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Systemic and local cardiac inflammation after experimental long bone fracture, traumatic brain injury and combined trauma in mice☆

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ABSTRACT

Background: Trauma is the leading cause of death and disability worldwide, especially in the young population. Cardiac injuries are an independent predictor for a poor overall outcome after trauma. The aim of the present study was to analyze systemic inflammation as well as local cardiac inflammation after experimental limb-, neuro- and combined trauma in mice.

Methods: Male C57BL/6 mice received either a closed tibia fracture (Fx), isolated traumatic brain injury (TBI) or a combination of both (Fx + TBI). Control animals underwent sham procedure. After 6 and 24 h, systemic levels of inflammatory mediators were analyzed, respectively. Locally, cardiac inflammation and cardiac structural alterations were investigated in left ventricular tissue of mice 6 and 24 h after trauma.

Results: Mice showed enhanced systemic inflammation after combined trauma, which was manifested by increased levels of KC, MCP-1 and G-CSF. Locally, mice exhibited increased expression of inflammatory cytokines (IL-1β, TNF) in heart tissue, which was probably mediated via toll-like receptor (TLR) signaling. Furthermore, mice demonstrated a redistribution of connexin 43 in cardiac tissue, which appeared predominantly after combined trauma. Besides inflammation and structural cardiac alterations, expression of glucose transporter 4 (GLUT4) mRNA was increased in the heart early after TBI and after combination of TBI and limb fracture, indicating a modification of energy metabolism. Early after combination of TBI and tibia fracture, nitrosative stress was increased, manifested by elevation of nitrotyrosine in cardiac tissue. Finally, mice showed a trend of increased systemic levels of cardiac troponin I and heart-fatty acid binding protein (HFABP) after combined trauma, which was associated with a significant decrease of troponin I and HFABP mRNA expression in cardiac tissue after TBI and combination of TBI and limb fracture.

Conclusion: Mice exhibited early cardiac alterations as well as alterations in cardiac glucose transporter expression, indicating a modification of energy metabolism, which might be linked to increased systemic- and local cardiac inflammation after limb-, neuro- and combined trauma. These cardiac alterations might predispose individuals for secondary cardiac damage after trauma that might compromise cardiac function after TBI and long bone fracture.

Translational potential statement: Injuries to the head and extremities frequently occur after severe trauma. In our study, we analyzed the effects of closed tibia fracture, isolated TBI, and the combination of both injuries with regard to the development of post-traumatic secondary cardiac injuries.

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Introduction

Trauma is the leading cause of death and disability worldwide, especially in young people [1]. Direct cardiac injuries are an independent predictor for a poor overall outcome after trauma and are mostly induced by mechanical cardiac damage. However, trauma-induced secondary cardiac injuries (TISCI) in the absence of direct mechanical trauma were also described in humans, and these correlate with long-term morbidity as well as mortality [2–4]. The development of secondary cardiac injuries after trauma are associated with adverse cardiac events (ACEs), multiple organ dysfunction syndrome (MODS) and mortality [5,6]. The development of TISCI in injured patients is linked to increased systemic inflammation and the pro-inflammatory cytokines interleukin-(IL)1β, IL-6 and tumor necrosis factor (TNF) are associated with the occurrence of ACEs after trauma [3,4]. Additionally, TISCI is characterized by the systemic release of markers of cardiac damage such as the heart fatty acid binding protein (HFABP), cardiac troponin I and the brain natriuretic peptide (BNP) [4].

Traumatic brain injury (TBI) affects over 1.7 million people in the United States annually and is accompanied by increased morbidity, mortality as well as by high economic burden [7,8]. TBI was recently linked to secondary organ damage, including the heart. Patients suffering severe TBI showed hemodynamic changes along with abnormalities in electrocardiography (ECG) such as prolonged QT intervals, ST segment and T wave changes, which were associated with an increased mortality [9,10]. Furthermore, patients with severe and moderate TBI showed cardiac dysfunction, correlating with in-hospital mortality [11]. Additionally, the systemic release of cardiac troponin I after TBI and of creatine kinase (CK-MB) was associated with abnormal echocardiography and myocardial damage [11,12]. Further clinical reports described a reduced ejection fraction of the left ventricle as well as regional wall motion abnormalities after TBI. Abnormal echocardiographic findings correlate with the severity of TBI based on the Glasgow Coma Scale (GCS) and were most intense after severe TBI [11]. The abnormal echocardiographic finding was an independent predictor of in-hospital mortality in TBI patients after adjusting age, head Abbreviated Injury Score (AIS) and gender [11]. Furthermore, patients with moderate-severe TBI showed systolic dysfunction which recovered after one week [13]. The development of myocardial injury post-TBI was linked to systemic enhanced inflammation, but also to excess catecholamines leading to neurogenic stunned myocardium [14–17]. Additionally, brain-heart interactions were also described after severe non-acute TBI neurological diseases [17–20]. However, local structural, cellular and molecular changes associated with these clinical observations are largely unknown.

Fractures of the extremities are frequent injury patterns in patients subjected to road accidents [21]. In our previous experimental studies, we described early myocardial damage (EMD) after isolated long bone fracture in pigs [22]. EMD damage after fracture manifested with impaired systolic and diastolic function as well as by valvular insufficiency and was further linked to systemic and local cardiac inflammation [22]. Multiple trauma involves injuries of at least two regions of the body and is a common consequence of automobile accidents [23]. Head injury combined with extremity fracture are a common result of high-energy trauma, such as motor-vehicle accidents, falls, and combat injuries. A recent study found that of 18,404 patients with a femoral shaft fracture, more than one-third sustained a concomitant head or neck injury [21,24,25].

In a previous study, mice showed increased systemic as well as local inflammation after combined trauma (tibia fracture and TBI) in the brain, which was associated with abnormal behavior as well as with severe brain damage [26]. Furthermore, specific brain-bone interactions were recently described. The concomitant occurrence of a fracture impaired neurologic recovery and worsened outcomes after TBI, whereas fracture healing itself was improved in presence of a TBI [26–28]. However, little is known about the effects of bone fracture combined with TBI on the heart. The aim of the present study was to investigate the effects of isolated closed tibia fracture, isolated TBI as well as the combination of both on the heart with regard to systemic and local inflammation.

Materials and methods

Animals

48 male C57BL/6 J mice (Jackson Laboratories) were included in the present study (age 10–12 weeks, mean weight 25–30 g). All experiments were approved by the local animal welfare committee (IACUC UCSF AN143402-03 B) and were performed in compliance with international regulations for laboratory animal welfare and handling (ARRIVE guidelines for animal experiments). The animals were subdivided in the following groups (n = 6): Tibia fracture (Fx) with 6 h follow-up, Fx with 24 h follow-up, traumatic brain injury (TBI) with 6 h follow-up, TBI with 24 h follow-up, combined trauma (TBI + Fx) with 6 h follow-up, combined trauma (TBI + Fx) with 24 h follow-up, sham 6 h follow-up, sham 24 follow-up. Mice were housed in groups of up to five animals with free access to standard mouse diet and water. The trauma procedure was conducted under general anesthesia with isoflurane and analgesia using 0.05 mg/kg Buprenex (s.c). Sham animals also received anesthesia and analgesia, but no trauma procedure was conducted.

Surgical procedure

The surgical procedures of tibia fracture and traumatic brain injury were described previously by Morioka et al. [24].

Tibia fracture

Anesthetized mice were placed pronate under a fracture apparatus. The critical components of the apparatus consist of a blunt two-pronged base to frame the tibia and a 2 mm-thick blunt arm connected to a guided, movable 500 g weight. The right tibia was centered on the base and under the arm before the weight is lifted to a distance of 5 cm above the tibia and then dropped to create a fracture via three-point bending. The skin does not break with this procedure, thereby preventing the possibility of infection from skin wounds. The fracture was not stabilized. The animals received pain medication every 6 h.

Traumatic brain injury (TBI)

Animals were anesthetized for surgery as described above and received enrofloxin antibiotic prior to the procedure. To produce TBI, controlled cortical contusions was produced on the right side of the brain (ipsilateral to the fracture) using a precision instrument designed to deliver a contusion to the mouse cortex by compressing the cortex 2 mm at a rate of 4 m/s for 150 ms using a 3 mm wide convex probe. Skin surrounding the target area of the brain was incised along the midline over frontal and parietal bones. The periosteum was removed and a 5 mm circular trephine was used to open the skull. With the head stabilized in a stereotaxic device, the probe of the contusion device was positioned perpendicular to the cortex and centered in the circular craniectomy window (2.5 mm lateral to the midline, and midway between bregma and lambda). The bone was removed without damage to the underlying dura. After contusion, the cortex was covered with saline soaked gelfoam and the wound was closed in separate anatomical layers using separate sterile sutures.

Sample collection

After the follow-up period of either 6 h or 24 h, mice were euthanized using carbon dioxide. Blood was taken immediately after euthanization by cardiac puncture. Serum samples were collected after centrifugation with 5 min (800×*g*, 4 °C) and a second centrifugation step for 2 min (13000×*g*, 4 °C). The serum samples were stored at −80 °C until analysis. Samples of the heart tissue was taken and either quick-frozen by liquid nitrogen or fixed in 4% paraformaldehyde for 48 h.

Multiplex analysis

For determination of systemic inflammatory mediators, the plasma from mice was analyzed by using the ProcartaPlex Immunoassay (ThermoFisher, Waltham, MA, USA). The plasma was analyzed for granulocyte-colony stimulating factor (G-CSF), interleukin (IL)-6, keratinocyte chemoattractant (KC), IL-10, tumor necrosis factor (TNF) and monocyte chemoattractant protein-1 (MCP-1). All procedures were performed according to manufacturer's instructions.

ELISAs

Systemic heart fatty-acid binding protein (HFABP) in mouse plasma was determined by using the mouse heart-fatty acid binding protein ELISA (Life Diagnostics, West Chester, PA, USA). Systemic troponin I was examined by using the mouse cardiac troponin I ELISA (Life Diagnostics, West Chester, PA, USA). All procedures were performed according to manufacturer's instructions.

RNA isolation

RNA from left ventricles was isolated using TRIzol RNA isolation reagent (ThermoFisher, Waltham, MA, USA).

Reverse transcribed quantitative polymerase chain reaction (RT-qPCR)

The respective RNA samples were reverse transcribed in cDNA using SuperScript™ IV VILO™ MasterMix with ezDNAse (Invitrogen, Carlsbad, CA, USA). For quantitative PCR the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Waltham, MA, USA) was used. All procedures were performed according to the manufacturer's instructions. For qPCR the QuantStudio3 system (Applied Biosystems, Waltham, MA, USA) was utilized. Quantitative mRNA expression of murine C5a receptor 1 (C5aR1), glucose transporter 4 (GLUT4), heart fatty acid binding protein (HFABP), interleukin-1β (IL-1β), toll-like receptor (TLR)4, TLR9, tumor necrosis factor (TNF) and troponin I (TnI) (primer sequences Supplemental Table 1) was examined and calculated by the cycle threshold method ΔΔCt. Respective genes were normalized using housekeeping gene glutaraldehyde-phosphate dehydrogenase (GAPDH). Results are presented as mean fold change.

Immunohistochemical analysis

Hematoxylin & eosin (H.E) staining

Formalin-fixed and paraffin embedded tissue from left ventricles was used. Tissue sections were dewaxed and rehydrated. Myocardial tissue sections were stained with hematoxylin & eosin staining kit (Morphisto, Frankfurt am Main, Germany). For quantification of myocardial damage, a heart injury score was defined as described previously [29,30]. For determination of the heart injury score, the H.E. sections of myocardial tissue were scored for 1) apoptosis, 2) contraction band necrosis, 3) neutrophil infiltration, 4) intramuscular bleeding, 5) rupture, 6) edema and 7) ischemia.

General procedure. For immunohistochemical analysis, formalin-fixed and paraffin embedded tissue from left ventricles was used. Tissue sections were dewaxed and rehydrated. Antigen unmasking was performed by boiling the sections in 10 mM citrate buffer (pH 6) at 100 °C. Non-specific binding sites were blocked by 10% goat serum.

Immunohistochemistry (IHC) and immunofluorescence (IF). Specific antigen binding was performed by incubating sections with the respective primary antibodies for C3a receptor (C3aR) (Bioss, Woburn, MA, USA), C5a receptor 1 (C5aR1) (Acris, Rockville, MD, USA), Nitrotyrosine (Merck, Darmstadt, Germany), Connexin 43 (Cell Signaling Technology, Danvers, MA, USA), Glucose transporter 1 (GLUT1) (abcam, Cambridge, UK) and glucose transporter 4 (GLUT4) (abcam, Cambridge, UK) for overnight at 4 °C. For immunohistochemical staining, a biotin-labelled secondary antibody was used for the detection of specific antibody binding (ThermoFisher, Waltham, MA, USA). Signal amplification was performed by using VECTASTAIN® ABC HRP Kit (Vector Laboratories Inc., Burlingame, CA, USA). Signal development was conducted by using VECTOR® NovaRED™ Peroxidase (HRP) Substrate Kit (Vector Laboratories Inc., Burlingame, CA, USA). Cell nuclei were counterstained with Hematoxylin according to Mayer. For immunofluorescence, an Alexa-Fluor488®-labelled secondary antibody was used for the detection of specific antibody binding (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cell nuclei were counterstained with Hoechst33342 (ThermoFisher, Waltham, MA, USA). The sections were investigated by bright field microscopy or by fluorescence microscopy using an Axio Imager M.2 microscope (Zeiss, Jena, Germany). To quantify epitope expression, imaging of three distinct, representative fields of view (40x magnification) were examined for each animal. For quantification of immunohistochemical and immunofluorescent staining, the ZEN 2.3 software (Zeiss, Jena, Germany) was used. A specific threshold for pixel density or fluorescence intensity was defined for each staining (C3aR, C5aR1, Connexin43, nitrotyrosine, GLUT1 and GLUT4) prior to the quantification. With the ZEN 2.3 software, each picture was analyzed independently with respect to the defined threshold for pixel density or fluorescence intensity. For each picture a specific mean value of pixel density (IHC) or fluorescence intensity (IF) was calculated by the software. Results are presented as mean pixel density (IHC) or mean fluorescence intensity (IF).

Statistical analysis. All values are expressed as mean ± SEM. The 6 and the 24 h groups were analyzed independently. The data were analyzed by two-way Analysis of Variance (ANOVA), followed by Sidak's multiple comparison test. Mean values of each trauma group (Fx, TBI or Fx + TBI) were compared to the mean values of the control group (sham). *$p \leq 0.05$ was considered as statistically significant. GraphPad Prism 8.0 software was used for the statistical analysis (GraphPad Prism Software Inc., San Diego, CA).

Results

Systemic inflammation. First, we analyzed the systemic levels of specific inflammatory mediators. After 6 h, the systemic G-CSF concentration significantly increased after TBI and after Fx + TBI (Fig. 1A). Also, the systemic KC levels significantly increased 6 h after Fx + TBI (Fig. 1C). The IL-6 levels increased after 6 and 24 h by a trend in all trauma groups (Fig. 1B). The IL-10 concentration increased significantly 24 h after Fx + TBI (Fig. 1D). The MCP-1 levels also increased 24 h after Fx + TBI (Fig. 1E). No significant differences were observed in systemic TNF levels (Fig. 1F).

Cardiac damage. Since the systemic concentration of inflammatory mediators increased after TBI and after Fx + TBI, we analyzed the plasma for HFABP and TnI, which are markers that are specific for cardiac damage. After 6 h, no differences were observed in systemic HFBAP levels, but after 24 h the HFABP levels trended higher within the combined trauma group (Fig. 2A). Also, there were no significant differences in systemic TnI concentrations after 6 h. However, after 24 h, the systemic TnI concentrations also trended higher within the combined trauma group (Fig. 2B). The cardiac expression of troponin I mRNA significantly decreased 6 h after TBI and Fx + TBI, as well as 24 h after tibia fracture (Fig. 2C). The local mRNA expression of HFABP significantly decreased 6 h after TBI and Fx + TBI as well as 24 h after tibia fracture (Fig. 2D). No considerable differences were present in myocardial tissue appearance (Supplemental Figure 1A) and no differences were observed in the heart injury score between the respective groups (Supplemental Figure 1B).

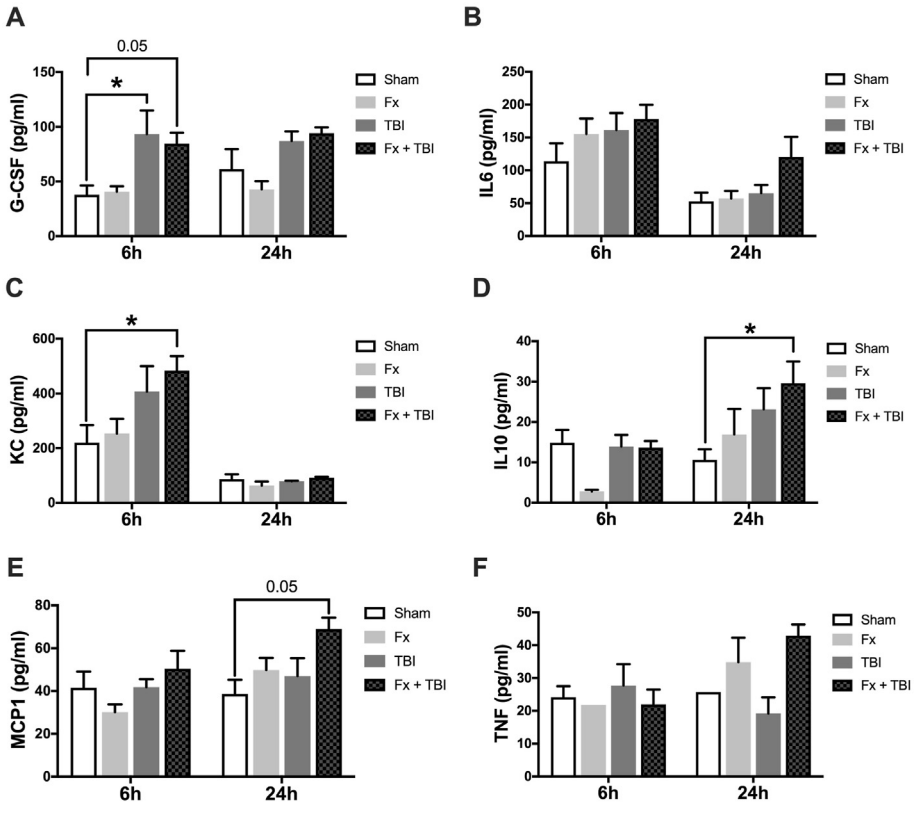

Figure 1. *Systemic inflammation after experimental limb-, neuro- and combined trauma in mice.* Mice received either closed tibia fracture (Fx) (light grey bars), traumatic brain injury (TBI) (dark grey bars), combined trauma (Fx + TBI) (squared bars) or sham treatment (white bars). Blood plasma was analyzed after 6 and 24 h, respectively. Systemic levels of granulocyte-colony stimulating factor (G-CSF) in pg/ml (**A**), interleukin-6 (IL-6) in pg/ml (**B**), keratinocyte chemoattractant (KC) in pg/ml (**C**), interleukin-10 (IL-10) in pg/ml (**D**), monocyte chemotactic protein-1 (MCP-1) in pg/ml (**E**) and tumor necrosis factor (TNF) in pg/ml (**F**). Data are presented as mean ± SEM. *p < 0.05 was considered as statistically significant.

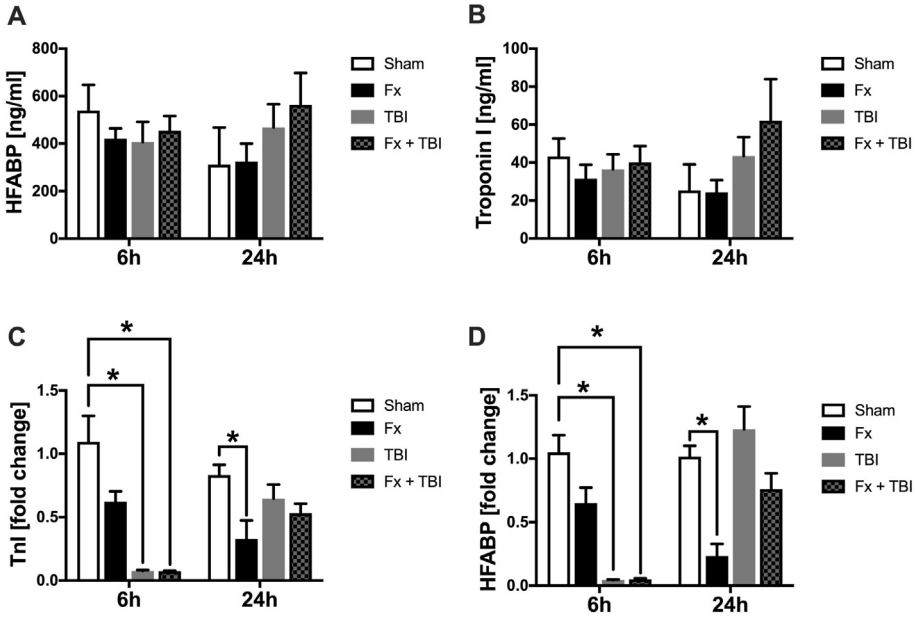

Figure 2. *Cardiac injury after experimental limb-, neuro- and combined trauma in mice.* Mice received either closed tibia fracture (Fx) (light grey bars), traumatic brain injury (TBI) (dark grey bars), combined trauma (Fx + TBI) (squared bars) or sham treatment (white bars). Blood plasma and cardiac tissue were analyzed after 6 and 24 h, respectively. Systemic release of heart fatty acid binding protein (HFABP) in pg/ml (**A**) and of cardiac troponin I in pg/ml (**B**). mRNA expression (fold change) of cardiac troponin I in left ventricles (**C**) and of cardiac HFABP in left ventricles (**D**). Data are presented as mean ± SEM. *p < 0.05 was considered as statistically significant.

Local inflammation. We also analyzed local cardiac inflammation, which is associated with cardiac damage after trauma. First, we analyzed the expression mRNA of the TLRs since these signaling pathways are associated with cardiac injury. The expression of TLR4 (Fig. 3A) and TLR9 (Fig. 3B) mRNA was significantly increased 24 h after tibia fracture. We also examined the expression of complement 3a (C3aR) and 5a receptors (C5aR), which may indicate local inflammation and complement activation. The expression of the C5aR1 mRNA was significantly increased after 24 h in all trauma groups (Fig. 3C), whereas no significant differences in C5aR1 protein expression was observed 24 h after trauma (Fig. 3D). Furthermore, no notable differences in cardiac C3aR protein expression were observed (Fig. 3E). Moreover, we analyzed the expression of IL-1b mRNA, since IL-1b is part of the NLRP3 inflammasome signaling pathway and is further associated with cardiac dysfunction. The expression of the IL-1b mRNA increased 24 h after tibia fracture (Fig. 3F). Also, expression of TNF mRNA in the left ventricle increased significantly 24 h after tibia fracture (Fig. 3G). To investigate local nitrosative stress we analyzed nitrotyrosine. This was significantly increased 6 h after Fx+TBI (Fig. 3H).

Local cardiac alterations. Finally, we analyzed cardiac alterations in

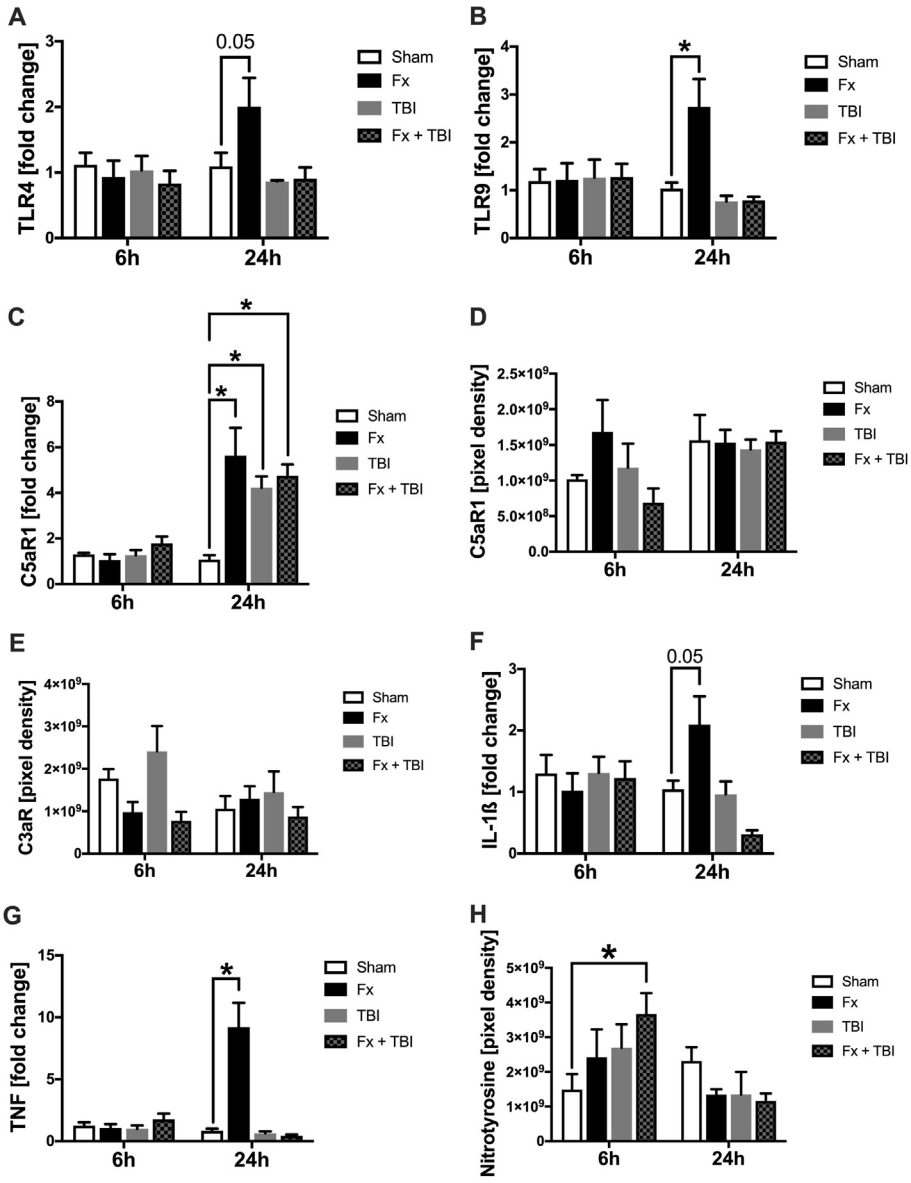

Figure 3. *Local cardiac inflammation after experimental limb-, neuro- and combined trauma in mice.* Mice received either closed tibia fracture (Fx) (light grey bars), traumatic brain injury (TBI) (dark grey bars), combined trauma (Fx + TBI) (squared bars) or sham treatment (white bars). Cardiac tissue were analyzed after 6 and 24 h, respectively. mRNA expression (fold change) of toll-like receptor 4 (TLR4) (**A**), TLR9 (**B**), C5a receptor 1 (C5aR1) (**C**), interleukin-1β (IL-1β) (**F**) and tumor necrosis factor (TNF) (**G**). Protein expression (pixel density) of C5aR1 (**D**), C3a receptor (C3aR) (**E**) and nitrotyrosine (**H**). Data are presented as mean ± SEM. *p < 0.05 was considered as statistically significant.

order to assess cardiac injury. We first examined the expression of proteins, which were involved in cardiac glucose- and fatty acid transport and analyzed the expression of GLUT4 mRNA. Alterations in this transporter and in cardiac metabolism were shown previously after trauma and were associated with impaired cardiac function. Expression of GLUT4 mRNA significantly increased 6 h after TBI and Fx + TBI (Fig. 4A). No significant differences were observed in myocardial GLUT4 protein expression (Fig. 4B). We further analyzed the expression of other GLUT isoforms. After 24 h, the GLUT2 mRNA expression increased in the TBI group but without reaching a level of significance (Fig. 4C). Moreover, the myocardial protein expression of GLUT1 was significantly reduced 6 h after TBI (Fig. 4D). We also analyzed the expression and localization of the cardiac gap junction protein connexin 43 (Cx43). In the left ventricle, Cx43 was translocated from the intercalated discs into the cytoplasm of the cardiomyocytes after 24 h on injury, without significant alterations in left ventricular Cx43 protein expression (Fig. 4E and F). The diffuse distribution of Cx43 in cytoplasm after 24 h seemed more pronounced in the Fx + TBI group (Fig. 4F).

Discussion

In the present study, we investigated systemic-as well as local cardiac inflammation 6 and 24 h after experimental limb-, brain and combined limb-brain trauma in mice. Systemic evidence for cardiac injury as well as local cardiac alterations were also examined to assess the link between inflammation and cardiac function. In summary, mice with combined trauma exhibited enhanced systemic inflammation after trauma, which manifest as increased levels of G-CSF, KC, and MCP-1. G-CSF was previously shown to be systemically elevated after TBI and was further described as biomarker for TBI in mice and humans, which is in accordance with the findings in the present study [31]. Moreover, systemic G-CSF levels correlate with the injury severity score (ISS) in humans and were likewise systemically enhanced in the present study after combined trauma [31]. MCP-1 and KC were also previously shown to be systemically elevated after fracture and TBI. Additionally, the release of both mediators was aggravated in a combined trauma model of fracture and thoracic trauma in mice [32,33]. KC, MCP-1 and G-CSF were recently

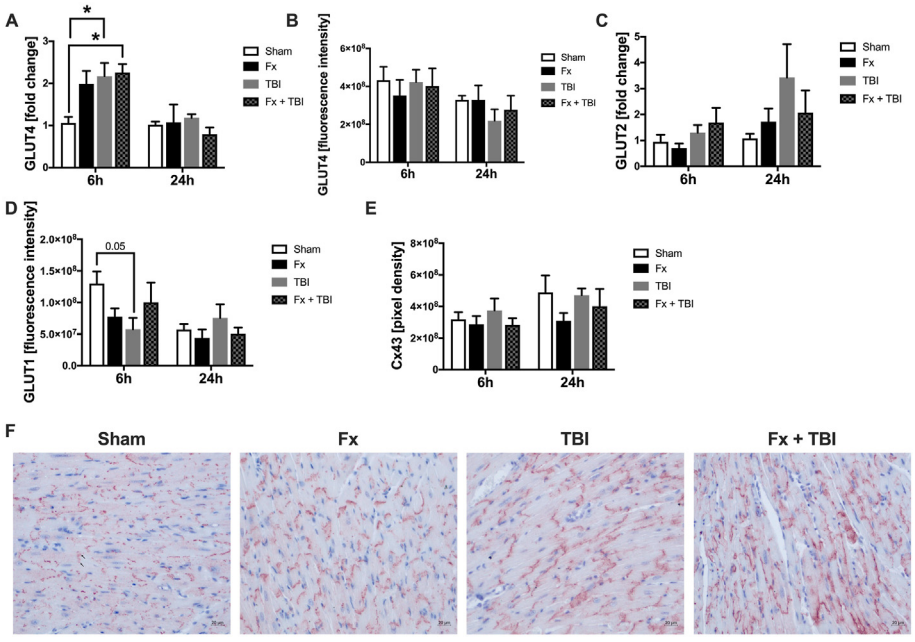

Figure 4. *Local cardiac alterations after experimental limb-, neuro- and combined trauma in mice.* Mice received either closed tibia fracture (Fx) (light grey bars), traumatic brain injury (TBI) (dark grey bars), combined trauma (Fx + TBI) (squared bars) or sham treatment (white bars). Cardiac tissue was analyzed after 6 and 24 h, respectively. mRNA expression (fold change) of glucose transporter 4 (GLUT4) (**A**), myocardial protein expression (fluorescence intensity) of GLUT4 (**B**), mRNA expression (fold change) of glucose transporter 2 (GLUT2) (**C**), myocardial protein expression (fluorescence intensity) of glucose transporter 1 (GLUT1) (**D**) and mRNA expression (fold change) of Connexin 43 (**E**). Representative images of Connexin 43 expression 24 h after Fx, TBI, Fx + TBI or sham procedure in left ventricles of mice (**F**). The black arrows demonstrate the myocardial expression of Cx43. Data are presented as mean ± SEM. *p < 0.05 was considered as statistically significant.

shown to impair cardiomyocyte function *in vitro* and were further associated structural cardiac alterations *in vivo* [30,34]. Interestingly, IL-10 levels were also elevated after combined trauma in blood plasma, which was previously shown to be associated with an anti-inflammatory response but also with a worse clinical outcome after trauma [35]. Overall, our data show that a combination of tibia fracture and TBI induced a systemic inflammatory response in mice.

To assess the impact of trauma on the heart, we analyzed the systemic occurrence of cardiac damage markers. We found that systemic HFABP and troponin I levels trended higher 24 h after trauma suggesting an impact on the heart. Interestingly, mice with combined trauma showed the highest systemic levels of both cardiac damage markers after 24 h. Expression of troponin I mRNA in the left ventricle significantly decreased after limb-, neuro- and combined trauma, which might be a compensatory effect due to enhanced systemic troponin I release. Troponin I is a reliable biomarker for cardiac injury and was previously shown to be elevated after multiple trauma in humans, pigs and mice, after blunt chest trauma in rats, after TBI in humans and after femur fracture in pigs, and this was associated with impaired cardiac function after these traumas [11,12,22,29,36,37]. The systemic release of HFABP is associated with cardiac damage and acute myocardial injury (AMI) [38]. Increased systemic HFABP levels were also demonstrated previously after experimental multiple trauma and after isolated long bone fracture, and these were also associated with post-traumatic cardiac dysfunction and the development of EMD [29,38] Interestingly, HFABP is also considered a reliable biomarker for stroke and TBI, which is elevated in cerebrospinal fluids (CSF) and blood, and these increases correlated with TBI severity [39–42]. However, due to the coincident occurrence of systemic troponin I, the HFABP in the present study might be released from both, brain and heart. We could not observe any anomalies in myocardial tissue appearance after combined trauma in mice. Consequently, the systemic elevation of troponin I as well as of HFABP after combined trauma might be induced by increased inflammation [3,43]. In the present study, mice showed enhanced local cardiac inflammation 24 h after closed tibia fracture, which was evidenced by increased expression of TLR4 and TLR9 mRNA. Both, TLR4 and TLR9 were shown to be involved in inflammatory-induced cardiac dysfunction [44,45]. Increased cardiac expression of TLRs after closed tibia fracture might be due to the enhanced systemic distribution of DAMPs and pro-inflammatory cytokines [22,46,47]. Furthermore, IL-1β and TNF mRNA expression increased in left ventricular tissue 24 h after closed tibia fracture, confirming local cardiac inflammation. Additionally, the cardiac expression of the pro-inflammatory cytokines IL-1β and TNF might amplify the systemic-as well as the local inflammatory response after fracture, resulting in an intensified post-traumatic immune response. Increased left ventricular expression of IL-1β was demonstrated previously during sepsis in mice as well as after isolated long bone fracture in pigs, and this was associated with impaired cardiac function, which was mediated via NLRP3 inflammasome signaling [22,48]. Apart from this, mice in the present study had increased cardiac expression of C5aR1 mRNA 24 h after limb-, neuro- and combined trauma. Increased cardiac expression of C5aR1, accompanied by an enhanced activation of the complement system were previously shown during sepsis and were associated with post-traumatic cardiac dysfunction [49,50]. Finally, mice showed increased local expression of nitrotyrosine 6 h after combined trauma, which was associated with increased nitrosative stress as well as with impaired cardiac function [29]. Together, these data show that mice exhibited enhanced local cardiac inflammation and increased nitrosative stress after limb-, neuro- and combined trauma, which might be associated with cardiac dysfunction. Further studies are needed to link these local and systemic findings to cardiac function and to unravel the reasons for the distinct differences in injury pattern between the different trauma types.

Mice showed alterations in the local expression of cardiac glucose- and fatty acid transporters after limb-, neuro- and combined trauma. In the present study, the GLUT4 mRNA expression was upregulated in left ventricles 6 h after TBI and combined trauma, indicating an enhanced demand for glucose to maintain proper cardiac function [51]. The alterations in GLUT4 mRNA expression might be due to increased inflammation rather than to the TBI-induced catecholamine release [51,52]. Furthermore, mice showed alterations in cardiac GLUT1 and GLUT2 expression after TBI, suggesting some important role of the cardiac glucose transport after neuro trauma. Additionally, the decreased cardiac expression of HFABP confirmed the switch from β-oxidation to anaerobic glycolysis in the context of the so-called myocardial hibernation [51,53]. Decreased cardiac expression of HFABP was previously demonstrated after isolated long bone fracture in pigs and were associated with impaired cardiac function [22,51]. Finally, in the present study mice showed a redistribution of the gap junction protein Cx43 24 h after limb-, neuro- and combined trauma, which was demonstrated previously after

experimental multiple trauma [29], after asphyxia and hemorrhage in newborns [54], after experimental blunt chest trauma [37], after chronic psychosocial stress [34] and after isolated long bone fracture [22]. In the present study, the Cx43 was translocated from the intercalated discs into the cytosol of the cardiomyocytes. Moreover, the diffuse Cx43 translocation seemed predominantly in mice after combined trauma. Redistribution of Cx43 after trauma was associated with disruption of the electrical communication of the cardiomyocytes, with severe arrhythmia as well as with post-traumatic cardiac dysfunction [29,55]. The redistribution of Cx43 in the present study might be also due to enhanced systemic inflammation. The *in vitro* exposure of murine HL-1 cells to KC, G-CSF, MCP-1 and IL-1β induced alterations in Cx43 mRNA expression, which were further associated with an impaired calcium signaling of the cells [34]. Summarized, the mice showed local cardiac alterations, which might be linked to increased inflammation after trauma and which might further predispose for secondary cardiac injury after limb-, neuro- and combined trauma. Therefore, future investigations targeting the inflammatory response might alleviate cardiac injury and might also decrease the morbidity and mortality after severe trauma.

Conclusion

In conclusion, mice showed distinct pattern of increased systemic-as well as local cardiac inflammation after limb- and neurotrauma, which was aggravated in a combination of both. Furthermore, cardiac alterations as well as the occurrence of systemic cardiac injury markers were observed in these mice, which were linked to the enhanced inflammation after trauma. Finally, these alterations might predispose for post-traumatic secondary cardiac damage. However, in order to manifest cardiac damage after limb-, neuro- and combined trauma, further investigations, including functional cardiac analysis has to be performed.

Declaration of competing interest

The authors have no conflicts of interests relevant to this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2020.12.003.

Refrences

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