

# Pathogenesis of Traumatic Brain Injury

## Introduction

Traumatic brain injury (TBI) is a major global health concern, as the most common cause of death for those less than 40 years of age in developed countries.<sup>1</sup> TBI has two distinct pathological stages: the initial traumatic impact and multiple cascades of secondary effects, with the end result being tissue degeneration and neurological impairment.<sup>2</sup> TBI can also be focal, diffuse, or both. Focal injury occurs in a specific location, while diffuse injury is more widespread.

The Centers for Disease Control (CDC) reports that approximately 1.7 million people in the United States experience TBI and 275,000 are hospitalized annually, with more than 50,000 deaths each year.<sup>3</sup> The injuries to armed forces members are not included in these figures. The CDC previously estimated that more than 80,000 Americans develop TBI-related long-term disability each year, and that 5.3 million people in the U.S., not including military veterans, are living with such disabilities.<sup>4</sup>

Contemporary therapeutic strategies utilize timely surgical intervention to prevent secondary injury, patient monitoring, and intensive care therapies that are goal-directed and targeted at pertinent parameters, such as intracranial pressure and cerebral perfusion pressure. However, potential gains in outcome improvement are limited without strategies that focus on the underlying pathological mechanisms that result from TBI.<sup>1</sup> The search for molecular mediators of post-TBI pathology has fostered a growing realization that inflammation plays a major role. This has led to efforts to better understand the inflammatory response and the mediators of neuronal injury. As part of these efforts, numerous examples of cytokine and growth factor production after TBI in rodent models have been documented by pre-clinical studies.<sup>1</sup>

## Cytokines as Inflammatory Mediators in TBI

Brain cells, including microglia, astrocytes, and neurons, produce cytokines which play a role in multiple central nervous system pathologies. Cytokines have pleiotropic actions, function in cascades, and act synergistically.<sup>1</sup> It is being increasingly recognized that cytokines also have physiological, neuromodulatory, and restorative roles, and when they are present at high concentrations, they contribute to neurodegeneration and tissue damage.<sup>1</sup> Therefore, cytokines can be classified as pro- or anti-inflammatory, and these roles often coexist in the brain.

Much of the research effort on TBI has focused on defining the role of cytokine inflammatory mediators by sampling blood, cerebrospinal fluid, microdialysis of brain tissue, and direct tissue samples taken from live or postmortem brain.<sup>1</sup> As a result, a coherent understanding of the pathological processes involved in TBI is beginning to emerge that may provide new and promising targets for therapeutic approaches. Due to the very large number of cytokines and chemokines that may be involved in TBI pathology, multiplex assays have been required to accurately elucidate the role that each of them plays in the neuropathology of TBI.

## Multiplex Assays are Essential for TBI Research

ELISA assays do not have the multiplex capability or dynamic range to rapidly and accurately measure very large increases in concentrations of a very large number of cytokines simultaneously. Reproducible, fast, and sensitive multiplexed assay systems with a large dynamic range, such as those based on Luminex® xMAP® Technology, are ideal solutions. They can simultaneously measure the levels of several cytokines per sample, across a large number of samples, using small sample volumes.

## Luminex xMAP Technology

Luminex internally dyes bead sets with precise concentrations of fluorescent dyes, resulting in 500 distinctly colored bead sets (Figure 1). Each bead set can be coupled with reagents for specific bioassays such as cytokines, antigens, antibodies, or oligonucleotides. Any combination of bead sets can be used in a single assay, enabling multiplexing of up to 500 analytes from a single reaction volume. The bead mixture is incubated with the sample and detected on a Luminex instrument using a reporter dye to quantify the amount of bound analyte.

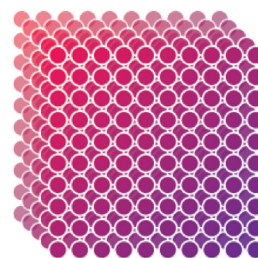


Figure 1: Luminex internally color-codes microspheres with precise concentrations of various fluorescent dyes, yielding up to 500 distinctly colored bead sets.

Depending upon the instrument used, up to 500 bead types can be used in each well of a 96- or 384-well plate, thus generating a high-throughput assessment of a large number of protein or oligonucleotide targets. This microsphere based “liquid array” system for measuring analytes is unique in its ability to provide both high-throughput and high-content data, and researchers are able to easily scale the number of analytes measured as well as customize both the assays and types of applications.

### TBI Studies in Animal Models

Many studies have been conducted in animal models to understand the role of cytokines and chemokines in the neuropathology of TBI. For example, fluid percussion injury (FPI) to the cortex in rats is used to induce TBI, and multiplex assays using xMAP Technology have been used to measure cytokine and chemokine levels following the injury. Using the MILLIPLEX® Rat Cytokine/Chemokine kit from EMD Millipore, a Luminex partner, a study was conducted to monitor the levels of 23 cytokines and chemokines following FPI.<sup>5</sup> In the ipsilateral cortex, cytokines such as TNF $\alpha$  and IL-6 increased, while IL-1 $\beta$  decreased after FPI. In contrast, IL-1 $\beta$  increased after FPI in the contralateral cortex, while IL-17, IL-10, IL-5, IP-10, and IFN $\gamma$  decreased.

Another study in rats used a modified lateral fluid percussion (LFP) procedure to induce mild traumatic brain injury (mTBI). Utilizing a multiplex cytokine assay based on xMAP Technology and produced by Bio-Rad®, a Luminex partner, acute increases in IL-1 $\alpha$  and  $\beta$  and TNF $\alpha$  were observed following the injury.<sup>6</sup> These increases were concurrent with macrophage/microglial and astrocyte cell activation, heightened cellular stress, and blood-brain barrier dysfunction that were observed as early as three to six hours after the injury. These findings were consistent with the researchers’ hypothesis that increased IL-1 $\alpha$  / $\beta$  and TNF $\alpha$  levels may trigger a cascade of inflammation with effects on glial activation, neuronal function impairment, and blood brain barrier (BBB) integrity.<sup>6</sup>

A second study of mTBI in rats using both FPI (diffuse injury) and mild controlled cortical impact (mCCI, focal injury) was conducted to identify molecular changes induced by these injury modalities and identify functional pathways altered by injury. These pathways could then be targeted for therapeutic intervention.<sup>7</sup> Using cytokine assay kits based on xMAP Technology and obtained from EMD Millipore, significant increases in macrophage inflammatory protein MIP-1 $\alpha$ , also known as CCL3, GRO-KC (CXCL1), IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6 were observed post-injury. In particular, marked increases in MIP-1 $\alpha$  and IL-1 $\beta$  occurred in the cortical vasculature and microglia, respectively, three hours after injury. This study demonstrated that many pathological processes occur in both focal and diffuse TBI that could be targets for therapeutic approaches.

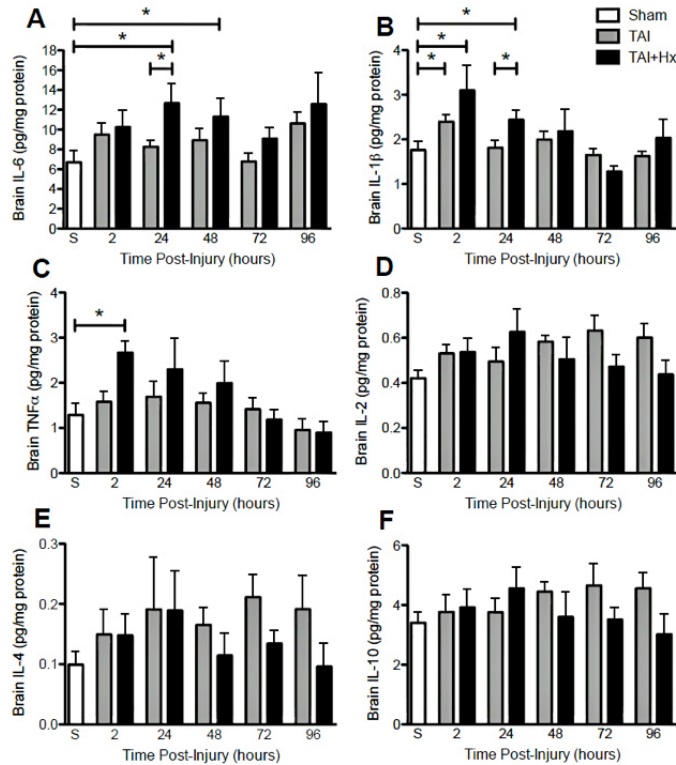
The chemokine CCL2, also known as MCP-1, is upregulated in patients and rodent models of TBI, and it has a powerful ability to mediate macrophage recruitment and migration to sites of inflammation. It has been reported that mice lacking the Ccl2 gene and subjected to experimental TBI exhibit reduced macrophage accumulation and tissue damage that corresponds with improved motor recovery.<sup>8</sup> However, Ccl2-deficient mice also unexpectedly experience a delayed but increased secretion of key proinflammatory cytokines in the injured cortex. A follow-up study was conducted to further

characterize the potential ability of CCL2 to modulate immunoreactivation of mouse astrocytes *in vitro* using stimulation of established Ccl2-deficient astrocyte cultures with various concentrations of IL-1 $\beta$  and lipopolysaccharide (LPS) for up to 24 hours. Cytokine secretion was then measured in culture supernatant using MILLIPLEX xMAP-based Mouse cytokine kits. The secretion of five key cytokines and chemokines was induced significantly by LPS in Ccl2 $^{-/-}$  astrocytes compared to wildtype cells. Stimulation by IL-1 $\beta$  also increased IL-6 production from Ccl2 $^{-/-}$  cultures. However, cytokine/chemokine production was not induced by the addition of recombinant CCL2 (rCCL2) alone to wildtype astrocyte cultures. In contrast, IL-6 levels were significantly reduced when wildtype astrocytes were pre-incubated with rCCL2, prior to stimulation with IL-1 $\beta$ . These data indicate that the exacerbated cytokine response previously observed *in vivo* post-injury in the absence of CCL2 is likely mediated by astrocytes. These data also suggest a novel, immunomodulatory role for CCL2 in acute neuroinflammation, because CCL2 inhibits cytokine production by astrocytes following IL-1 $\beta$  stimulation. These findings may have implications for therapeutic strategies that target CCL2-mediated leukocyte infiltration following TBI.<sup>8</sup>

Post Traumatic Epilepsy (PTE) can occur after TBI, which is one of the few known factors contributing to PTE. Five percent of all epileptic cases and 20% of symptomatic epilepsies are attributable to PTE.<sup>9</sup> This form of epilepsy is one of the most difficult to treat, as it is often resistant to treatment. Alterations in the hippocampus have been associated with TBI pathogenesis and the development of PTE in animal models, but the early time course of inflammation and neuroplasticity has not been fully characterized. A recent study in a rat FPI model of PTE rats was undertaken to examine inflammation and neuropathology after TBI.<sup>9</sup> Cytokine analysis was performed using the MILLIPLEX Rat Cytokine/Chemokine kit based on xMAP Technology. Changes in astrocyte morphology were also assessed. The results revealed a rapid inflammatory and astrocytic response in the ipsi- and contralateral hippocampus following FPI. In the ipsilateral hemisphere, several cytokines and chemokines were significantly increased at 24 hours after FPI, including GRO-KC (CXCL1), CCL2 (MCP-1), CCL3 (MIP-1 $\alpha$ ), and TNF $\alpha$ . However, in the contralateral hemisphere a significant decrease in the levels of several cytokines was observed, including IL-17, IL-2, IL-1 $\alpha$ , IL-10, IFN $\gamma$ , IL-13, IL-5, GM-CSF, IP10, VEGF, and G-CSF. The results of the study demonstrated that following TBI, inflammation and neuroplasticity to hippocampal interneurons may contribute to consequent pro-epileptogenic alterations to hippocampal circuitry function.

Almost 50% of human patients with severe head trauma also experience brain hypoxia, often related to brainstem injury.<sup>2</sup> While this hypoxic state results in additional brain damage, the precise processes that mediate this increased damage are little understood. A study was conducted to determine the effect of post-traumatic hypoxia on the intensification of secondary brain damage using a rat model of diffuse traumatic axonal injury (TAI).<sup>2</sup> After induction of diffuse TAI using the Marmarou impact acceleration model, followed by 30 minutes of hypoxic ventilation, rats were examined for behavioral and sensorimotor deficits, increased brain production of inflammatory cytokines, cerebral edema, brain metabolism changes and enlargement of the lateral ventricles. Using a Bio-Rad cytokine kit based on xMAP

Technology, it was determined that hypoxia increased cortical production of the pro-inflammatory cytokines IL-6, IL-1 $\beta$ , and TNF (Figure 2). This boost in cytokine production was accompanied by intensified behavioral deficits and considerable metabolic depression, but no increase in edema. The data supported the conclusion that the behavioral deficits observed with TAI and hypoxia may be due to increased neuroinflammation and a prolonged period of metabolic dysfunction.



**Figure 2. Cytokines IL-6, IL-1 $\beta$ , and TNF are increased in rats after traumatic axonal injury with additional hypoxia.** The concentration (pg/mg protein) of cytokines (A) IL-6, (B) IL-1 $\beta$ , (C) TNF, (D) IL-2, (E) IL-4, and (F) IL-10 was measured in cortical homogenates of sham (S), TAI (traumatic axonal injury) alone, and TAI with hypoxia (TAI+Hx) animals by multiplex assay over four days. \* indicates significant differences between groups. Note the significant increases of IL-6 and IL-1 $\beta$  in TAI+Hx vs TAI rats. TNF did not increase after TAI alone, and was only evident at two hours in TAI+Hx rats. Data shown as mean  $\pm$  SEM, n=6 per group per time point. Data was analyzed by 1-way ANOVA with Bonferroni post hoc test, with a p-value of <0.05 considered significant. This work is licensed under a Creative Commons Attribution 2.0 Generic License. doi:10.1371/journal.pone.0087572.g007.

In order to manage brain swelling and prevent death after head trauma, decompressive craniectomy is performed and the resultant bone flap is put back in place after the swelling is gone. Unfortunately, this significantly increases the risk of infection of the bone flap, which ranges from 0.8% to 15%.<sup>10</sup> The risk is heightened by the fact that a disturbing number of infections are caused by methicillin-resistant *Staphylococcus aureus* (MRSA). This high infection rate exposes patients to at least two additional surgical procedures and multiple complications. A novel mouse model of *S. aureus* cranial bone flap infection that mimics several aspects of human disease has recently been developed to aid in the identification of important

immunologic and therapeutic mechanisms involved in persistent bone flap infection.<sup>10</sup> Using a MILLIPLEX Mouse Cytokine and Chemokine kit based on xMAP Technology, altered chemokine expression patterns in the ipsilateral galea and cortex were observed during the process of infection. Expression of the chemokines CXCL2 (MIP-2) and CCL5 (RANTES), which are potent neutrophil and monocyte/T-cell chemokines, was minimal in the ipsilateral cortex. However, their expression was prominent in the galea. The T-cell chemokine CXCL10 (IP-10) was detected mainly in the parenchymal compartment. Taken together, these data demonstrate the existence of two microenvironments that respond individually to *S. aureus*. Significant immunologic and therapeutic mechanisms relevant to persistent bone flap infection in humans can thus be elucidated using this mouse model.

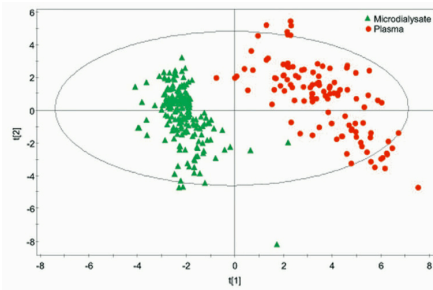
### Human TBI Research

Although a number of cytokines and chemokines have been implicated in human TBI, the research has focused on a fairly small number of them.<sup>1</sup> However, Luminex xMAP Technology is enabling studies that can investigate the role that a large number of different cytokines and chemokines play in TBI.

For example, the MILLIPLEX Multi-Analyte Profiling Human Cytokine/Chemokine kit based on xMAP Technology has been used to analyze the production of 42 cytokines after severe TBI.<sup>11</sup> Using cerebral microdialysis and paired arterial and jugular bulb plasma sampling, sixteen cytokines exhibited a temporal peak that was at least twice the median value for that cytokine over the monitoring period. On day one, that peak occurred in TNF $\alpha$ , IL-7, IL-8, MIP-1 $\alpha$  (CCL3), soluble CD40 ligand, GRO (CXCL1), IL-1 $\beta$ , PDGF-AA, MIP-1 $\beta$  (CCL4), and RANTES (CCL5). The increases continued through days four to five, when IL-12p70 and IL-10 levels peaked. The concentrations of 19 cytokines were significantly higher in brain extracellular fluid concentrations than in plasma. In addition, no clear arterio-jugular venous gradients were observed. These data support the conclusion that these cytokines are produced in the cerebrum and demonstrate a stereotyped temporal pattern after TBI.

In an effort to identify biomarkers for the pathobiological processes that occur following TBI, a statistical analysis was conducted of the large number of human cytokines that are produced during this condition.<sup>12</sup> Using the MILLIPLEX Multi-Analyte Profiling Human Cytokine/Chemokine kit based on xMAP Technology, the levels of 42 cytokines were measured in the brains and peripheral blood of twelve individuals with diffuse severe TBI over multiple days. Following principal component analysis (PCA) and least squares discriminant analysis (LSDA), groups of cytokines and chemokines were identified that are produced at defined time points and differentiate temporal phases of the inflammatory response. The data also revealed that there are tissue-specific (brain versus blood) differences in the patterns of production of the cytokines that are produced as a result of TBI (Figure 3).

Prevention and reduction of secondary insults that occur shortly after severe TBI are the focus of a large part of patient management. Intracranial hypertension (ICH) and cerebral hypoperfusion (CH) are two of the major secondary insults, and they often result in worse outcomes in respect to both mortality and functional outcome.<sup>13</sup> Predicting



**Figure 3. Paired microdialysis and plasma samples partial least squares discriminant analysis.** Paired microdialysate and plasma derived cytokine data from 12 patients. The source of the sample (microdialysate vs. plasma) has been used as a supervising variable within a partial least squares discriminant analysis to identify two principal components that maximize the differentiation between the two biological compartments. The cytokine patterns from the two sample types, brain microdialysate and plasma, can be completely differentiated. This work is licensed under a Creative Commons Attribution 2.0 Generic License. doi:10.1371/journal.pone.0087572.g007.

which patients are at risk of ICH or CH is difficult at best, and a predictive test based on biomarkers of their clinical development would be very useful. A study has been conducted to identify such biomarkers in serum.<sup>13</sup> Because neuroinflammation is characteristic of secondary injury after severe TBI, the study focused on cytokines that may mediate this process. Specifically, IL-8 and TNF $\alpha$  were monitored, as earlier work by the same research group had identified them as potential candidate markers for development of ICH and CH.<sup>14</sup> Using MILLIPLEX Human Cytokine/Chemokine kits based on xMAP Technology, serum levels of IL-8 and TNF $\alpha$  were monitored in 68 TBI patients and correlated with the onset of ICH and CH. Serum levels of both cytokines were found to be predictive of both ICH and CH with specificities of 81 to 95%, suggesting that they may be used to predict imminent events following TBI, before they are manifested clinically.

A separate study of the onset of elevated intracranial pressure (ICP) in humans following TBI was conducted to determine whether interleukin levels in serum can be predictive of intracranial pressure levels.<sup>15</sup> Cytokine kits from R&D Systems, a Luminex partner, were used to measure the levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and IL-1ra in TBI patients. Increased levels of IL-6 were observed regardless of ICP status. However, significantly higher IL-6 levels were observed in patients with ICP  $\geq$ 25 mm Hg within the first 17 hours of injury. Cutoff levels for IL-6 were established for healthy patients (<5 pg/mL, 100% identification), for those who consequently developed elevated ICP (>128 pg/mL correctly identified 85%), and patients whose ICP remained  $\leq$ 20 mm Hg throughout the study period (range of 5 pg/mL to 128 pg/mL, 75% identification). For patients with TBI and poly-trauma, the marker had no value in predicting elevated ICP. Significant IL-6 increases were also found in patients with orthopedic injury and no TBI, but the levels were not as high as those seen with TBI.

The development of Alzheimer's disease (AD) has been linked to severe or repeated TBI by epidemiological evidence.<sup>16</sup> Following TBI, amyloid precursor protein (APP) accumulates with high frequency, especially in injured axons, and APP may be cleaved to amyloid- $\beta$  (A $\beta$ ) peptides that play significant pathophysiological roles in AD. A recent study employed cerebral microdialysis to determine whether interstitial A $\beta$  levels are in fact changed following TBI in humans.<sup>17</sup> The study also attempted to determine whether these levels could be correlated with the injury type, cerebral energy metabolism, age of the

patient, and level of consciousness. The A $\beta$  peptide levels were measured using the INNO-BIA A $\beta$  forms xMAP assay from Innogenetics, a Luminex partner. Compared with patients with focal TBI at days 1–6 post-injury, the levels of both A $\beta$ 40 and A $\beta$ 42 were consistently higher in patients with primarily diffuse axonal injury. Specifically, A $\beta$ 42 levels significantly increased in axonal TBI patients at 113–116 hours after the injury. However, there was no correlation between A $\beta$  levels and interstitial energy metabolic situation, age of the patient, or the level of consciousness. Based on these results, particularly in cases of axonal injury, interstitial generation of potentially toxic A $\beta$  species may occur following human TBI.

### Potential Therapeutic Approaches

A variety of studies in modes of systemic inflammation have shown taurine to be associated with potent anti-inflammatory effects.<sup>17</sup> Specifically, the production of cytokines such as IL-1 $\beta$ , IL-6 and TNF $\alpha$  is reduced by taurine in spinal cord injury and ischemia reperfusion models. A study has been conducted to determine if taurine could counter inflammation, increased astrocyte activation, and cerebral edema in rats.<sup>17</sup> Using the MILLIPLEX RCYTOMAG-80K-23 Rat Cytokine/Chemokine kit based on xMAP Technology, the levels of 23 cytokines were measured in the cortex of rats subjected to FPI and then injected with taurine at one and seven days post-injury. Compared to the TBI control rats that did not receive taurine, taurine-treated rats exhibited markedly decreased levels of 17 cytokines and chemokines. Only MIP-1 $\alpha$  (CCL3) increased in concentration over the seven-day period. The results suggest that the severity of brain damage in TBI can be effectively alleviated with taurine administration, which diminishes the increase in pro-inflammatory cytokines, as well as astrocyte activity and edema.

There is an endogenous competitive antagonist (IL-1ra) at the interleukin-1 type-1 receptor (IL-1R) that has been shown in several rodent models of neuronal injury to provide neuroprotection. A single center, phase II, open label, randomized-control study of recombinant human IL-1ra (rhIL-1ra, anakinra) has been conducted for severe TBI.<sup>18</sup>

Cerebral microdialysis and the MILLIPLEX Human Cytokine/Chemokine kit were used to directly ascertain brain extracellular concentrations of IL-1ra and 41 cytokines and chemokines. The Interleukin-1 receptor antagonist (IL-1ra) penetrated into plasma and the brain extracellular fluid and was shown to be safe. Statistical analysis of the cytokine data by PCA revealed that rhIL-1ra administration induced changes in the brain cytokine profiles. Macrophage-derived chemokine (MDC/CCL22) was significantly lower in the rhIL-1ra-treated group, making it a candidate biomarker. The results of this trial provide promising data for rhIL-1ra as a therapeutic neuroprotective candidate by demonstrating for the first time modification of the neuroinflammatory response to TBI in humans.

While TBI outcomes are worsened by hypotension and hypoxemia, hyperoxic resuscitation is controversial. A study to determine whether hyperoxia would in fact improve hemodynamics and neuronal survival has been conducted using a mice model for TBI and hemorrhagic shock.<sup>19</sup> Using the MILLIPLEX MAP Mouse Cytokine/Chemokine kit, brain tissue cytokines and chemokines were shown to increase about 2 to 20 fold following CCI plus hemorrhagic shock (35

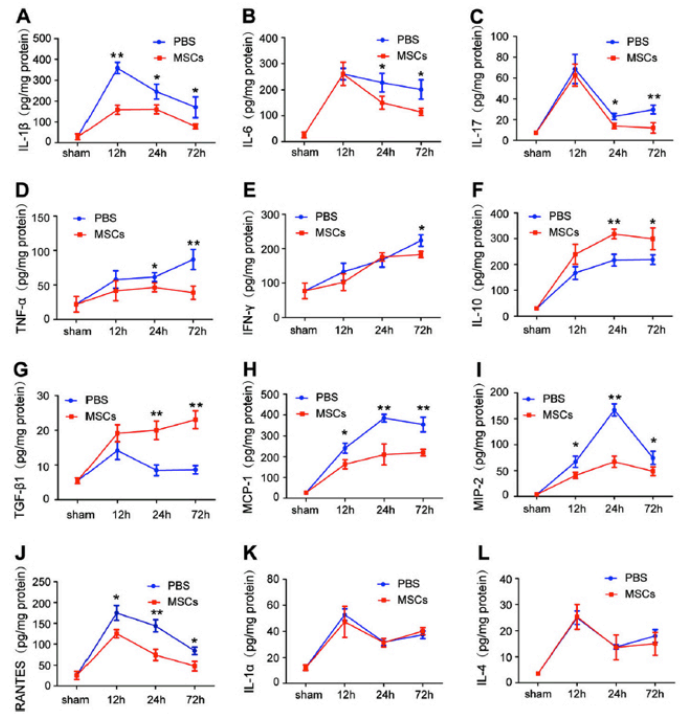
minutes at 25 to 27 mm Hg mean arterial pressure). However, following resuscitation with oxygen, cytokine levels were shifted toward a pro-inflammatory profile. The overall effect of oxygen was to increase hippocampal IL-2 and IL-6 (pro-inflammatory) levels and decrease hippocampal IL-5 and IL-7 and cortical IL-4 and IL-5 (anti-inflammatory), levels versus room air. However, despite increased neuroinflammation and ascorbate depletion, hyperoxic resuscitation increased brain oxygen tension and neuronal survival in the hippocampus.

Although moderate hypothermia has been shown to reduce neutrophil accumulation and ICP after TBI, its impact on cerebrospinal fluid (CSF) cytokine levels has been the subject of conflicting reports.<sup>20</sup> A comprehensive analysis of CSF cytokine levels after severe traumatic brain injury (TBI) in children has been performed to resolve this issue.<sup>20</sup> After maintaining moderate hypothermia (32–33°C) for 48 hours, each patient was slowly re-warmed from 48–72 hours. A MILLIPLEX (LINCOplex) Human Cytokine/Chemokine assay based on xMAP technology was used to measure the levels of 21 cytokines and chemokines collected over multiple days following hypothermic treatment. The levels of chemokines IL-8 and MIP-1 $\alpha$ , anti-inflammatory IL-10 and pro-inflammatory IL-1 $\beta$ , IL-6, and IL-12p70 increased after TBI compared to controls. However, the hypothermia protocol did not reverse or diminish these increases in CSF cytokine levels after TBI, versus normothermia.

The beneficial effects of mesenchymal stem cell (MSC) transplantation in central nervous system injuries such as TBI have been demonstrated in previous studies.<sup>21</sup> Few of these have focused on the impact of MSC transplantation on immune cells and cytokines that mediate inflammation, particularly in an experimental TBI model. Thus, a study was undertaken that monitored the levels of 12 cytokines after MSC transplantation in a rat TBI model.<sup>21</sup> Using the Bio-Rad Rat Bio-Plex assays based on xMAP Technology, the levels of several pro-inflammatory cytokines were shown to decrease after MSC treatment, including IL-1 $\beta$ , IL-17, TNF $\alpha$ , and IFN $\gamma$ . During the same time period after MSC treatment, the levels of anti-inflammatory cytokines IL-10 and TGF- $\beta$ 1 increased (Figure 4). The data from this study thus suggest that the beneficial effects of MSCs observed in central nervous system injuries may be due in part to reduction of the TBI-induced inflammatory responses.

### Luminex xMAP Technology

Luminex provides the only flexible and open multiplexing technology that is used by several market leaders to provide assays for both gene and protein expression. Unlike conventional technologies that can only measure one or a few biomarkers, researchers have the capability to easily scale up or down the number of biomarkers measured and to customize assays. xMAP Technology combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology. Featuring a flexible, open-architecture design, xMAP Technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately. Focused, flexible multiplexing of 1 to 500 analytes meets the needs of a wide variety of applications, including the multiplex analysis of large numbers of cytokines.



**Figure 4. Influence of MSC (mesenchymal stem cell) treatment on cytokine concentrations.** Levels of the proinflammatory cytokines IL-1 $\beta$  (at 12, 24 and 72h), IL-6 (at 24 and 72h), IL-17 (at 24 and 72h), TNF $\alpha$  (at 24 and 72h), and IFN $\gamma$  (at 72h) were significantly decreased in the MSC-treatment group compared with the PBS group (A–E). Levels of the anti-inflammatory cytokines IL-10 and TGF- $\beta$ 1 (at 24 and 72h after TBI) (F, G) were increased in the MSC-treatment group compared with the PBS group. The chemokines MCP-1, MIP-2, and RANTES were reduced at 12, 24, and 72h after TBI in the MSC group compared with the PBS group (H–J). There were no significant differences in levels of the cytokines IL-1 $\alpha$  and IL-4 (K, L) between the two groups.  $n = 6$  in each time point per group. Data are presented as the mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ . This work is licensed under a Creative Commons Attribution 2.0 Generic License. doi:10.1371/journal.pone.0087572.g007.

All of the microsphere bead assays described in this white paper were developed using xMAP Technology to provide unique multiplexed assays for cytokines, chemokines and amyloid- $\beta$  peptides. The open architecture of the system made it feasible for the researchers to create their own assays, or obtain commercially available kits. An ever-expanding menu of assays for other applications is also available from Luminex and its commercial partners.

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