burn/CLP and Sham/CLP groups by contour plot of the dimension reduction map. **(B)** Clustering by FlowSOM. **(C)** Height-adjusted histograms showing differences among the three metaclusters (MC-03, MC-14, and MC-15). CD, cluster of differentiation; CLP, cecal ligation and puncture; CX3CR1, C-X-3C chemokine receptor 1; Ly6, lymphocyte antigen 6; MC, metacluster.

The non-overlapping area is shown as MC-14 (surrounded by the dashed line in Figure 8B) in the metacluster scatter plot. The scatter plot revealed the existence of another non-overlapping metacluster (MC-15, surrounded by the solid line in Figure 8B) on the neutrophil island, which was not apparent in the contour plot when the two groups were overlaid. These two metaclusters, MC-14 and MC-15, which did not overlap between the two groups, were found to be present in the Sham/CLP group but absent in the Burn/CLP group. To characterize both metaclusters, histograms of each cell surface marker were generated, and each metacluster was found to express high levels of CD68 (Figure 8C) when compared to the surrounding largest neutrophil metacluster (shown as MC-03 in Figure 8B). They also exhibited slightly lower CD11b and Ly6G expression when compared to MC-03 cells. Further, MC-15 showed higher expression of C-X-3C chemokine receptor 1 (CX3CR1), CD205, and Ly6A (stem cell antigen-1: Sca-1) than MC-03 or MC-14 cells. We also generated another metacluster map to compare the abundance of CD68 expressions between macrophages (MC-mac in Figure 9A), CD68 positive neutrophils (MC-14 and MC-15 in Figure 9A), and CD68 negative neutrophils (MC-03 in Figure 9A). In Figure 9B, histograms show that MC-mac, MC-14, and MC-15 had higher expression of CD68 when compared to MC-03, but MC14 and MC15 had lower CD68 expression compared to MC-mac which indicates a macrophage island. To confirm that MC-14 and MC-15 are indeed neutrophils and not macrophages, a scatterplot with the Z-channel set to macrophage scavenger receptor (MSR) (Figure 9C) and a histogram showing MSR expression (Figure 9D) are demonstrated. MC-mac had high expression of MSR, while MC-14, MC-15, and MC-03 lacked MSR expression.

Discussion

In this study, we investigated neutrophil phenotypes implicated in the pathogenesis of post-traumatic sepsis. Histopathological analysis revealed a suppression of the immune response and progressive organ ischemia in the Burn/CLP group, which were consistent with the lower survival rate. We found that the Burn/CLP group exhibited differences in the expression of CD11b, SIRPα, Siglec-F, and CD68 in neutrophils compared to the Sham/CLP group. These phenotypic differences in neutrophils may contribute to the dysregulation of immune homeostasis during post-traumatic sepsis, thus contributing to increased organ damage and reduced survival rates.

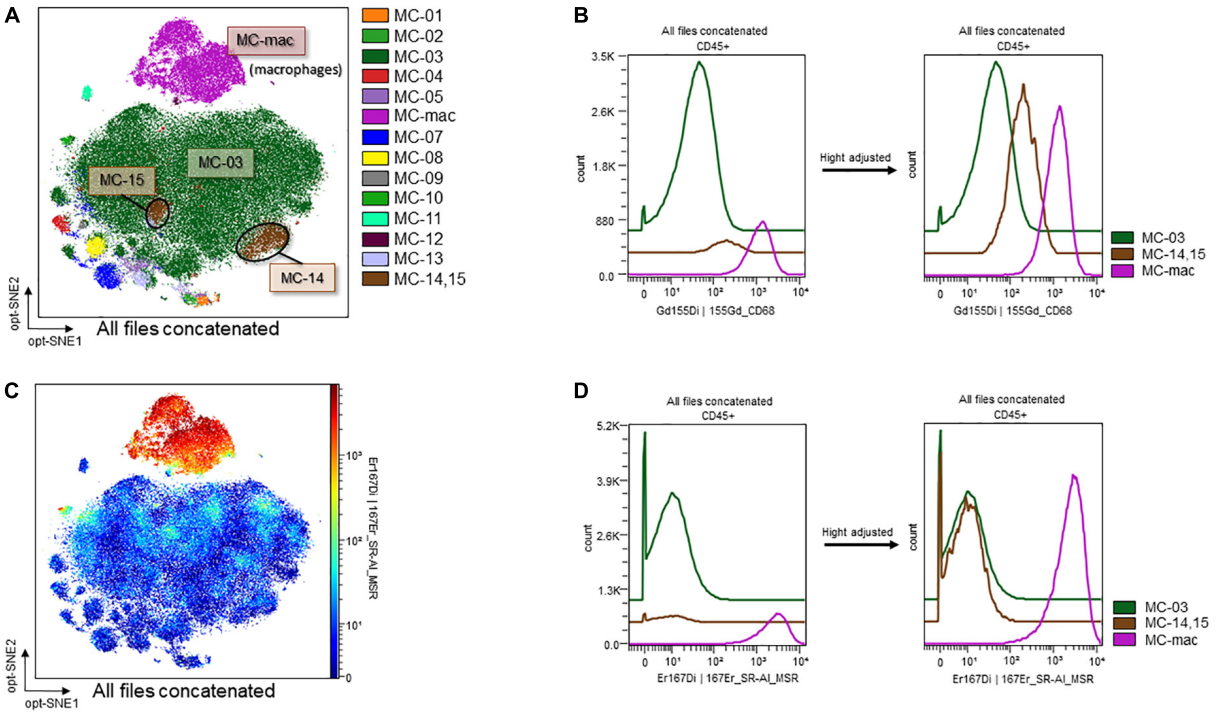

FIGURE 9
CD68 and MSR expression on macrophages and CD68-high/low neutrophils. **(A)** Metaclusters re-created with emphasis on CD68 expression. MC06: macrophages; MC14 and MC15: CD68-positive neutrophils; MC03: CD68-negative neutrophils. **(B)** MSR expression on an opt-SNE map. **(C)** Histograms of CD68 expression. **(D)** Histograms of MSR expression. CD, cluster of differentiation; MC, metacluster; MSR, macrophage scavenger receptor; opt-SNE, optimized parameters for T-distributed stochastic neighbor embedding.

The decreased numbers of macrophages in the spleen suggest attenuated immune responses in the Burn/CLP compared to Sham/CLP groups. Hepatocyte damage around the central hepatic vein, acute tubular necrosis in the kidneys, and lung congestion were indicative of more advanced organ ischemia and greater tissue damage in the Burn/CLP group when compared with the Sham/CLP group. These observations were consistent with the decreased survival rate and overall lower cytokine release in the Burn/CLP group. The higher TGF-β1 levels detected in the blood of the Burn/CLP group could be attributed to its role in wound healing (40). TGF-β1 is also known to play an immunosuppressive role by inhibiting cell proliferation and suppressing cytokine release (41), and may therefore contribute to the suppressed immune response observed following trauma. In our study, both pro- and anti-inflammatory cytokines were generally suppressed in the Burn/CLP group. Pro-inflammatory cytokines, such as IFNγ, which are normally released in response to CLP-induced sepsis, may have been suppressed by TGF-β1, resulting in an impaired immune response and undesirable outcome. Figure 3 shows the variation in each mouse. This result is expected because outbred mice, which can have uneven immune responses, were used to generate clinically useful evidence. Furthermore, it is possible that differences in the size of the appendix and the amount of feces in the intestinal tract of each mouse may have influenced the results in the process of creating the CLP-induced sepsis model used in this study. However, we believe it is meaningful that there were clear differences between the two groups despite such variations.

Neutrophils are among the key cellular components of the innate immune system and are mobilized during the earliest stages of infection to directly eliminate target microorganisms through phagocytosis, the production of reactive oxygen species (ROS), the generation of neutrophil extracellular traps (NETs), and apoptosis (9, 42). Furthermore, neutrophils are known to have a significant impact on subsequent adaptive responses through the production of specific cytokines (25, 26). In our study, CD11b expression on neutrophils in both blood and ascitic fluid was higher in the Burn/CLP group, as previously reported for CD11b expression after trauma (9, 43). CD11b plays an important role in the adhesion and migration of neutrophils by forming integrins together with CD18. A previous report has demonstrated that CD11b/CD18 activation by integrin agonists improves inflammatory nephritis *via* enhancement of leukocyte adhesion and suppression of leukocyte migration to the tissues (44). Trauma-induced increased expression of CD11b may lead to

the systemic attenuation of neutrophil migration, contributing to poorer outcomes in the Burn/CLP groups compared to the Sham/CLP groups, while this does not indicate a causal relationship.

Signal-regulatory protein alpha, which has recently attracted attention as a candidate target of immune checkpoint inhibitors in the field of cancer (45–48), was highly expressed on neutrophils, monocytes, and macrophages in the Burn/CLP group. SIRPα is a transmembrane protein that is expressed on the surface of phagocytic cells. When CD47 on target cells bind to SIRPα on phagocytes, their phagocytic activity is suppressed as a result of the generated "don't eat me" signal (47). Therefore, the high expression of SIRPα in the Burn/CLP group suggests a decreased phagocytic capacity and may reflect a compromised immune response.

Recently, Siglec-F has been recognized as a surface marker of neutrophils, in addition to its well-established role as an eosinophil marker. Siglec-F-positive neutrophils were previously detected in nasal lavage fluid under inflammatory conditions (49), in myocardial tissue after acute myocardial infarction (50), and in lung cancer tissue (51, 52). This neutrophil subpopulation has thus been considered to promote tumorigenesis (52). Siglec-F-positive neutrophils have been reported to arise from conventional Siglec-F-negative neutrophils under the influence of TGF-β1 and/or granulocyte-macrophage colony-stimulating factor (GM-CSF) released from damaged tissues (53). Siglec-F-positive neutrophils are rarely detected in the bone marrow, spleen, or peripheral blood, with many reports of their presence in inflamed tissues. Siglec-F-positive neutrophils are known to be hyper-segmented neutrophils and can survive for several days, as opposed to conventional neutrophils, which survive for up to several hours (51). Previous studies have also shown that Siglec-F-positive neutrophils have an enhanced ability to produce ROS and form NETs when compared to Siglec-F-negative neutrophils (49, 53). Although there have been various reports on Siglec-F-positive neutrophils, their characteristics have not yet been fully elucidated, and the significance of their abundance in ascitic fluid of the Burn/CLP group remains elusive.

CD68 is often used as a marker for monocytes or macrophages, but CD68-positive cells from other cell types are also known (54). A previous report described CD68-positive neutrophils in the colon mucosal tissue of patients with inflammatory bowel disease (55). The inflammatory stimuli-induced upregulation of CD68 on macrophages has also been reported (56). Macrophages in CD68-knockout mice have an equivalent phagocytic capacity (57). Thus, the details of the role of CD68 on macrophages/monocytes in immune function have not been clarified, and the same has not been clarified for CD68 on neutrophils. We indicated that CD68-high neutrophils were deficient in the Burn/CLP group, but further study is needed to interpret these results with regard to neutrophil immunocompetence.

Conclusion

In the present study, we characterized the histological and immunological findings in a murine model of post-traumatic sepsis. We noted apparent changes in the expression of CD11b, SIRPα, Siglec F, and CD68 on neutrophils in model mice. Our results suggest that elevated serum TGF-β1 levels may influence subsequent cytokine suppression and neutrophil phenotype in the pathogenesis of post-traumatic sepsis, facilitating an immunosuppressive state. The functional modulation of neutrophils may represent a potential therapeutic target for the post-traumatic immunosuppressed state in sepsis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by The Hokkaido University Animal Experiment Regulations and the Institutional Ethical Review Board at Hokkaido University (Approval number: 19-0167).

Author contributions

AM contributed to the experimentation, analysis, and manuscript preparation. TW contributed to the research concept and oversaw the study. TT contributed to the performance of experiments. YO and ST contributed to the provision of histopathological photographs and pathological evaluation. KK and KY contributed to the immunological evaluation through sample measurements. KY contributed to the revision of the manuscript. All authors read and approved the final version of the manuscript prior to submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.982399/full#supplementary-material

References

1. CDC. *Injury Prevention and Control. Data and Statistics*. (2021). Available online at: https://www.cdc.gov/injury/wisqars/?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Finjury%2Fwisqars%2Foverview%2Fkey_data.html (accessed May 3, 2022).

2. Ministry of Health, Labour and Welfare. *Statistics Information and White Paper*. (2009). Available online at: https://www.mhlw.go.jp/toukei_hakusho/index.html (accessed May 3, 2022).

3. Roberts I, Shakur H, Coats T, Hunt B, Balogun E, Barnetson L, et al. The CRASH-2 trial: a randomised controlled trial and economic evaluation of the effects of tranexamic acid on death, vascular occlusive events and transfusion requirement in bleeding trauma patients. *Health Technol Assess.* (2013) 17:1–79. doi: 10.3310/hta17100

4. CRASH-3 trial collaborators. Effects of tranexamic acid on death, disability, vascular occlusive events and other morbidities in patients with acute traumatic brain injury (CRASH-3): a randomised, placebo-controlled trial. *Lancet.* (2019) 394:1713–23. doi: 10.1016/S0140-6736(19)32233-0

5. Jaunoo SS, Harji DP. Damage control surgery. *Int J Surg.* (2009) 7:110–3. doi: 10.1016/j.ijsu.2009.01.008

6. Guo Z, Kavanagh E, Zang Y, Dolan SM, Kriynovich SJ, Mannick JA, et al. Burn injury promotes antigen-driven Th2-type responses in vivo. *J Immunol.* (2003) 171:3983–90. doi: 10.4049/jimmunol.171.8.3983

7. Shelley O, Murphy T, Paterson H, Mannick JA, Lederer JA. Interaction between the innate and adaptive immune systems is required to survive sepsis and control inflammation after injury. *Shock.* (2003) 20:123–9. doi: 10.1097/01.shk.0000079426.52617.00

8. Stoecklein VM, Osuka A, Lederer JA. Trauma equals danger—damage control by the immune system. *J Leukoc Biol.* (2012) 92:539–51. doi: 10.1189/jlb.0212072

9. Janicova A, Relja B. Neutrophil phenotypes and functions in trauma and trauma-related sepsis. *Shock.* (2021) 56:16–29. doi: 10.1097/SHK.0000000000001695

10. Park MY, Kim HS, Lee HY, Zabel BA, Bae YS. Novel CD11b+ Gr-1+ Sca-1+ myeloid cells drive mortality in bacterial infection. *Sci Adv.* (2020) 6:eaax8820. doi: 10.1126/sciadv.aax8820

11. Silvestre-Roig C, Fridlender ZG, Glogauer M, Scapini P. Neutrophil diversity in health and disease. *Trends Immunol.* (2019) 40:565–83. doi: 10.1016/j.it.2019.04.012

12. Yang P, Li Y, Xie Y, Liu Y. Different faces for different places: heterogeneity of neutrophil phenotype and function. *J Immunol Res.* (2019) 2019:8016254. doi: 10.1155/2019/8016254

13. Tsuda Y, Takahashi H, Kobayashi M, Hanafusa T, Herndon DN, Suzuki F. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant *Staphylococcus aureus*. *Immunity.* (2004) 21:215–26. doi: 10.1016/j.immuni.2004.07.006

14. Silvestre-Roig C, Hidalgo A, Soehnlein O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. *Blood.* (2016) 127:2173–81. doi: 10.1182/blood-2016-01-688887

15. Zhou J, Nefedova Y, Lei A, Gabrilovich D. Neutrophils and PMN-MDSC: their biological role and interaction with stromal cells. *Semin Immunol.* (2018) 35:19–28. doi: 10.1016/j.smim.2017.12.004

16. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell.* (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017

17. Puhl SL, Steffens S. Neutrophils in post-myocardial infarction inflammation: damage vs. Resolution? *Front Cardiovasc Med.* (2019) 6:25.

18. García-Culebras A, Durán-Laforet V, Peña-Martínez C, Moraga A, Ballesteros I, Cuartero MI, et al. Role of TLR4 (toll-like receptor 4) in N1/N2 neutrophil programming after stroke. *Stroke.* (2019) 50:2922–32. doi: 10.1161/STROKEAHA.119.025085

19. Seshadri A, Brat GA, Yorkgitis BK, Keegan J, Dolan J, Salim A, et al. Phenotyping the immune response to trauma: a multiparametric systems immunology approach. *Crit Care Med.* (2017) 45:1523–30. doi: 10.1097/CCM.0000000000002577

20. Wanke-Jellinek L, Keegan JW, Dolan JW, Guo F, Chen J, Lederer JA. Beneficial effects of CpG-oligodeoxynucleotide treatment on trauma and secondary lung infection. *J Immunol.* (2016) 196:767–77. doi: 10.4049/jimmunol.1500597

21. Yamakawa K, Tajima G, Keegan JW, Nakahori Y, Guo F, Seshadri AJ, et al. Trauma induces expansion and activation of a memory-like Treg population. *J Leukoc Biol.* (2021) 109:645–56. doi: 10.1002/JLB.4A0520-122R

22. O'Sullivan ST, Lederer JA, Horgan AF, Chin DH, Mannick JA, Rodrick ML. Major injury leads to predominance of the T helper-2 lymphocyte phenotype and diminished interleukin-12 production associated with decreased resistance to infection. *Ann Surg.* (1995) 222:482–90; discussion490–2. doi: 10.1097/00000658-199522240-00006

23. Ni Choileain N, MacConmara M, Zang Y, Murphy TJ, Mannick JA, Lederer JA. Enhanced regulatory T cell activity is an element of the host response to injury. *J Immunol.* (2006) 176:225–36. doi: 10.4049/jimmunol.176.1.225

24. Hanschen M, Tajima G, O'Leary F, Ikeda K, Lederer JA. Injury induces early activation of T-cell receptor signaling pathways in CD4+ regulatory T cells. *Shock.* (2011) 35:252–7. doi: 10.1097/SHK.0b013e3181f489c5

25. Hazeldine J, Hampson P, Lord JM. The impact of trauma on neutrophil function. *Injury.* (2014) 45:1824–33. doi: 10.1016/j.injury.2014.06.021

26. Leliefeld PH, Wessels CM, Leenen LP, Koenderman L, Pillay J. The role of neutrophils in immune dysfunction during severe inflammation. *Crit Care.* (2016) 20:73. doi: 10.1186/s13054-016-1250-4

27. Mortaz E, Zadian SS, Shahir M, Folkerts G, Garssen J, Mumby S, et al. Does neutrophil phenotype predict the survival of trauma patients? *Front Immunol.* (2019) 10:2122. doi: 10.3389/fimmu.2019.02122

28. Yang S, Stepien D, Hanseman D, Robinson B, Goodman MD, Pritts TA, et al. Substance P mediates reduced pneumonia rates after traumatic brain injury. *Crit Care Med.* (2014) 42:2092–100. doi: 10.1097/CCM.0000000000000486

29. Hsieh T, Vaickus MH, Stein TD, Lussier BL, Kim J, Stepien DM, et al. The role of substance P in pulmonary clearance of bacteria in comparative injury models. *Am J Pathol.* (2016) 186:3236–45. doi: 10.1016/j.ajpath.2016.08.014

30. Abdullahi A, Amini-Nik S, Jeschke MG. Animal models in burn research. *Cell Mol Life Sci.* (2014) 71:3241–55. doi: 10.1007/s00018-014-1612-5

31. Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock–a review of laboratory models and a proposal. *J Surg Res.* (1980) 29:189–201. doi: 10.1016/0022-4804(80)90037-2

32. Hubbard WJ, Choudhry M, Schwacha MG, Kerby JD, Rue LW, Bland KI, et al. Cecal ligation and puncture. *Shock.* (2005) 24 Suppl. 1:52–7. doi: 10.1097/01.shk.0000191414.94461.7e

33. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc.* (2009) 4:31–6. doi: 10.1038/nprot.2008.214

34. Niiyama S, Takasu O, Sakamoto T, Ushijima K. Intraperitoneal adipose tissue is strongly related to survival rate in a mouse cecal ligation and puncture model. *Clin Transl Immunol.* (2016) 5:e64. doi: 10.1038/cti.2016.3

35. Vincent JL, Marshall JC, Namendys-Silva SA, François B, Martin-Loeches I, Lipman J, et al. Assessment of the worldwide burden of critical illness: the intensive care over nations (ICON) audit. *Lancet Respir Med.* (2014) 2:380–6. doi: 10.1016/S2213-2600(14)70061-X

36. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. *Lancet.* (2018) 392:75–87. doi: 10.1016/S0140-6736(18)30696-2

37. Angus DC, van der Poll T. Severe sepsis and septic shock. *N Engl J Med.* (2013) 369:840–51. doi: 10.1056/NEJMra1208623

38. Kirihara Y, Takechi M, Kurosaki K, Kobayashi Y, Kurosawa T. Anesthetic effects of a mixture of medetomidine, midazolam and butorphanol in two strains of mice. *Exp Anim.* (2013) 62:173–80. doi: 10.1538/expanim.62.173

39. Iskander KN, Vaickus M, Duffy ER, Remick DG. Shorter duration of post-operative antibiotics for cecal ligation and puncture does not increase inflammation or mortality. *PLoS One.* (2016) 11:e0163005. doi: 10.1371/journal.pone.0163005

40. Lichtman MK, Otero-Vinas M, Falanga V. Transforming growth factor beta (TGF-β) isoforms in wound healing and fibrosis. *Wound Repair Regen.* (2016) 24:215–22. doi: 10.1111/wrr.12398

41. Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. *Immunity.* (2019) 50:924–40. doi: 10.1016/j.immuni.2019.03.024

42. Balamayooran G, Batra S, Fessler MB, Happel KI, Jeyaseelan S. Mechanisms of neutrophil accumulation in the lungs against bacteria. *Am J Respir Cell Mol Biol.* (2010) 43:5–16. doi: 10.1165/rcmb.2009-0047TR

43. Hazeldine J, Naumann DN, Toman E, Davies D, Bishop JRB, Su Z, et al. Prehospital immune responses and development of multiple organ dysfunction syndrome following traumatic injury: a prospective cohort study. *PLoS Med.* (2017) 14:e1002338. doi: 10.1371/journal.pmed.1002338

44. Maiguel D, Faridi MH, Wei C, Kuwano Y, Balla KM, Hernandez D, et al. Small molecule-mediated activation of the integrin CD11b/CD18 reduces inflammatory disease. *Sci Signal.* (2011) 4:ra57. doi: 10.1126/scisignal.2001811

45. Logtenberg MEW, Scheeren FA, Schumacher TN. The CD47-SIRPα immune checkpoint. *Immunity.* (2020) 52:742–52. doi: 10.1016/j.immuni.2020.04.011

46. Feng M, Jiang W, Kim BYS, Zhang CC, Fu YX, Weissman IL. Phagocytosis checkpoints as new targets for cancer immunotherapy. *Nat Rev Cancer.* (2019) 19:568–86. doi: 10.1038/s41568-019-0183-z

47. Zhang W, Huang Q, Xiao W, Zhao Y, Pi J, Xu H, et al. Advances in anti-tumor treatments targeting the CD47/SIRPα axis. *Front Immunol.* (2020) 11:18. doi: 10.3389/fimmu.2020.00018

48. Yanagita T, Murata Y, Tanaka D, Motegi SI, Arai E, Daniwijaya EW, et al. Anti-SIRPα antibodies as a potential new tool for cancer immunotherapy. *JCI Insight.* (2017) 2:e89140. doi: 10.1172/jci.insight.89140

49. Matsui M, Nagakubo D, Satooka H, Hirata T. A novel Siglec-F+ neutrophil subset in the mouse nasal mucosa exhibits an activated phenotype and is increased in an allergic rhinitis model. *Biochem Biophys Res Commun.* (2020) 526:599–606. doi: 10.1016/j.bbrc.2020.03.122

50. Vafadarnejad E, Rizzo G, Krampert L, Arampatzi P, Arias-Loza AP, Nazzal Y, et al. Dynamics of cardiac neutrophil diversity in murine myocardial infarction. *Circ Res.* (2020) 127:e232–49. doi: 10.1161/CIRCRESAHA.120.317200

51. Pfirschke C, Engblom C, Gungabeesoon J, Lin Y, Rickelt S, Zilionis R, et al. Tumor-promoting Ly-6G+ SiglecFhigh cells are mature and long-lived neutrophils. *Cell Rep.* (2020) 32:108164. doi: 10.1016/j.celrep.2020.108164

52. Engblom C, Pfirschke C, Zilionis R, Da Silva Martins J, Bos SA, Courties G, et al. Osteoblasts remotely supply lung tumors with cancer-promoting SiglecFhigh neutrophils. *Science.* (2017) 358:eaal5081. doi: 10.1126/science.aal5081

53. Ryu S, Shin JW, Kwon S, Lee J, Kim YC, Bae YS, et al. Siglec-F-expressing neutrophils are essential for creating a profibrotic microenvironment in the renal fibrosis. *J Clin Invest.* (2022) 132:e156876. doi: 10.1172/JCI156876

54. Chistiakov DA, Killingsworth MC, Myasoedova VA, Orekhov AN, Bobryshev YV. CD68/macrosialin: not just a histochemical marker. *Lab Invest.* (2017) 97:4–13. doi: 10.1038/labinvest.2016.116

55. Amanzada A, Malik IA, Blaschke M, Khan S, Rahman H, Ramadori G, et al. Identification of CD68(+) neutrophil granulocytes in in vitro model of acute inflammation and inflammatory bowel disease. *Int J Clin Exp Pathol.* (2013) 6:561–70.

56. Rabinowitz SS, Gordon S. Macrosialin, a macrophage-restricted membrane sialoprotein differentially glycosylated in response to inflammatory stimuli. *J Exp Med.* (1991) 174:827–36. doi: 10.1084/jem.174.4.827

57. Song L, Lee C, Schindler C. Deletion of the murine scavenger receptor CD68. *J Lipid Res.* (2011) 52:1542–50. doi: 10.1194/jlr.M015412

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