

ORIGINAL
ARTICLETumor necrosis factor- α synthesis inhibitor,
3,6'-dithiothalidomide, reverses behavioral
impairments induced by minimal traumatic brain
injury in miceRenana Baratz,* David Tweedie,† Vardit Rubovitch,* Weiming Luo,†
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National Institutes of Health, Baltimore, Maryland, USA§Department of Neurosurgery, Case Western Reserve University School of Medicine, Cleveland, Ohio,
USA**Abstract**

Mild traumatic brain injury (mTBI) patients do not show clear structural brain defects and, in general, do not require hospitalization, but frequently suffer from long-lasting cognitive, behavioral and emotional difficulties. Although there is no current effective treatment or cure for mTBI, tumor necrosis factor- α (TNF- α), a cytokine fundamental in the systemic inflammatory process, represents a potential drug target. TNF- α levels increase after mTBI and may induce or exacerbate secondary damage to brain tissue. The present study evaluated the efficacy of the experimental TNF- α synthesis inhibitor, 3,6'-dithiothalidomide, on recovery of mice from mTBI in a closed head weight-drop model that induces an

acute elevation in brain TNF- α and an impairment in cognitive performance, as assessed by the Y-maze, by novel object recognition and by passive avoidance paradigms at 72 h and 7 days after injury. These impairments were fully ameliorated in mice that received a one time administration of 3,6'-dithiothalidomide at either a low (28 mg/kg) or high (56 mg/kg) dose provided either 1 h prior to injury, or at 1 or 12 h post-injury. Together, these results implicate TNF- α as a drug target for mTBI and suggests that 3,6'-dithiothalidomide may act as a neuroprotective drug to minimize impairment.

Keywords: etanercept, neuroinflammation, thalidomide, thiothalidomide, TNF- α , traumatic brain injury.

J. Neurochem. (2011) 10.1111/j.1471-4159.2011.07377.x

Notwithstanding some recent improvements in the management of mild traumatic brain injury (mTBI), as yet no effective treatment is available for those afflicted, and thus morbidity and mortality are significant (Cernak and Noble-Haeusslein 2010). mTBI-associated brain damage can be segregated into two phases: an initial primary phase induced by the injury, itself, that is immediate, irreversible and only minimized by preventive strategies, followed by an extended second phase, which is initiated at the time of original injury and persists over subsequent days, weeks and, possibly, months. This delayed phase involves an array of molecular,

Received January 20, 2011; revised manuscript received July 1, 2011; accepted July 2, 2011.

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Abbreviations used: ARE, adenylate-uridylylate-rich element; LPS, lipopolysaccharide; mTBI, mild traumatic brain injury; PBS, phosphate-buffered saline; SCI, spinal cord injury; TNF- α , tumor necrosis factor- α ; 3'-UTR, 3'-untranslated region.

cellular, and physiological reactions focused towards restoring the homeostasis of damaged tissue, which, if not appropriately regulated, can lead to additional injury. The frequent occurrence of secondary brain injury provides an opportunity in which therapeutic strategies with neuroprotective properties can be administered. However, to develop effective treatment strategies for TBI, it is clearly necessary to understand the molecular and biochemical cascades that induce secondary injury (Tweedie *et al.* 2007a; Stoica and Faden 2010). In this regard, a fundamental pathway recognized to be triggered in response to mTBI is cellular and humoral inflammation (Cederberg and Siesjö 2010; Svetlovey *et al.* 2009; Ziebell and Morganti-Kossmann 2010).

Neuroinflammation within the injured brain is widely considered to exacerbate damage induced by mTBI, as it plays a critical role in the initiating processes of a wide variety of other neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis (Frankola *et al.* 2011). Although inflammation represents a first line of defense against injury and infection, an exaggerated inflammatory response can induce additional injury to neural tissues (Tweedie *et al.* 2007b; Tansey 2010). Within the CNS, glial cells, specifically microglia and astrocytes, represent the primary source of inflammatory reactions. Under normal conditions, these cells provide supportive functions to optimize neuronal activity and maintenance of homeostasis. Under stress, however, glial cells proliferate, become activated and generate the production of neurotoxic molecules, including free radical species and proinflammatory cytokines (Lecca *et al.* 2008; Reale *et al.* 2009; Yan *et al.* 2009).

Key amongst the proinflammatory cytokines, tumor necrosis factor- α (TNF- α) is central both in initiating and regulating the cytokine cascade during an inflammatory response (Tweedie *et al.* 2007b; Frank-Cannon *et al.* 2009; Tansey 2010; Frankola *et al.* 2011). Generated as a membrane-bound 26-kDa precursor molecule, it is cleaved by TNF- α converting enzyme (ADAM17) to a soluble protein that homotrimerizes to provide the active ligand. TNF- α exerts its pharmacological action via two transmembrane-spanning receptors, TNFR1 and TNFR2, which differ in their expression profiles, ligand affinity and downstream signaling pathway activation (Tansey and Szymkowski 2009; Tansey 2010). An early transient elevation in the mRNA expression of TNF- α has been reported in rodents following closed head injury, and precedes the appearance of the ensuing cytokine (Shohami *et al.* 1997; Lu *et al.* 2009; Yang *et al.* 2010). Furthermore, numerous studies of TBI models have reported commensurate TNF- α protein up-regulation in brain within a few hours of trauma (Taupin *et al.* 1993; Shohami *et al.* 1994; Knoblich *et al.* 1999). Manipulating TNF- α levels may thus substantiate its role in mTBI and define its value as a potential treatment target.

Although a reduction in systemic TNF- α can be effectively achieved by antibodies or fusion proteins capturing and clearing TNF- α prior to its receptor activation, exemplified by the utility of infliximab and etanercept in the treatment of rheumatoid arthritis (Taylor 2010), neither drug significantly enters the brain (Tweedie *et al.* 2007b; Zhou *et al.* 2011), which limits their use in neurological conditions (Andersson *et al.* 2006; Tobinick 2010). Consequently, in this report, a novel experimental TNF- α synthesis inhibitor, 3,6'-dithiothalidomide (Zhu *et al.* 2003; Greig *et al.* 2004; Tweedie *et al.* 2009), was time-dependently administered prior to and after mTBI and brain biochemistry and behavioral correlates were then evaluated. This lipophilic compound is an analog of the classic, orally active neurological drug, thalidomide (*N*-phthaloylglutimide), that has been shown to lower TNF- α protein levels by post-transcriptional mechanisms (Sampaio *et al.* 1991; Moreira *et al.* 1993).

Materials and methods

Materials

3,6'-Dithiothalidomide and thalidomide

3,6'-Dithiothalidomide was synthesized according to a published procedure (Luo *et al.* 2008) to greater than 99.8% chemical purity, and was freshly prepared prior to each study. Thalidomide was purchased from Sigma (St Louis, MO, USA). In cell culture studies, both agents were prepared freshly in tissue culture grade dimethylsulfoxide (100%), whereas in animal studies, 3,6'-dithiothalidomide was prepared as a suspension in 1% carboxymethyl cellulose to provide a final concentration of 28 or 56 mg/kg (0.1 mL/10 g and 0.1 mL/100 g body weight injection in mice and rats, respectively), and was administered by the intraperitoneal (i.p.) route. These concentrations of 3,6'-dithiothalidomide are equimolar to 25 and 50 mg/kg of thalidomide.

Cellular studies

Validation of 3,6'-dithiothalidomide-induced TNF- α lowering in a RAW 264.7 cell model of inflammation

The ability of 3,6'-dithiothalidomide to ameliorate a lipopolysaccharide (LPS)-induced inflammatory response in cultured RAW 264.7 cells was quantified, using equimolar thalidomide as a positive control (Tweedie *et al.* 2009, 2011). RAW 264.7 cells were purchased from ATCC (Manassas, VA, USA), grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum; penicillin 100 U/mL; streptomycin 100 μ g/mL and maintained at 37°C and 5% CO₂. For all experiments, RAW 264.7 cells were seeded at a density of 200×10^3 cells per well in 24 well plates. Cells were allowed to equilibrate for 24 h after plating; 1–2 h prior to the initiation of any pharmacological study the seeding media was replaced with fresh media and the cells were allowed to equilibrate at 37°C and 5% CO₂. To initiate the study, RAW 264.7 cells were pre-treated with thalidomide or 3,6'-dithiothalidomide (0, 10 or 30 μ M, in 100% dimethylsulfoxide; Sigma). Both drugs were then diluted in media to a 1 in 200 working solution one hr prior to the addition of LPS (Sigma, serotype 055:B5) at 60 ng/mL. At 24 h

following the addition of LPS, conditioned media was harvested and used for further analysis. The levels of TNF- α protein secreted into the media were measured by ELISA (BioLegend, San Diego, CA, USA). Nitrite levels in culture media were measured by use of the Griess Reagent System (Promega, Madison, WI, USA), as per the manufacturer's guidelines. The optical density of unknown samples were read at 520 nm λ and compared with a sodium nitrite standard curve (1.5–100 μ M). The concentration of nitrite measured in the media was expressed as μ M units. As an assessment of drug-induced toxicity, cell viability was assessed by use of the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), a reduction in absorbance at 490 nm λ is indicative of cell death.

Animal studies

Rodents for these studies included male ICR mice (6–8 weeks age and 30–40 g weight: initially purchased from HSD Jerusalem, Israel, and thereafter bred and raised within the vivarium of the University of Tel Aviv, Tel Aviv, Israel) and F344 rats (3 months old and 200–225 g weight: Taconic Farms, Germantown, NY, USA). Experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committees of Tel Aviv University (M-10-006) and the Intramural Research Program, National Institute on Aging (ASP 331-LNS-2012). Animals were housed two (rats) and five (mice) per cage with *ad libitum* access to food and water on a 12 : 12 light/dark cycle at $22 \pm 1^\circ\text{C}$, with lighting during the light phase kept constant. All experimental manipulations were undertaken during the light phase of the cycle. A minimum number of animals were used and all efforts were made to minimize potential suffering. Each animal was used for only one experiment.

Procedures

Validation of systemic and brain TNF- α lowering activity of 3,6'-dithiothalidomide in rodent

To verify TNF- α synthesis lowering action of 3,6'-dithiothalidomide in rodents, the agent was administered to animals to lower systemic and brain TNF- α protein levels elevated by LPS challenge. Specifically, F344 rats were anaesthetized (50 mg/kg sodium pentobarbital, 1 mL/kg vol. i.p., $n = 6$) and the right femoral artery was cannulated to permit blood sampling. 3,6'-Dithiothalidomide (56 mg/kg, i.p.), followed 1 h later by LPS (1 mg/kg i.p., *Escherichia coli* 055:B5; Sigma), were administered, and serial blood samples then were collected over a subsequent 4 h period. At 4 h, the animal was killed by excess sodium pentobarbital and a CNS sample collected. Plasma was derived immediately by centrifugation ($8000 g \times 5 \text{ min}$) and, together with the CNS sample, frozen at -80°C for subsequent analysis of TNF- α by ELISA (BioLegend). For determination of secreted levels of brain TNF- α , the brain was briefly perfused with phosphate-buffered saline (PBS) to clear the vascular system prior to freezing. At a later time, 50 μ m brain slices were cut by cryostat and then incubated (2 h, 37°C) in 250 μ L cytoprotectant/PBS solution. This solution was then quantified for TNF- α levels by ELISA.

Close head mTBI injury and treatment

Mice were anesthetized lightly with Isoflurane and then placed under a weight drop device. This device consisted of a 50 g weight, a cylindrical-shaped piece of metal with a slight spherical tip, which

was dropped through a vertical metal guide tube (13 mm in diameter and 80 cm long). Each mouse was positioned with the temporal right side of its head, between the corner of the eye and the ear, positioned under the tube whereas the head was manually supported and immobilized by a sponge. The weight then was released from the top of the tube, and the sponge supported the head of the mouse allowing some anterior/posterior motion in the absence of any rotational head movement at the moment of impact (Pan *et al.* 2003; Milman *et al.* 2005; Zohar *et al.* 2003). Sham mice underwent an identical procedure as described for mTBI, but in the absence of dropped weight. Treatment comprised of 3,6'-dithiothalidomide at a low (28 mg/kg) or high (56 mg/kg) dose, or vehicle administered as a single dose at one of three separate times: 1 h prior to injury or 1 and 12 h post-injury. Thereafter, mice were assessed in behavioral tests 72 h or 7 days following the injury. In a separate series of animals, brain TNF- α levels together with other markers of apoptosis were time-dependently quantified from brain slices following mTBI.

Validation of elevated brain TNF- α levels following mTBI

To verify the occurrence of TNF- α elevation in our mTBI model and define its time-dependence, mice were subjected to mTBI and killed at specific times thereafter (1 to 18 h; $n = 3\text{--}5$ per time). Animals were perfused with PBS (0.1 mM, pH 7.4) to remove blood, and the brain immediately frozen to -70°C . Brain sections (50 μ m) were later cut by cryostat, and individually placed (5 sections, per time point, per animal) into separate wells of a 96-well plate containing PBS. Following incubation (2 h at 37°C), PBS/cytokine admixture was harvested and quantified for TNF- α by ELISA assay (BioLegend).

Validation of brain entry of 3,6'-dithiothalidomide in mice

To define whether actions of 3,6'-dithiothalidomide were centrally mediated, the brain uptake of the agent was assessed at an early time point (1 min) following administration to avoid potential confounds due to metabolism. Specifically, three mice were administered 56 mg/kg 3,6'-dithiothalidomide and brain and plasma samples were collected after exactly 1 min and analyzed by HPLC to define a brain/plasma ratio. Samples were quantified for 3,6'-dithiothalidomide on an Agilent 1100 series PHLC (Foster City, CA, USA), equipped with an Agilent Zorbax column (Eclipse xDB-c18, $4.8 \times 250 \text{ mm}$, 5 μ m particle size) and detected at 297 nm λ , which was determined to be a peak of its UV absorbance. The mobile phase was acetonitrile/methanol/20 mM phosphate buffer (15 : 42 : 43), run isocratically at a flow rate of 1.0 mL/min. Briefly, plasma samples (200 μ L) were mixed with internal standard (50 μ L revlimid, 0.1 mg/mL) and trichloro acetic acid (200 μ L) was then added, vortexed, and the sample centrifuged ($2500 g \times 10 \text{ min}$). The supernatant (400 μ L) was removed and 10 μ L injected on to the HPLC. Brain (300 mg) was sonicated in 700 μ L RIPA buffer, internal standard (revlimid 0.1 mg/mL) was added, and the sample centrifuged ($20\,000 g \times 15 \text{ min}$). To the supernatant (800 μ L), 20% trichloro acetic acid (400 μ L) was added, and the sample again centrifuged ($2500 g \times 10 \text{ min}$). Thereafter, 10 μ L of the supernatant (1000 μ L) was injected on to the HPLC.

Behavioral tests

At 72 h and 7 days after the mTBI procedure, animals were assessed into three behavioral tests: Y-maze, object recognition and passive

avoidance. Passive avoidance was designated as the last performed test as, otherwise, its aversive nature could potentially impact performance in the other tests. Tests were undertaken at 24 h intervals. All studies were undertaken in the same sound-insulated room that had constant illumination.

Y maze paradigm

The Y maze test was used to assess spatial memory. This task takes advantage of the preference of rodents to explore novel rather than familiar places. Comprising three arms ($8 \times 30 \times 15$ cm at an angle of 120° from the others), with each distinguished by the presence of a different visual cue (triangle, square, or circle), the Y maze was constructed of black Perspex (Dellu *et al.* 1992). From the three arms, one was randomly selected as the 'start' arm. Each animal was placed twice in the 'start' arm. During the initial trial, of 5 min duration, one of the two remaining arms was randomly selected to be closed off whereas on the second trial, of 2 min, both arms were open. These trials were separated by a 2-min interlude, during which the mouse was returned to its home cage and the maze was cleaned (70% ethanol solution; v/v) and dried.

The time spent in each of the arms was quantified. Discrimination of spatial novelty was assessed by a preference index (Dix and Aggleton 1999) determined as: $(\text{time in the new} - \text{time in the old arm}) / (\text{time in the new} + \text{time in the old arm})$.

Novel object recognition paradigm

An object recognition task was used to appraise recognition memory, as described by Messier (1997). This task takes advantage of a propensity of rodents to discriminate a familiar from a new object. Mice were initially individually habituated to an open field box ($59 \times 59 \times 20$ cm) for 5 min, 24 h before the test. During the acquisition phases, two objects (A and B) of identical material, which were sufficiently heavy and high to ensure that mice could neither move nor climb over them, were placed in a symmetric position within the chamber for 5 min duration. At 24 h after acquisition phase training, one of these objects (A or B randomly) was substituted by a novel one (C), and exploratory behavior was again evaluated for 5 min. All objects were thoroughly cleansed (70% ethanol) between sessions to preclude odor recognition. Exploration of an object was characterized as rearing on it or sniffing it at a distance of less than 2 cm and/or touching it with the nose. Successful recognition was revealed by preferential exploration of the novel object. Discrimination of visual novelty was assessed by a preference index (Dix and Aggleton 1999), determined as: $(\text{time near the new} - \text{time near the old object}) / (\text{time near the new} + \text{time near the old object})$.

Passive avoidance paradigm

The passive avoidance task was used to evaluate simple non-spatial memory ability. The passive avoidance apparatus (San Diego Instruments, San Diego, CA, USA) was created from black Perspex ($48 \times 22 \times 22$ cm) and possessed two separate chambers, one light-illuminated the other dark, of equal dimensions. These chambers were joined by a door that was shut at the start of each trial and could be opened by the experimenter. The test involved two sessions separated by a 24-h interval. Animals were positioned within the illuminated chamber and, after 30 s, the connecting

door was opened. On crossing into the dark compartment, the door was closed, and the animal received a mild electric foot shock (1 mA for 5 s). Following the electric shock, animals remained for further 5 s within the apparatus and then were returned to their home cage. After a 24-h interval, animals were evaluated for retention of the passive avoidance response to the shock by again placing them in the illuminated compartment. Memory was operationally defined as a failure to enter the dark compartment within 3 min (Milman *et al.* 2005). No shock was delivered during the second day.

Data analysis

All results are presented as mean \pm SEM and were analyzed either by with SPSS 15 software (Genius Systems, Petah Tikva, Israel) or GraphPad InStat Version 3.05 (GraphPad Software, San Diego, CA, USA). The number (*n*) of studies performed and/or animals is provided in parentheses. One-way ANOVA's were performed to compare between all groups, followed by LSD *post hoc* tests. ANOVA's were used to analyze the results of the Y maze and the object recognition test. Results of the passive avoidance test were evaluated using the non-parametric chi-squared test. Statistical comparisons of TNF- α levels in cell culture and animal studies were undertaken by use of a Student's *t*-test with appropriate Bonferroni corrections for multiple comparisons, as necessary. *P* values of ≤ 0.05 are considered to be of statistical significance.

Results

Amelioration of LPS-induced cellular inflammation by 3,6'-dithiothalidomide

3,6'-Dithiothalidomide, but not thalidomide, dose-dependently lowered secreted levels of both TNF- α and nitrite in LPS-challenged RAW 264.7 cells, as assessed at 48 h incubation post-LPS (Fig. 1). In contrast, 10 μM thalidomide elevated nitrite levels with no impact on TNF- α , although higher thalidomide concentrations (100 μM) have been reported to lower TNF- α levels in LPS-challenged cultured human peripheral blood mononuclear cells (Zhu *et al.* 2003). Cell viability, assessed by MTS assay, was unaltered by either drug.

Amelioration of elevated central and systemic LPS-induced TNF- α levels in rodents by 3,6'-dithiothalidomide

3,6'-Dithiothalidomide induced a time-dependent lowering of TNF- α plasma levels in anesthetized rats challenged with LPS to induce increased TNF- α synthesis and secretion (Fig. 2). As assessed at 4 h, 3,6'-dithiothalidomide likewise lowered CNS TNF- α levels, confirming the compound's ability to act as a TNF- α synthesis inhibitor in cellular and *in vivo* models of inflammation.

3,6'-Dithiothalidomide brain/plasma ratio

With a measured brain/plasma concentration ratio of 1.34 ± 0.63 , the neurological actions of 3,6'-dithiothalidomide were likely centrally mediated.

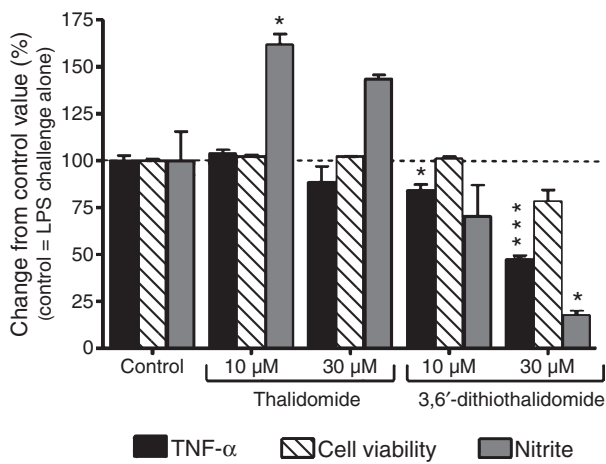


Fig. 1 3,6'-Dithiothalidomide lowers TNF- α protein and nitrite generation in LPS-challenged RAW 264.7 cells without loss of viability. The effects of 3,6'-dithiothalidomide (3,6'-DT) and equimolar thalidomide (TH) on cell viability and LPS-induced elevation of TNF- α protein levels and nitrite generation in RAW 264.7 cells. Cells were incubated for 48 h after administration of LPS with and without drug. Whereas 3,6'-dithiothalidomide (10 and 30 μ M) significantly lowered TNF- α protein and nitrite generation, thalidomide (10 μ M) significantly elevated generation of nitrite. Control refers to 100% dimethylsulfoxide (DMSO) ($n = 4$ observations; *, *** refers to $p \leq 0.05$ and $p \leq 0.001$ compared with control, respectively; Dunnett's t -test).

mTBI-induced elevation in TNF- α

As illustrated in Figure S1, compared with control animals whose brain TNF- α were minimal, mTBI induced an acute rise (up to 4.5-fold) in TNF- α that peaked at 12 h and returned to resting levels within 18 h.

mTBI-induced behavioral impairments and their amelioration by 3,6'-dithiothalidomide

Y-maze paradigm

72-h test. As illustrated in Fig. 3 (upper), mTBI mice demonstrated a slight memory deficit, compared with the sham group, that did not reach significance in all three time-dependent studies (1 h prior to, and 1 and 12 h post-injury, respectively) [$F(5, 94) = 7.09$, N.S.; $F(5, 53) = 6.806$, N.S.; $F(5, 55) = 8.41$, N.S.]. This trend was not evident in mTBI mice treated with 3,6'-dithiothalidomide at either the lower or higher dose (28 and 56 mg/kg).

7-day test. As illustrated in Fig. 3 (lower), mTBI mice displayed significantly reduced spatial memory compared with all other groups (1 h prior to, and 1 and 12 h post-injury, respectively) [$F(5,84) = 4.09$, $p < 0.005$; $F(5,51) = 4.102$, $p < 0.05$; $F(5,59) = 3.660$, $p < 0.05$]. *Post hoc* analyses confirmed that the mTBI group was significantly different from all other (in all experiments; $p < 0.05$

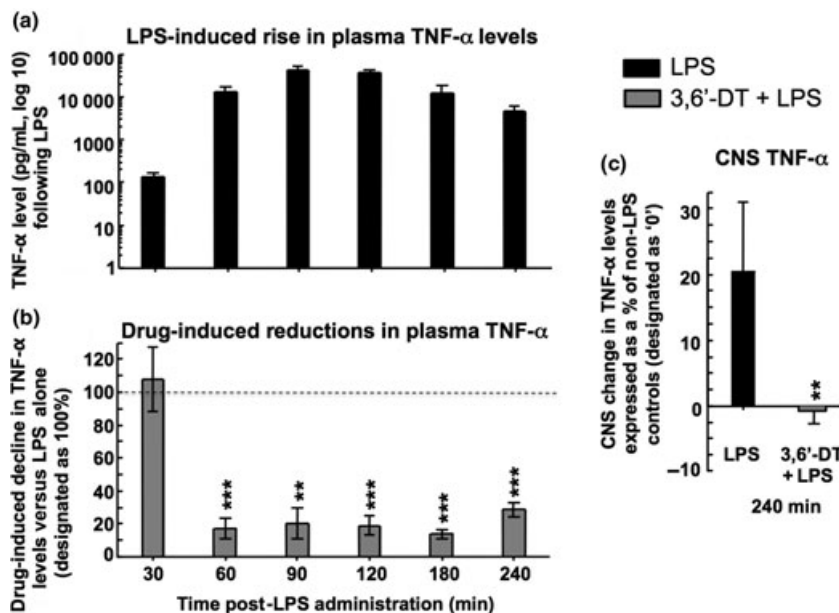


Fig. 2 3,6'-Dithiothalidomide lowers LPS-induced TNF- α levels in plasma and CNS. (a) Systemically administered LPS induced a rapid, time-dependent 3 log elevation in plasma TNF- α levels that peaked at 90 min. (b) This LPS-induction in plasma TNF- α levels was substantially (up to 80%) inhibited by prior administration of 3,6'-dithiothalidomide (3,6'-DT, 56 mg/kg) – levels are expressed as a percent of those determined at the same time in LPS alone animals. (c) Sys-

temically administered LPS elevated CNS levels of TNF- α by 20% compared with basal levels in pure controls (no LPS). This CNS LPS-induced TNF- α rise was fully blocked by prior administration of 3,6'-dithiothalidomide (56 mg/kg) ($n = 6$). **, *** refers to $p \leq 0.01$ and $p \leq 0.001$ compared with LPS alone determined at the same time points, respectively; paired Student's t -test).

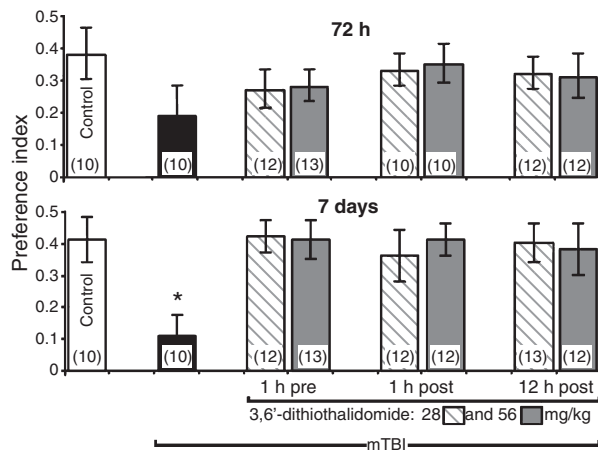


Fig. 3 mTBI induces impairment in performance in a Y maze preference index paradigm that is fully ameliorated by 3,6'-dithiothalidomide. Performance of mice was quantitatively assessed in a Y maze paradigm at 72 h and 7 days following mTBI as a preference index, calculated as (time new – time old)/(time new + time old). Values are mean \pm SEM. (Upper) 72 h post-injury the mTBI group displayed a trend toward lower memory ability that did not reach significance (one-way ANOVA showed no differences; N.S.) but this trend was not evident in mTBI drug-treated mice. (Lower) 7 days post-injury, one-way ANOVA showed that mTBI animals had a deficit in spatial memory performance compared with all the other groups ($*p \leq 0.05$). No differences were found between any of the other groups, suggesting complete amelioration by 3,6'-dithiothalidomide.

compared with all groups). 3,6'-Dithiothalidomide at both low and high doses fully reversed this injury-induced effect, whether administered before or after mTBI. *Post hoc* analyses revealed that 3,6'-dithiothalidomide abolished mTBI-induced behavioral deficits seen in this Y-maze paradigm.

Novel object recognition

72-h test. As illustrated in Fig. 4 (upper), mTBI mice demonstrated a trend towards a lower visual memory, compared with the sham group, which was not statistically significant in all time-dependent studies (1 h prior to, and 1 and 12 h post-injury, respectively) [$F(5, 60) = 1.17$, N.S.; $F(5, 53) = 3.17$, N.S.; $F(5, 52) = 4.15$, N.S.]. This trend was not as evident in 3,6'-dithiothalidomide-treated mice.

7-day test. Reduced visual memory was clearly evident in mTBI mice compared with all other groups, in all three time-dependent studies (1 h prior to, and 1 and 12 h post-injury, respectively) [$F(5, 73) = 7.107$, $p < 0.001$; $F(5, 52) = 4.102$, $p < 0.001$; $F(5, 57) = 4.472$, $p < 0.001$] (Fig 4, lower). *Post hoc* analyses showed statistical significance ($p < 0.05$ compared with all groups). Mice administered either dose of 3,6'-dithiothalidomide prior to or post-mTBI performed similar to the sham group. *Post hoc*

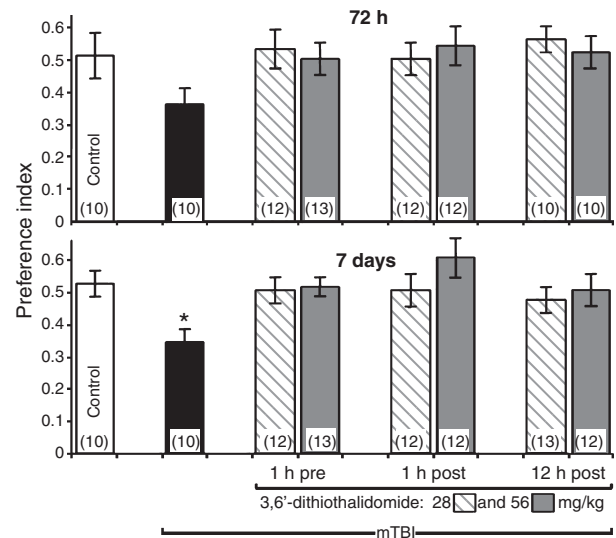


Fig. 4 mTBI induces impairment in performance in a novel object recognition preference index paradigm that is fully ameliorated by 3,6'-dithiothalidomide. Performance of mice was quantitatively measured in a novel object recognition paradigm at 72 h and 7 days post-mTBI as a preference index, calculated as (time new – time old)/(time new + time old). Values are mean \pm SEM. (Upper) 72 h post-injury the mTBI group displayed a trend toward a lower preference index that did not reach significance (one-way ANOVA showed no differences; N.S.) but this trend was not evident in mTBI drug-treated mice. (Lower) 7 days post-injury, one-way ANOVA showed that mTBI animals demonstrated a deficit in spatial memory performance compared with all the other groups ($*p \leq 0.05$). No differences were found between any of the other groups, suggesting a complete amelioration by 3,6'-dithiothalidomide.

analyses showed no differences between all other groups, indicating complete amelioration of this mTBI-induced behavioral deficit by 3,6'-dithiothalidomide.

Passive avoidance

72-h test. As illustrated in Fig. 5 (upper), pair-wise comparisons revealed no differences in memory ability between all groups in all three time-dependent experiments (1 h prior to, and 1 and 12 h post-injury, respectively) [$\chi^2(73) = 12.31$; N.S.; $\chi^2(54) = 11.27$; N.S.; $\chi^2(51) = 14.35$, N.S.].

7-day test. Pair-wise comparisons revealed that the difference between the groups did not approach significance all three experiments (1 h prior to, and 1 and 12 h post-injury, respectively) [$\chi^2(79) = 10.31$, $p = 0.06$; $\chi^2(64) = 9.14$; N.S.; $\chi^2(61) = 11.27$, N.S.]; however, a trend towards reduced memory was evident in mTBI animals that was not noticeable in those treated with 3,6'-dithiothalidomide (Fig. 5, lower).

In conclusion, across the three behavioral paradigms studied, a general pattern emerged. The mTBI groups presented a consistently (in all the behaviors) inferior performance, as compared with the sham mice. However,

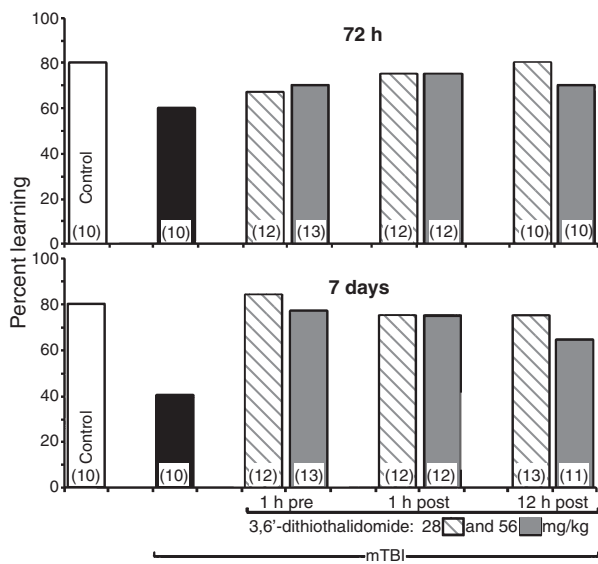


Fig. 5 mTBI induces a trend towards impairment in learning as assessed by passive avoidance that is not evident in mice administered 3,6'-dithiothalidomide. Performance of mice was measured in a passive avoidance paradigm at 72 h and 7 days post-mTBI with learning defined as the percent of animals that did not enter the dark compartment on the second day. Values are in percents. (Upper) 72 h post-injury chi-squared tests revealed no differences between all groups. (Lower) 7 days post-injury chi-squared tests revealed no differences between all groups, although a trend towards decreased learning was evident in mTBI mice that was not seen in animals treated with 3,6'-dithiothalidomide prior to or post-injury.

this reached statistical significance only at 7 days post-injury. 3,6'-dithiothalidomide proved to be well tolerated in mice challenged with mTBI at both low (28 mg/kg) and high (56 mg/kg) doses, and these mice performed at levels that were not statistically different from the sham group in all behaviors. Hence, this compound fully ameliorated mTBI-induced behavioral deficits at both doses.

Discussion

mTBI has become a major and increasing public health concern and represents the most frequent cause of mortality and disability in young adults. Additionally, associated with battlefield injury, blast-TBI is currently of particular concern. At present, no effective pharmaceutical therapies are available for mTBI and existing treatment principally involves optimized intensive care management after injury occurrence (Gamer and Brett 2007; Moppett 2007). The pathology resulting from head injury is becoming better characterized. Mechanical forces induce shearing and compression of neuronal and vascular tissue at the time of impact. A cascade of neurochemical events may then ensue that result in additional damage. Such secondary injury makes the brain vulnerable to further injury but may be amenable to

intervention. mTBI has hence been considered a gateway to the development of neuropsychiatric disorders, such as depression and anxiety (Hesdorffer *et al.* 2009; Bryant *et al.* 2010), and also chronic neurodegenerative diseases exemplified by Alzheimer's disease and Parkinson's Disease (Uryu *et al.* 2003; Kiraly and Kiraly 2007; Johnson *et al.* 2010).

Our closed head murine injury model attempts to mimic mTBI in humans, and results of the present study support previous work showing a decline in performance in cognitive tests after mTBI (Milman *et al.* 2005; Zohar *et al.* 2003; Baratz *et al.* 2010; Edut *et al.* 2010) that are also evident in humans (Vakil 2005; Ghajar *et al.* 2008). Studies of the neurochemical changes induced after injury suggests that mTBI represents a complex neurological condition, encompassing numerous molecular and cellular pathways, including inflammation (Raghupathi 2004; Lu *et al.* 2009), resulting in the induction of diffuse neuronal dysfunction and apoptosis (Tweedie *et al.* 2007a; Tashlykov *et al.* 2009; Rubovitch *et al.* 2010). The inflammatory cascades triggered by mTBI are mediated by the generation of both pro- and anti-inflammatory cytokines that, although barely detectable in healthy brain, are rapidly up-regulated in response to mTBI (Israelsson *et al.* 2009). In particular, an early transitory rise in TNF- α mRNA expression of up to 30-fold, with similar elevations in IL-1 β and IL-6, has been described in rodent closed head injury, and preceded the subsequent arrival of cytokine activity and leukocyte recruitment (Shohami *et al.* 1997; Israelsson *et al.* 2009; Yang *et al.* 2010). Likewise, TNF- α , IL-1 β and IL-6 protein up-regulation in brain has been described in diverse mTBI models as well as in human CSF within a few hours of trauma (Taupin *et al.* 1993; Shohami *et al.* 1994; Knobloch *et al.* 1999). As evident in Figure S1, an acute and rapid rise in brain TNF- α levels was seen in our mTBI murine model, occurring within an hour, and increasing to 4.5-fold above control levels at 12 h. Such an elevation supports the use of this model to assess agents that lower brain TNF- α . In this respect, prior studies focused on lowering TNF- α with a TNF- α binding protein and the competitive non-selective phosphodiesterase inhibitor, pentoxifylline, that additionally lowers TNF- α transcriptionally, have been reported to ameliorate mTBI over an initial 24 h (Shohami *et al.* 1996, 1997), when these agents were administered i.v. immediately following trauma.

The biosynthesis of inflammatory cytokines like TNF- α are tightly regulated post-transcriptionally at the level of mRNA stability via their 3'-untranslated region (3'-UTR) (Moreira *et al.* 1993), which allows rapid changes in protein expression to adapt to environmental cues. The occurrence of adenylate-uridylylate-rich elements (AREs) within the 3'-UTR of TNF- α mRNA performs a key function in post-transcriptional repression, targeting it for rapid degradation or inhibition of translation (Khera *et al.* 2010). p38 MAPK has been characterized as a primary signaling cascade

impacting TNF- α stability via its 3'-UTR ARE *cis* elements, mediated through interactions with RNA binding proteins (Patil *et al.* 2008). Proteins such as HuR have been associated with promoting transcript stabilization. Following export to the cytoplasm, HuR binds and stabilizes ARE-containing transcripts and aids convey them to translational machinery. In contrast, interaction with RNA-binding proteins such as, tristetraprolin and alike proteins, can accelerate the degradation of bound mRNAs (Khera *et al.* 2010; Stamou and Kontoyiannis 2010). Challenges such as LPS extends the half-life of TNF- α mRNA, allowing release of its translational repression. Whereas, administration of agents such as thalidomide have been shown to increase translational blockade and, thereby, reduce TNF- α mRNA half-life from 30 min to 17 min (Moreira *et al.* 1993); thereby lowering its rate of protein synthesis (Sampaio *et al.* 1991). As assessed by following a luciferase element within the 3'-UTR of TNF- α mRNA, 3,6'-dithiothalidomide appears to likewise regulate mRNA stability (Zhu *et al.* 2003; Greig *et al.* 2004).

Although thalidomide is a controversial drug (Melchert and List 2007), several groups have generated analogs of significant clinical interest (Zhu *et al.* 2003; Knight 2005; Aragon-Ching *et al.* 2007; Luo *et al.* 2008). As illustrated in Figs 1 and 2, the compound 3,6'-dithiothalidomide is a more potent TNF- α synthesis lowering analog than thalidomide in cell culture models. Indeed, a 10- μ M thalidomide concentration, which compares favorably with plasma levels observed after a routine 200 mg dose in humans (Teo *et al.* 2004), was found to mildly elevate nitrite levels in culture. This finding is in accord with others demonstrating thalidomide-induced elevations rather than in biological markers (Shannon and Sandoval 1996; Tadesse *et al.* 2006). In contrast, 3,6'-dithiothalidomide elicited a time-dependent decline in TNF- α levels in the plasma and CNS of rodents following a marked, up to 3 log, induction of TNF- α by LPS. As assessed by its brain/plasma ratio of 1.34, which is in accord with its log *D* value of -0.56 (Zhu *et al.* 2003; Greig *et al.* 2004), a measure of its balanced aqueous solubility/lipophilicity, 3,6'-dithiothalidomide appears to readily enter the brain. In light of elevations in TNF- α apparent in our mTBI mouse model, 3,6'-dithiothalidomide was administered as a single dose either an hour before or after mTBI to define its ability to lower TNF- α synthesis prior to and immediately after its induction by mTBI. These are time points when TNF- α levels in brain would be basal and sub-maximally elevated, respectively. In addition, and more related to clinical use, the agent was assessed when administered as a single dose 12 h post-mTBI, coinciding with the peak expression of TNF- α in brain after mTBI (Figure S1). Our chosen doses of 3,6'-dithiothalidomide (28 and 56 mg/kg, equipotent to 25 and 50 mg/kg thalidomide) compare favorably with those of thalidomide used in humans, where doses of up to 1200 mg are administered.

Consistent with prior cognitive loss previously reported in our mTBI model (Milman *et al.* 2005; Zohar *et al.* 2003;

Baratz *et al.* 2010), a reduced memory ability was seen following mTBI in both the Y maze and novel object recognition paradigms that was evident at 72 h and reached significance at 7 days. A trend towards impairment was also apparent in the passive avoidance test. 3,6'-dithiothalidomide, administered in either a single low or high dose prior to or up to 12 h after injury, fully ameliorated all animal performance deficits. There are few prior studies of thalidomide and analogs in traumatic brain and spinal cord injury (SCI) in humans and animal models. The immediate administration of a combination of thalidomide (100 mg/kg) and the phosphodiesterase 4 inhibitor, rolipram (3 mg/kg), ameliorated functional loss following SCI in rats, with thalidomide alone showing a trend toward improvement (Koopmans *et al.* 2009). In contrast, no behavioral or morphological improvements were evident following either single or repeated doses of thalidomide (100 mg/kg), administered 10 min, 4 and 24 h after SCI in a study by Reyes-Alva *et al.* (2009). Moreover, in other animal models of brain injury, such as ischemia, thalidomide has demonstrated efficacy over a narrow dose range when administered prior to injury (Lee *et al.* 2007; Hyakkoku *et al.* 2009). By comparison, in the present study, 3,6'-dithiothalidomide compares favorably with the results of past studies of its parent compound, and the full reversal of mTBI-induced cognitive impairments induced by both studied doses, suggest that still lower doses may prove effective and warrant future assessment. A reexamination and reinterpretation of the novel object recognition test has recently been published (McTighe *et al.* 2010). These investigators suggest, rather than a loss of memory for the familiar object, there is a 'false memory' of the novel object as familiar after perirhinal cortex damage.

An evaluation of the behavioral and histopathological outcome of TBI in mice deficient in TNF- α (TNF- α -/-) and their wild-type (wt) littermates has provided insight into the time-dependent roles of TNF- α in brain injury and repair (Scherbel *et al.* 1999), albeit that the TBI model studied, induced by cortical impact injury, was far more severe than ours. TNF- α -/- mice suffered significantly less behavioral (assessed at a week) and neuromotor deficits (assessed at 2 days) than wt mice. However, whereas the latter demonstrated some recovery over 4 weeks post-TBI, the TNF- α -/- lacked improvement, and their cortical brain damage (assessed histologically at 4 weeks) proved greater (Scherbel *et al.* 1999). Such results suggest that TNF- α has time-dependent actions, playing a detrimental role during the acute phase within the traumatized brain, but providing beneficial actions in the delayed, chronic reparative phase, and are in accord with results of more deleterious effects in TNF- α receptor knockout mice following TBI (Sullivan *et al.* 1999; Stahel *et al.* 2000); thereby emphasizing the importance of defining a treatment window.

In conclusion, the results of the current study support 3,6'-dithiothalidomide as a potent TNF- α synthesis inhibitor and a

promising drug in the treatment of experimental mTBI. Administration within the initial 12 h window following injury resulted in full amelioration of behavioral deficits. Additionally, such protection supports a role for elevated TNF- α levels in the development of mTBI, and its targeting as a treatment strategy (Esposito and Cuzzocrea 2009; Frankola *et al.* 2011).

Acknowledgements

This research was supported in part by the Sackler School of Medicine, Tel-Aviv University, and the Intramural Research Programs of both the National Institute on Aging and National Institute on Drug Abuse, National Institutes of Health. This work was performed in partial fulfillment of the requirements for a Ph.D. degree of Renana Baratz, Sackler Faculty of Medicine, Tel Aviv University, Israel.

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. mTBI induces a time-dependent increase in brain levels of TNF- α .

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