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HEALTH SCIENCES

Taurine Chloramine decreases cell viability and cytokine production in blood and spleen lymphocytes from septic rats

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Abstract: Taurine (Tau) is an abundant amino acid in polymorphonuclear leukocytes that react with hypochlorous acid to form taurine chloramine (TauCl) under inflammatory conditions. We investigated potential interactions between lymphocytes and TauCl in rats submitted to cecal ligation. Animals were divided into sham or CLP groups (24 or 120 h) to isolate lymphocytes from blood and spleen. Lymphocytes were cultured at a concentration of 1×106 cells/mL and activated by concanavalin A. Tau and TauCl were added at 1, 10, and 100 µM. Cells were incubated with MTT to evaluate cell viability and cytokine concentration in the supernatant was determined. TauCl decreased lymphocyte viability and altered the secretion pattern of important inflammatory mediators in non-specific-phenotype manner. The effort to a is elucidate mechanisms of immune cell (dys) function in sepsis is important to better understand the complex regulation of immune system during sepsis development, and further studies are necessary to confirm TauCl as potential target in this context.

Key words: lymphocyte, sepsis, taurine, taurine chloramine, viability.

INTRODUCTION

The Third International Consensus Definitions Task Force defined sepsis as life-threatening organ dysfunction caused by dysregulated host response to infection (Seymour et al. 2016). Sepsis remains the leading cause of death in intensive care units (ICUs), despite remarkable advances in treatment of critical illnesses and progress in general intensive care medicine (Perner et al. 2017).

Although polymorphonuclears (PMNs) are mostly related to innate immunity, they also co-localized in T cells at sites of persistent infections, chronic inflammations or tumors (Müller et al. 2009). There is increasing evidence for mutual interaction between PMNs and T cells (Müller et al. 2009); thus, we aimed to evaluate possible interactions among chloramine and lymphocytes. Although MPO and Tau are separated in mature PMNs, the formation of TauCl inside PMNs is possible after reactive oxygen species (ROS)-driven permeabilization of azurophilic granules to the cytosol. This may occur during bacteria-induced apoptosis in PMNs Blomgran et al. 2007).

In recent years, great attention has been focused on the mechanisms of immune cell death during sepsis. The early phase of sepsis is dominated by a hyperinflammatory state, which is mediated by systemic production of inflammatory cytokines, including interleukin 1 (IL-1), IL-6, tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ). This

hyperinflammatory response may lead to organ damage and death in a subset of septic patients (Döcke et al. 1997, Heidecke et al. 1999, Rittirsch et al. 2008). To balance the pro-inflammatory response, the anti-inflammatory cytokines, including IL-10, are produced. As a result, the majority of patients that survive the initial phase of pro-inflammatory sepsis progress to a protracted period of immune suppression (Hotchkiss et al. 2001, Rittirsch et al. 2008).

More recently IL-17, a pro-inflammatory cytokine expressed by activated memory $CD4^+$ T cells was demonstrated to be important in sepsis (Jekarl et al. 2015). In particular, IL-17A is a mediator of neutrophil stimulation and mobilization by T lymphocytes (Kolls & Linden 2004, Kasten et al. 2010). Later stages of T_H17 cell differentiation rely on the expression of IL-23, which is a potent inducer of IL-17 (Murphy et al. 2003, Langrish et al. 2005). Although innate IL17 responses might eradicate most pathogens, it can lead to hyperinflammatory responses; moreover, innate immune cells activated by IL17 could damage the surrounding tissue (Hirota et al. 2012).

Taurine chloramine (TauCl) is an oxidative product released during the inflammatory response. Taurine (Tau) is generated from cysteine and the most abundant free amino acid in mammalian monocytes and tissues (Vinton et al. 1986, Learn et al. 1990), found in all animal cells at millimolar concentrations, in the plasma and extracellular fluids in much lower ranges (Shuller-Levis & Park 2003) and at the site of inflammation reaches micromolar concentrations (Jang et al. 2009).

Tau protects activated neutrophils from inflammatory injury by detoxifying the highly oxidizing hypochlorous acid (HOCl/OCl^-) produced from hydrogen peroxide (H_2O_2) (Thomas et al. 1986a). Some evidences showed that Tau can act as an antioxidant by enhancing expression and activities of defense enzymes such catalase, superoxide dismutase and glutathione peroxidase (Jang et al. 2009). Neonates, aging process and some specific conditions like trauma and sepsis limit the biosynthetic capacity of humans to produce Tau (Marcinkiewicz & Kontny 2014). It has been reported that Tau may protect immune cells against attack by chlorinated oxidants, besides protect tissues and inflammation sites (Cantin 1994). In contrast, a study in synoviocyte cultures showed that Tau does not affect the functions of these cells *in vitro* (Kontny et al. 2003)

Taurine removes excessive HOCl by reacting readily with HOCl/OCl^- and forming TauCl, which is more stable and weaker oxidant (Park et al. 1995). Despite its lower reactivity it was shown that TauCl could induce apoptosis in lymphocytes (Klamt & Shacter 2005) and inhibits inducible nitric oxide synthase and TNF-α gene expression (Barua et al. 2001). TauCl has been described as an oxidant with anti-inflamatory effects, with capacity of suppress production of mediators like cytokines, chemokines, nitric oxide and prostaglandin E2 (Marcinkiewicz et al. 1998, Kontny et al. 2000). Thus, we hypothesized that lymphocytes from septic animals are susceptible to the effects of TauCl and this could be one of the mechanisms of immunosuppression during sepsis.

The objective of this study was to assess the effect of Tau and TauCl on the viability of blood and splenic lymphocytes. Considering that Tau has a redundant role, we hypothesized that these compounds, mainly TauCl, could be act on the pathways that explain, at least in part, a pathophysiologic mechanism on the complex balance of stimulatory and inhibitory pathways in sepsis.

MATERIALS AND METHODS

Animals

Animal experiments were conducted according to protocols approved by the Animal Experimental Ethics Committee of Universidade do Extremo Sul Catarinense, Criciúma, Brazil (protocol number: 89/2012). Eight-week-old male *Wistar* rats (250-300g) were housed at 18°C-22°C, in 12 h/12 h light/dark conditions and 55-65% relative humidity. *Wistar* rats were divided randomly into two groups: sham (n=12) and sepsis (n =16).

Cecal and ligation puncture (CLP) model of sepsis

Wistar rats were subjected to CLP as previously described (Fink & Heard 1990). Briefly, the animals were randomized and anesthetized using a mixture of intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg). Under aseptic conditions, a 3-cm midline laparotomy was performed to expose the cecum and adjoining intestine. The cecum was ligated with a 3.0 silk suture at its base, below the ileocecal valve and punctured once with a 14- gauge needle. The cecum was then squeezed gently to extrude a small amount of feces through the perforation site. The cecum was then returned to the peritoneal cavity, and the laparotomy was closed with 4.0 silk sutures. Animals were resuscitated with regular saline (50mL/kg) subcutaneously (s.c.) immediately and 12h after CLP. All animals received antibiotics (ceftriaxone at 30 mg/kg) every 6h s.c. for 3 days and dipyrone sodium (80 mg/kg) (s.c.) immediately and 12h after CLP. To minimize variability between different experiments, the CLP procedure was always performed by the same investigator.

Collection, isolation and stimulation of blood and splenic lymphocytes

Blood from both sham and sepsis groups was collected through cardiac puncture and stored in EDTA tubes in 24 and 120 hours after CLP surgery. Spleens were simultaneously collected. Blood and spleen lymphocytes were isolated by Ficoll-Hypaque solution gradient separation (Sigma-Aldrich Co, St. Louis, MO, USA), using standard protocols (Boyum 1977, Mishell & Dutton 1967). Cells were suspended in RPMI 1640 media (Sigma-Aldrich) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), penicillin (100 UI/mL, Sigma-Aldrich), streptomycin (100 µg/mL, Sigma-Aldrich) and processed for further staining with Giemsa stain (Sigma-Aldrich) and Trypan blue (Sigma-Aldrich). Cells were counted in a Neubauer chamber, and the viability was determined using 94% by Trypan blue exclusion dye. T cells were suspended in 96-well plates in the presence of concanavalin A (Con A, Sigma-Aldrich) at 2 µg/mL (Fazzino et al. 2010). The final volume was 200µl with density of 1×10^6 cells/ml. The plates were incubated at 37°C in 5% CO_2, for 24 hours.

Preparation of Tau and TauCl

TauCl was frechly prepared according standard protocols (Thomas et al. 1986a, b) with modifications. Briefly, 25 mM sodium hypochlorite (Sigma-Aldrich) was added to 100 mM Tau (Sigma-Aldrich) in phosphate buffered saline (PBS) (pH 7,8-8,0). Each preparation of TauCl was monitored by ultraviolet absorption (252nm, 429 $M^{-1}cm^{-1}$) to confirm the presence of the chloramine. Tau was prepared in PBS (pH 7,4). Stock solutions of Tau (1; 10 and 100 µM TauCl) were used.

Citotoxicity assay and measurement of cell viability

Twenty-four hours after culture, lymphocytes were incubated with Tau or TauCl (at 1; 10; 100 µM concentrations) for 1 hour (Ogino et al. 2009). After this period, supernatants were collected and frozen at –80°C. Cell viability was assessed by the mitochondria-dependent reduction of thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich) to formazan crystals (Mosmann 1983). After 4 hours incubation with MTT (500 µg/mL) 100 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added. The absorbance of DMSO-dissolved MTT crystals was measured at 560-630 nm with a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, California, USA).

Cytokine measurements

To measure IL-17A, IL-10, TNF-α, IL-6, IFN-γ and IL-23, sandwich ELISAs were performed (Peprotech Inc. Connecticut, USA). The concentration for each cytokine was calculated using a recombinant protein as the standard. The linear ranges were: 15,6 to 1000 pg/mL (IL-23); 16 to 1000 pg/mL (IL-17A); 39 to 2500 pg/mL (IL-10); 17 to 3000 pg/mL (TNF-α); 32-8000 pg/mL (IL-6); 25-4000 pg/mL (IFN-γ).

Statistical analysis

All experiments were performed in triplicate and analyzed with the SPSS statistical package (SPSS 20.0 software). Values were represented as means ± SD. ANOVA with *post-hoc* Tukey test were performed for ELISAs and Multi-factorial ANOVA tests were used for MTT assays. P values < 0.05 were considered significant.

RESULTS

Cell viability by MTT assay

In general, cell viability alteration was more often seen at 24 hours than 120 hours both in blood and spleen in non-dependent dose manner and did not follow any specific pattern. Tau and Tau-Cl treatments (1, 10 and 100 µM) reduced blood cell viability after 24 hours when compared to the corresponding sham group (Figure 1a). A different pattern was seen in blood lymphocytes at 120 hours (Figure 1b), when Tau did not reduce viability. However, cell viability decreased upon exposure to 10 and 100 µM TauCl.

Splenic lymphocytes were less sensitive than blood cells 24 hours after treatment with both Tau and Tau-Cl. Only 1 µM TauCl decreased splenic cell viability after 24 hours when compared to the relative sham group (Figure 1c). After 120 hours, no sensitivity to Tau and TauCl in CLP group was observed.

Cytokine secretion

As cytokine production by adherent and non-adherent mononuclear leukocytes plays a critical role in inflammation, a lymphocyte cytokine production profile in the presence of TauCl was analyzed. We did not investigate the effect of Tau in cytokine production because it did not influence the production of these mediators, as described previously (Marcinkiewicz et al. 1995, 1999). TauCl did not modify IL-17 secretion was decreased in splenic lymphocytes treated with 10 µM TauCl 120 hours after sepsis induction (Figure 2a and b). The secretion of IL-23, which is a potent inducer of IL-17 (Murphy et al. 2003, Langrish et al. 2005), was altered only at 1 µM TauCl, as compared to untreated cells (Figure 3a and b). Both blood and splenic lymphocytes increased the secretion of IFN-γ when treated with TauCl (Figure 4a and b). However, 10 µM

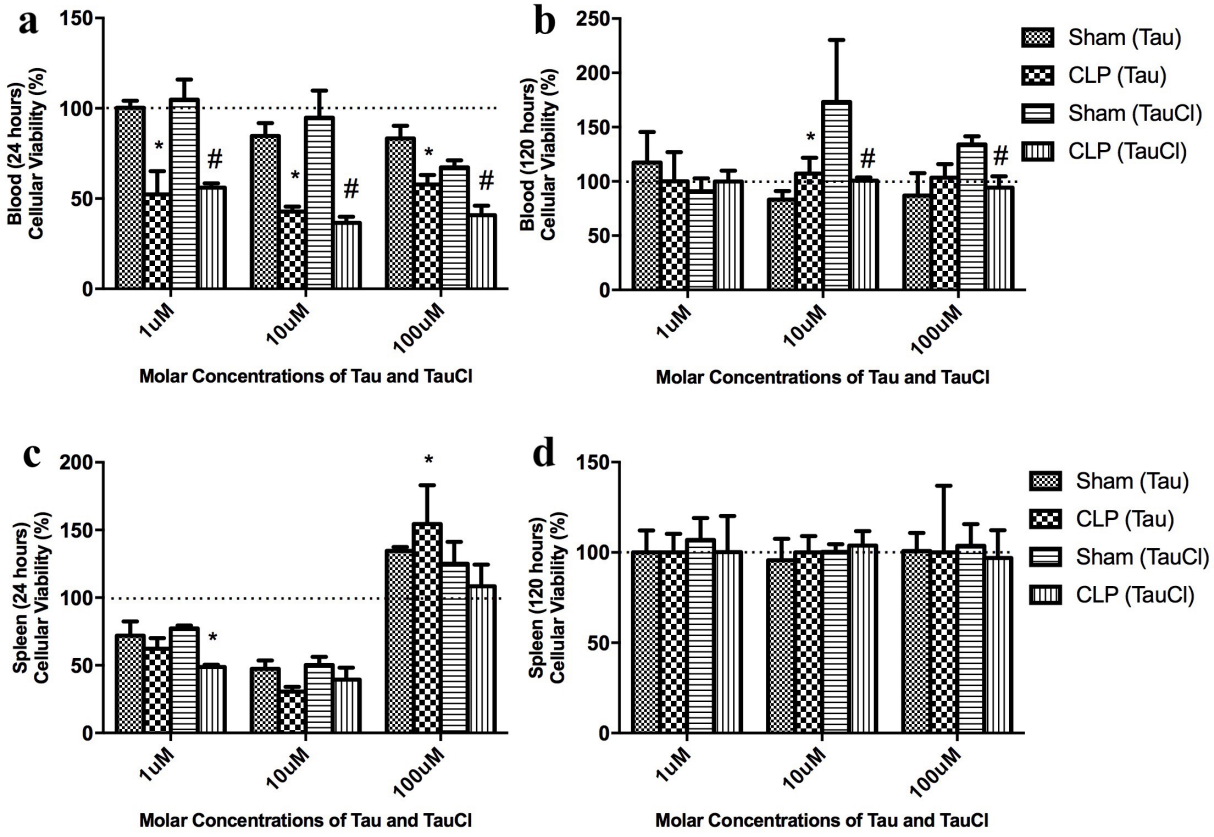


Figure 1. **Cell viability measurement by MTT assay. Viability of lymphocytes treated with Tau and TauCl in different molar concentrations. Rats were submitted to CLP or Sham, and 24 or 120 hours after the surgery their blood and spleens were collected, the lymphocytes were isolated, cultured and the cell viability was measured by MTT assay. (a) blood, 24 hours; (b) blood, 120 hours; (c) spleen, 24 hours; (d) spleen, 120 hours. * = P < 0.05 for CLP compared with sham group (Tau-treated). # = P < 0.05 for CLP compared with Sham group (TauCl-treated). (n = 5).**

TauCl reduced the production of IFN-γ secretion in splenic lymphocytes.

TNF-α secretion by spleen decreased significantly after 24 hours, as compared to sham (Figure 5a and b). The last pro-inflammatory cytokine evaluated was IL-6, an important mediator released during sepsis, however, the effect of TauCl in IL-6 secretion was not consistent (Figure 6a and b).

It is also important to analyze an anti-inflammatory mediator that acts as an immune regulator in sepsis. TauCl decreased IL-10 secretion primarily in spleen cells (Figure 7) in the doses of 1 and 10 μM.

DISCUSSION

Determine the effects to TauCl on production of important inflammatory mediators such as cytokines by lymphocytes seems to be fundamental to understanding its role in immune dysfunction associated with sepsis.

Here it was demonstrated that cell viability did not show a consistent pattern of alteration. Tau decreased cell viability at 24 hours, similarly to TauCl, and this is the opposite from what was expected, since Tau could exert a physiological scavenging potential against reactive species generated by oxidative metabolism (Oliveira et al. 2010). In addition, sepsis has been associated

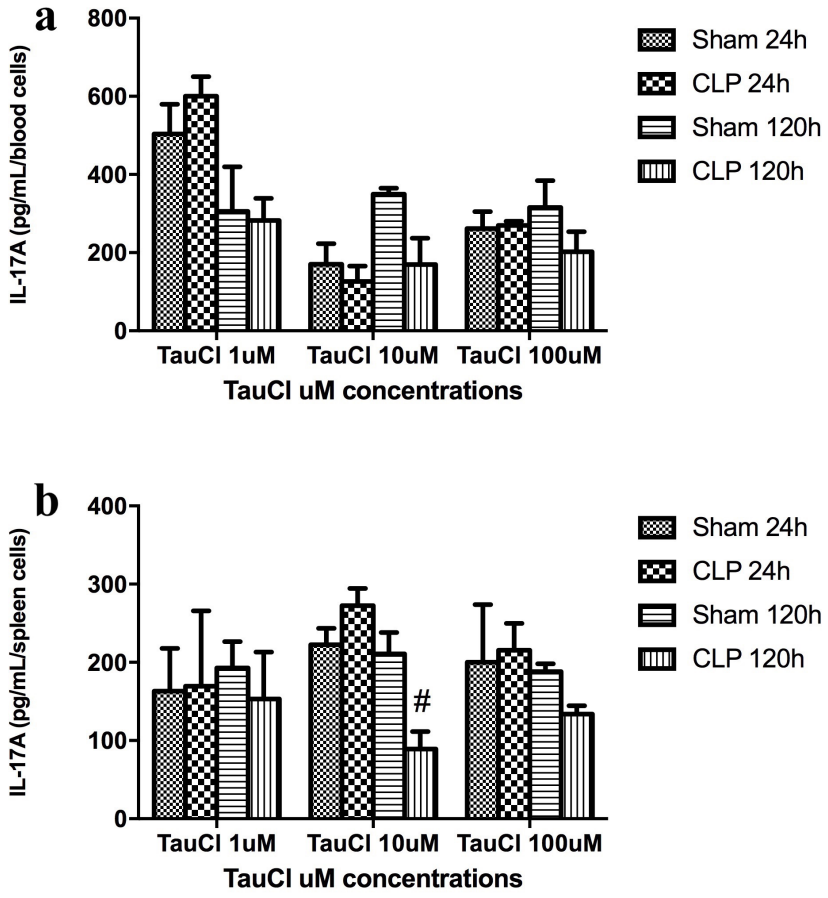


Figure 2. The effect of TauCl on the production of the pro-inflammatory mediator, IL-17A. Lymphocytes (1x106 cells/mL) were preincubated with TauCl (1, 10 or 100 uM) for 1 hour. After this, supernatants were collected and IL-17A was measured by ELISA for (a) blood and (b) spleen. Results are expressed as means ± SD. # = P < 0.05, compared with sham rats at 120 hours (n=5).

with increased oxidant levels, depleted antioxidants, and accumulated markers of oxidative stress (Zhang et al. 2000). Ritter et al. (2003) demonstrated that superoxide dismutase (SOD) is predictive of mortality. The SOD/Catalase (CAT) imbalance seems to be an important factor in the oxidative stress during the letal sepsis (Andrades et al. 2005) and plasma SOD activity can be used as a biomarker in human sepsis (Guerreiro et al. 2010). This Tau potencial could be related to the functional impairment of taurine transporter (taut) caused by peroxynitrite. This is likely due to the nitrosylation and/or nitration of amino acids residues of taut, leading to less intracellular transport of Tau (Koo et al. 2012). Ther is a considerable body of evidence supporting the modification and functional alteration of proteins by nitrogen oxides (NOs) (Dimmeler et al. 1994, Kuhn & Arthur 1996, Mohr et al. 1996). In a previous study, the functional activities of secondary active transport systems, including the Tau uptake system, were reduced at the barrier between systemic circulation and the cerebrospinal fluid after an induction of inflammation. In addition, *in vitro* Tau uptake activity was reduced, suggesting that this process is sensitive to NOs (Han et al. 2002). In contrast, Park et al. (2002) conducted a study that cell viability was unaffected by 0.1mM and 0.4 mM TauCl.

It was demonstrated that taurine haloamines, such as TauCl, inhibit the production of pro-inflammatory mediators like TNF-α, IL-1B and IL-6 (Marcinkiewicz et al. 1995, Park et al. 1995,

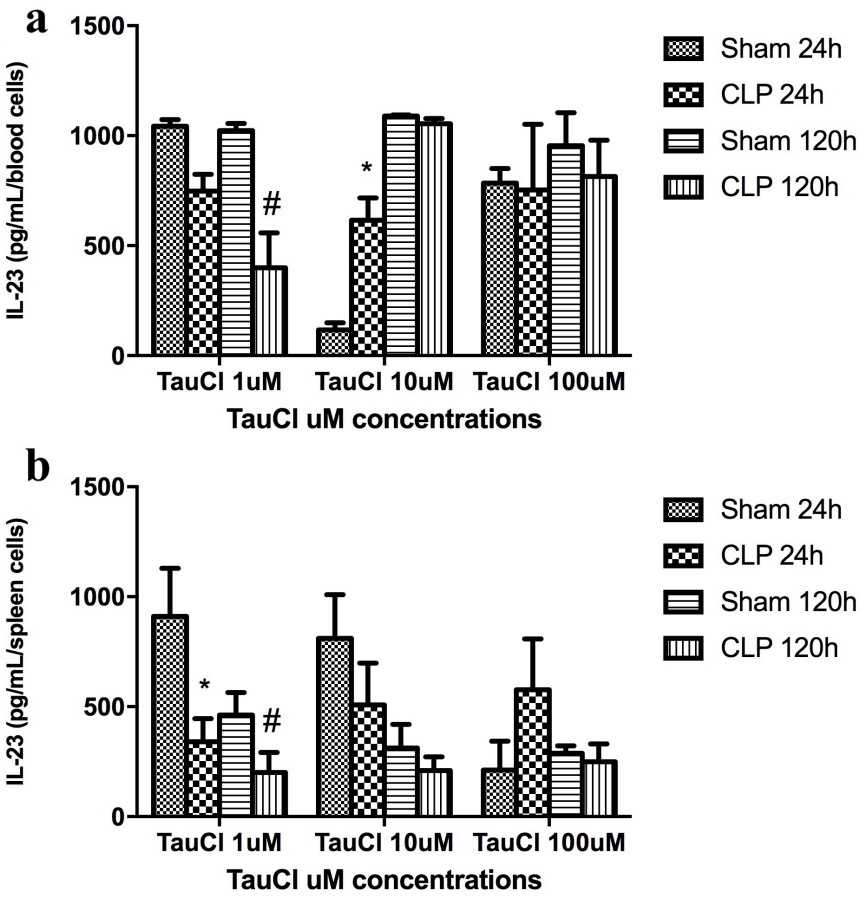


Figure 3. The effect of TauCl on the production of the pro-inflammatory mediator, IL-23. Lymphocytes (1x106 cells/ mL) were preincubated with TauCl (1, 10 or 100 uM) for 1 hour. After this, supernatants were collected and IL-23 was measured by ELISA for (a) blood and (b) spleen. Results are expressed as means ± SD. * = P < 0.05 compared with sham rats at 24 hours. # = P < 0.05 compared with sham rats at 120 hours (n=5).

Barua et al. 2001). Klamt & Shacter (2005) suggest that TauCl may be involved in the resolution and termination of acute inflammation. Besides that, Kontny et al. (2003) proposed that TauCl may represent negative regulatory loop to damp inflammation during inflammatory response and low TauCl concentration can lead to a pathological chronic status and affirmed that TauCl mediated downregulatory response is not effective enough to prevent transition from the acute to the chronic phase in an experimental of cells stimulated with lipopolysaccharide (LPS) (Chorazy et al. 2002).

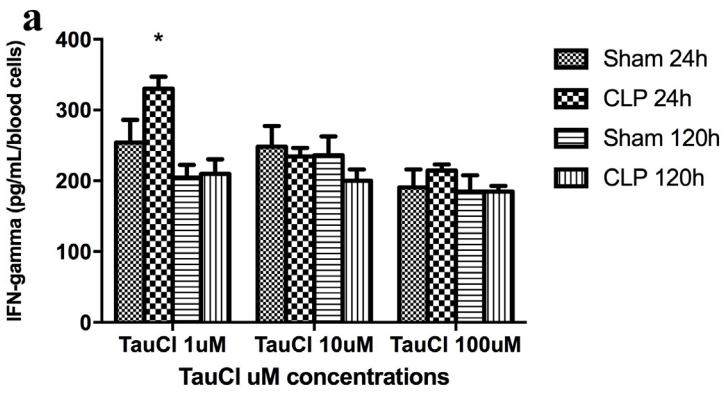
Lymphocytes at 120 hours were more sensitive to TauCl treatment. Increase in spleen lymphocyte apoptosis has been shown to reduce survival in experimental animals with sepsis and they are the immune cells most affected by deregulated apoptotic cell death during sepsis (Wagner et al. 2007, Wesche-Soldato et al. 2007, Tinsley et al. 2003). Both extrinsic negative regulatory pathways and cell-intrinsic negative regulatory pathways have key roles in T cells exhaustion (Wherry 2011). TauCl could be implicated in this context. Although functional effector T cells can transiently express inhibitory receptors during activation, prolonged and/or high expression of multiple inhibitory receptors is a key feature of the exhaustion of CD8+ and CD4+ T cells both in animal models and in humans (Virgin et al. 2009). Thus, TauCl can act as a molecule that signal to down-regulation of lymphocytes after sepsis.

Our experiments analyzed two stages of sepsis development – 24 hours representing the acute inflammatory response and 120

hours representing a hypo-inflammatory response. In an attempt to explain these stages, Marcinkiewicz et al. (1998) suggested that in acute phase of inflammation TauCl contributes to the inflammatory function of cells like neutrophils, and the increase in its levels is also associated with a secondary down-regulation of the inflammatory process. Peripheral blood mononuclear cells (PBMCs) isolated from patients that succumbed to sepsis exhibit a reduction in cytokine secretion when stimulated in vitro, supporting the hypothesis that functional immune cell impairment may predispose a patient to a poorer outcome (Boomer et al. 2011). However, in our study it was not possible to observe a reduction in all of cytokines when CLP cells were TauCl-treated, at least in lymphocytes.

The proliferative response of peripheral blood mononuclear cells in septic patients after stimulation with mitogens was also markedly decreased in comparison with that in healthy individuals (Venet et al. 2009). The fact that TauCl decreases these cytokines production in lymphocytes from septic animals, particularly at 120 hours, could compromise important host defenses, in a lymphocyte population non-specific manner. Studies have indicated that a shift from a T_H1 to a T_H2 response occurs that contributes to the late immunosuppression seen during sepsis and this study contributes to show that T_H17 also can be involved in sepsis. Multiple inhibitory mechanisms were identified in immunosuppressive phase of sepsis, which encompasses the 120 hours timepoint, including the dominance of inhibitory over activating receptors, expansion of suppressive cell types and induction of inhibitory ligands on both APCs and tissue parenchymal cells. The presence of apoptotic cells inducing $CD8^+$ T_{REGS} mediates suppression by producing TNF-related apoptosis-inducing ligand (TRAIL) (Unsinger et al. 2010).

As a product of the chlorinating system of neutrophils, TauCl can act as an immune modulator of other cells, including lymphocytes, trying to promote changes in the hyper/hypo inflammatory axis. TauCl may therefore play a role in maintaining the delicate balance between mounting an effective immune response on one hand, and minimizing the destrictive effect of the inflammatory cells on the other (Marcinkiewicz et al. 1998). In a study with rheumatoid arthritis cells, due to prolonged activation, neutrophils accumulating in rheumatoid synovial fluid exhibit features indicative of partial functional "exhaustion" and these cells generate less TauCl *in vitro* than their peripheral blood counterparts, suggesting that the local concentration of TauCl in the local of inflammation is probably too low to exert anti-inflammatory effects (Kontny et al. 2002). When activated adherent and non-adherent monocuclear leukocytes were treated with TauCl, production of cytokines tested – IL-6, IL-8 and IL-12 – was significantly down-regulated, except for TNF-α (Park et al. 2002). In contrast, our results showed significantly decrease of TNF-α at 24 hours, and this could be a feedback inhibition of TH1 responses during sepsis (Biron 2001). In the initial phase of immune response TauCl (present at low concentration) may favors the resolution of protective inflammation by supporting systhesis of TNF-α (Chorazy et al. 2002). As the inflammatory process progress a local accumulation of TauCl occurs, and this compound could prevent perpetuation of chronic inflammation by a gradual down-regulation of IL-6 (Tilg et al. 1994). Another possible mechanism to control inflammation could be an increase in IL-10, but we and others (Marcinkiewicz & Kontny 2014) could not observe any effect of TauCl on IL-10 secretion.



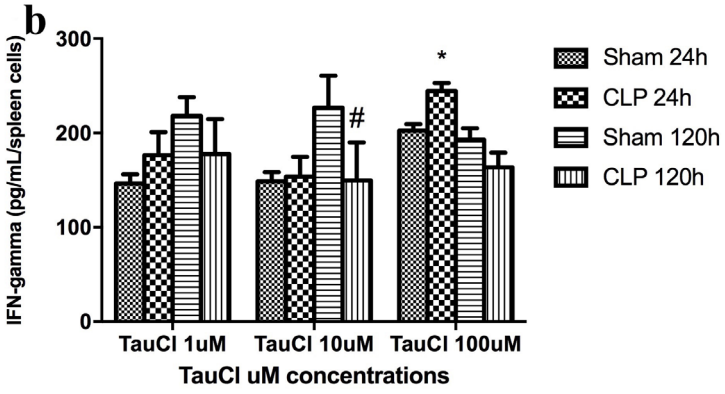
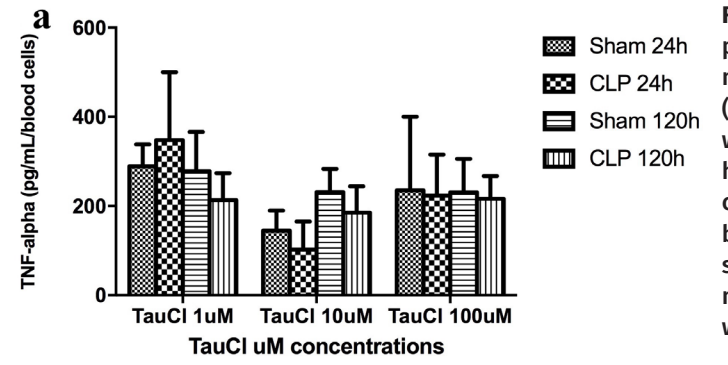


Figure 4. The effect of TauCl on the production of the pro-inflammatory mediator, IFN-gamma. Lymphocytes (1x106 cells/mL) were preincubated with TauCl (1, 10 or 100 uM) for 1 hour. After this, supernatants were collected and IFN-gamma was measured by ELISA for (a) blood and (b) spleen. Results are expressed as means ± SD. * = P < 0.05 compared with sham rats at 24 hours. # = P < 0.05 compared with sham rats at 120 hours (n=5).



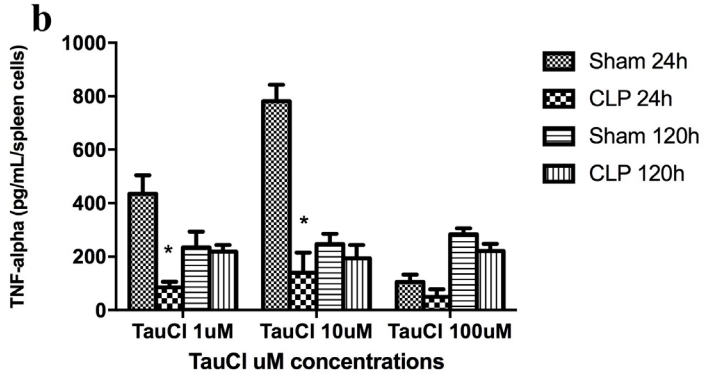


Figure 5. The effect of TauCl on the production of the pro-inflammatory mediator, TNF-α. Lymphocytes (1x106 cells/mL) were preincubated with TauCl (1, 10 or 100 uM) for 1 hour. After this, supernatants were collected and TNF-α was measured by ELISA for (a) blood and (b) spleen. Results are expressed as means ± SD. * = P < 0.05 compared with sham rats at 24 hours (n=5).

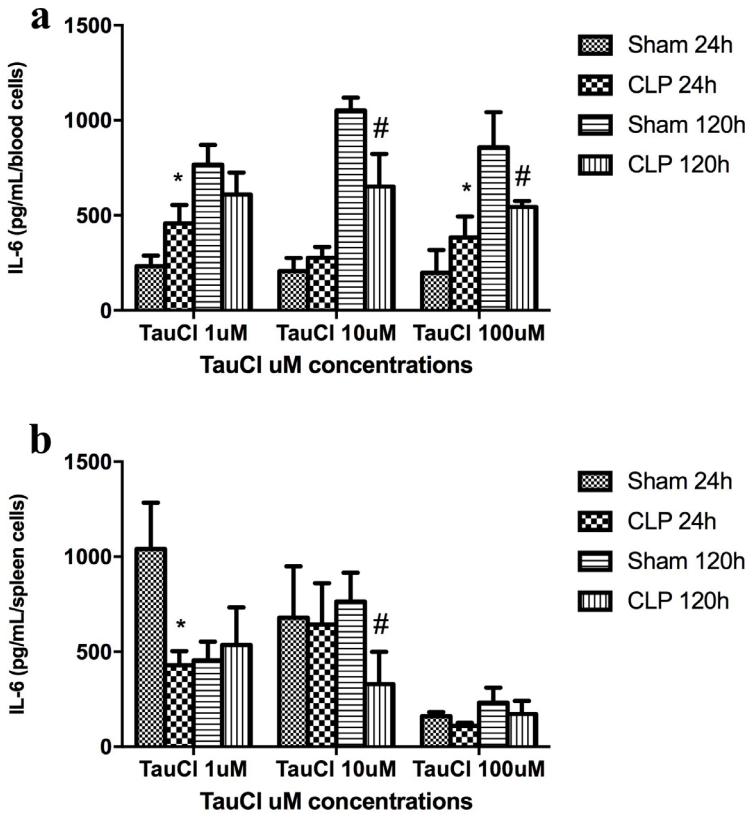


Figure 6. The effect of TauCl on the production of the pro-inflammatory mediator, IL-6. Lymphocytes (1x106 cells/mL) were preincubated with TauCl (1, 10 or 100 uM) for 1 hour. After this, supernatants were collected and IL-6 was measured by ELISA for (a) blood and (b) spleen. Results are expressed as means ± SD. * = P <0.05 compared with sham rats at 24 hours. # = P < 0.05 compared with sham rats at 120 hours (n=5).

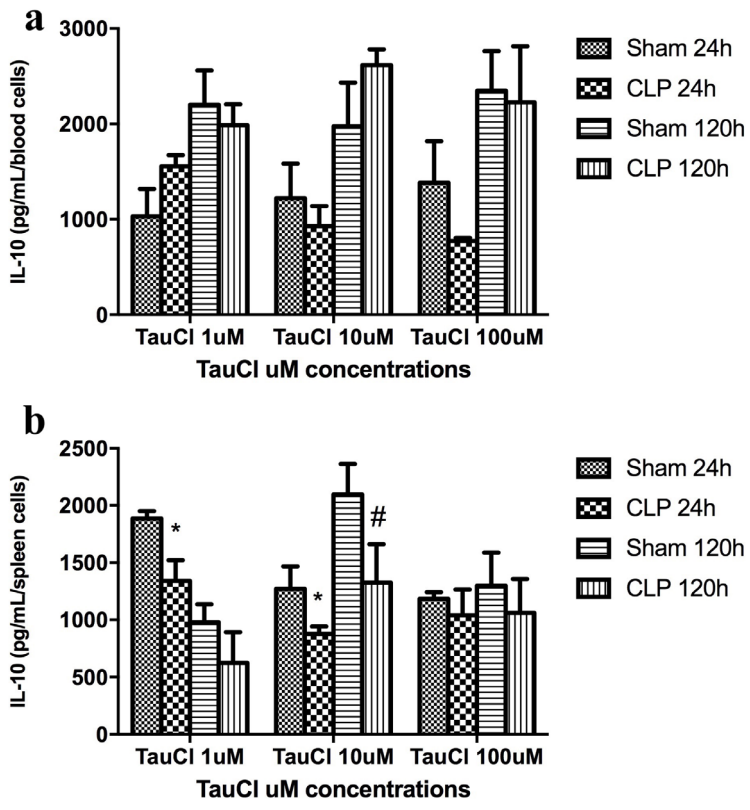


Figure 7. The effect of TauCl on the production of the pro-inflammatory mediator, IL-10. Lymphocytes (1x106 cells/mL) were preincubated with TauCl (1, 10 or 100 uM) for 1 hour. After this, supernatants were collected and IL-10 was measured by ELISA for (a) blood and (b) spleen. Results are expressed as means ± SD. * = P < 0.05 compared with sham rats 24 hours. # = P < 0.05 compared with sham rats 120 hours (n=5).

CONCLUSION

Our data suggest that TauCl could contribute to immune modulation during sepsis by decreasing lymphocyte viability, especially at early stages (24 hours) and by altering secretion pattern of important inflammatory mediators. The effort to elucidate mechanisms of immune cell (dys)function in sepsis is important to better understand the complex regulation of immune system during sepsis development, and futher studies are necessary to confirm TauCl as a potential target in this context.

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