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Temporal profile of M1 and M2 responses in the hippocampus following early 24 h of neurotrauma

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ABSTRACT

Traumatic brain injury (TBI) elicits complex inflammatory assets (M1 and M2 responses) in the brain that include the expression of various cytokines/chemokines and the recruitment of blood cells, contributing secondary injury cascades (SIC), and also recovery processes. The modulation of such inflammatory assets might be a therapeutic option following TBI. The present study assesses a temporal profile of various molecular markers of M1 and M2 response in the hippocampus after TBI. Following a unilateral controlled cortical impact (CCI) on young rats, hippocampal tissues of each brain were harvested at 2, 4, 6, 10, and 24 h post trauma. Including shams (craniotomy only), half of the rats were assessed for gene expression and half for the protein of various markers for M1 [interferon-gamma (IFNγ), tumor necrosis factor-α (TNFα), interleukin (IL)-1-β (IL-1β), and IL-6] and M2 [IL-4, IL-10, IL-13, arginase 1 (Arg1), YM1, FIZZ1, and mannose receptor C-1 (MRC1)] responses. Analysis revealed that molecular markers of M1 and M2 responses have heterogeneous injury effects in the hippocampus and that "time-post-injury" is an important factor in determining inflammation status. With the heterogeneous gene expression of pro-inflammatory cytokines, M1 response was significantly elevated at 2 h and declined at 24 h after TBI, however, their levels remained higher than the sham rats. Except IFNγ, proteins of M1 cytokines were significantly elevated in the first 24 h, and peaked between 2–6 h [TNFα (2 h), IL-1β (6 h), and IL-6 (4–6 h)]. With the heterogeneous relative gene expression of Arg1, YM1, FIZZ1, and MRC1, levels of M2 cytokines were peaked at 24 h post TBI. IL-10 and IL-13 expression appeared biphasic in the first 24 h. Protein values of IL-4 and IL-13 peaked at 24 h and IL-10 at 6 h post injury. Results suggest that the M1 response rises rapidly after injury and overpowers the initial, comparatively smaller, or transient M2 response. A treatment that can modulate inflammation, reduce SIC, and improve recovery should be initiated early (within 10 h) after TBI.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of neurological disorders and death among healthy populations. Millions of individuals suffer from TBI each year, resulting in long term disabilities, often requiring hospitalization [49]. Most TBI sufferers have costly health problems including cognitive loss, epilepsy, and depression [30,54]. As the hippocampus is an important brain region involved in cognitive function, it is important to investigate its involvement after TBI. Following a mechanical trauma, delayed pathology involves multiple secondary injury cascades/mechanisms (SIC) that include altered cell signal transduction and cytoskeletal damage, resulting in neuronal dysfunction or death. Various cellular and molecular changes play a dynamic role in these cascades including ionic imbalance [77], mitochondrial dysfunction [83], oxidative stress [4,7], excitotoxicity [15,77], and inflammation [25,51,71,76]. (See Table 1.)

TBI causes a complex series of inflammatory responses with the expression and release of several cytokine/chemokines [25,34,41,65,76] and the production of free radicals [7,16,51]. Our previous studies have demonstrated that the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 [71] and oxidative stress are significantly increased in the cerebral cortex after TBI [4,5,7,71]. Together, enhanced free radicals, cytokine/chemokine expression, and excitotoxicity increase the complexity of SIC following TBI.

Inflammation is a complex phenomenon, when multiple perspectives of its origin, progression, and potential are taken into consideration. In tissue, inflammation is characterized by the presence of perivascular infiltrates arising from the adoptive immune response [13,68,72]. The process involves many pro-inflammatory and anti-

Abbreviations: TBI, traumatic brain injury; SIC, secondary injury cascades; CCI, controlled cortical impact; M1, classical activation of microglia/macrophages; M2, alternative activation of microglia/macrophages; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; rRNA, ribosomal ribonucleic acid; cDNA, complementary deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; Arg-1, arginase-1; MRC-1, mannose receptor C type-1; FIZZ-1, found in inflammatory zone protein (Resistin-like molecule alpha); IFNγ, interferon-gamma; TNFα, tumor necrosis factor-α; IL-1β, interleukin-1-β.

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Table 1
shows the genes tested along with their PMID and TaqMan ID.

Gene	Primary inflammatory phenotype	PMID	TaqMan ID
IFNγ	M1	NM_138880.2	Rn00594078_m1
TNFα	M1	NM_012675.3	Rn01525859_g1
IL-1β	M1	NM_031512.2	Rn00580432_m1
IL-6	M1	NM_012589.2	Rn01410330_m1
IL-4	M2	NM_201270.1	Rn01456866_m1
IL-10	M2	NM_012854.2	Rn00563409_m1
IL-13	M2	NM_053828.1	Rn00587615_m1
Arg1	M2	NM_017134.3	Rn00691090_m1
YM1	M2	NM_053560.1	Rn01490608_m1
FIZZ1	M2	NM_053333.1	Rn00584229_m1
MRC1	M2	NM_001106123.2	Rn01487342_m1

inflammatory factors derived from both blood infiltrates and local cells [72]. Although a precise definition for inflammation in the brain is still unclear [29], but, it is well known that inflammation plays essential roles in the neuropathological conditions and is associated with cognitive impairment [2,86,89,90].

Recently, inflammation in the brain has been associated with activation (polarization) of microglial cells, referred to as resident macrophages, and immune cells in the central nervous system (CNS) [9,27,32,41,61]. Overall, the process of inflammation is demonstrated in two states of microglia/macrophage polarization (M1 and M2 responses) [43,55,57,91]. The expression of various cytokines, receptors, receptor ligands, enzymes, and matrix proteins [57,78,82,89] plays a pivotal role in the secondary injury as well as recovery/regenerative processes. M1 response is a "classical activation" of microglia/macrophage that produces a number of pro-inflammatory substances, contributes to the cleaning of damaged/dead cells, and activates the immune system. An M1 response is typically induced by the exposure of microbial lipopolysaccharide or pro-inflammatory cytokines, such as interferon-γ; IFN-γ, TNF-α, IL-1β, and IL-6. The M2 response is an "alternative activation" of microglia/macrophage that down-regulates inflammation and shut-off the immune system. M2 response is induced by anti-inflammatory cytokines (e.g. IL-4, IL-10, and IL-13), based on the different phenotypic expressions further classified as M2a, M2b, and M2c states [37,55].

Growing evidence suggests that M1 and M2 responses (expression of different M1 and M2 phenotypes) occur in the brain [25,34,41,65,76], exhibiting both harmful and beneficial roles [9,18,38]. CNS injury activates glial cells (called gliosis) at the sites of damage. Activated microglia become a source of cytokines/chemokines [33,65] and other reactive components [16] that lead to SIC [59,66] as well as participating in the repair/recovery processes [3,14,39,70]. Such inflammation in the CNS is also referred as neuroinflammation [59], however, it is difficult to explain how glial activation manages the corresponding complex cell signal mechanisms in both neurons and the immune systems [20,29]. Glial activation contributes to immunological functions [38], synaptic turnover [14,66,70,79], and recovery processes after CNS injury [3,39]. Together microglial activation infiltrated (immigrated) blood cells in the brain are also participating in these inflammatory responses. For instance, infiltration of blood cells into the brain [3,74,75] and bimodal increase in resident microglia recently observed in the brain for 4 weeks after TBI [44]. Head injury induces microglia/microphage polarization and generates a mixture of M1 and M2 responses [25,41,76,88] that have dual influences on the structural and functional integrity of the CNS. For example, after an acute brain injury, suppression of microglial activation is neuroprotective [31,94] and their removal exacerbates injury [48].

Therefore, inflammation is an important tool for controlling injury effects and enhancing recovery after TBI. Recently, various studies have demonstrated that the M1 response is rapidly induced at the site of injury and sustained for an extended period after injury. It initially predominates over a comparatively smaller M2 response [32,41,61,85,88], and enhances neuropathology [2,24,62]. The suppression of M1 and the up-regulation of M2 response prevent secondary injury and promote repair processes in the brain [12,42,46,80]. If modulation of such inflammatory responses is protective/regenerative after injury, then detailed information of the initial changes in both the M1 and M2 phenotypes is required to find a window of opportunity. The current study investigates an early time course of both the M1 and M2 responses (phenotypic changes) in the hippocampus following TBI. Our results may aid new therapeutic strategies by demonstrating the window of opportunity to modulate microglia/macrophage polarization toward M2 response early after head injury.

2. Material and methods

2.1. Animals and surgical procedure

All experimental protocols involving animals in this study were approved by the University of Kentucky Animal Use and Care Committee. Young adult male Sprague–Dawley (SD) rats (n = 72, 280–300 g) purchased from Harlan Labs (Indianapolis, IN) were housed in cages (2 rats per cage) on a 12 hour light/dark cycle with free access of food and water. The rats were randomly assigned to one of the six groups (n = 12) of the study.

Surgical procedure and cortical contusions were made under isoflurane anesthesia (2%) as previously described [7]. Rats were subjected to a unilateral moderate controlled cortical impact (CCI) of 2.0 mm depth at 3.5 m/s and 500 ms dwell time using the TBI 0310, a pneumatic impacting device (Precision Systems & Instrumentation, Fairfax Station, VA) with a hard stop Bimba cylinder (Bimba Manufacturing, Monee, IL). The size of beveled impactor was 5 mm. All craniotomies were placed midway between the bregma and lambda on the left hemisphere of the brain. Following injury, animals received a subcutaneous injection of analgesic buprenorphine (0.05 mg/kg), maintained at 37 °C, and allowed to survive for 2, 4, 6, 10, or 24 h post injury. Sham animals received a craniotomy without CCI. Rats were rapidly killed and the brain removed to isolate both the ipsilateral and contralateral hippocampus. Tissues were immediately kept into a dry ice container and then transferred into −80 °C freezer. Half of the animals of each group were analyzed for gene expression and half for protein levels of pro- and anti-inflammatory cytokines.

2.2. Analysis of gene expression: quantitative real-time reverse transcription (qRT)-PCR

Ipsilateral and contralateral hippocampi from each animal (six rats per group) were used to isolate RNA with Trizol plus RNA Purification Kit (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Isolated RNA was quantified using a BioSpace Nano spectrophotometer (Shimadzu Scientific Instruments, Addison, IL, USA). The cDNA of each sample was prepared using a High Capacity cDNA kit (Life Technologies) according to the manufacturer's instructions. qRT-PCR was performed using the 384-well microfluidic Custom TaqMan® Gene Expression Array Card containing TaqMan Gene Expression probes for our genes of interest (Applied Biosystems, Grand Island, NY, USA) as previously described [81]. All genes were normalized to 18S rRNA. Relative gene expression (fold change) in various M1 and M2 phenotypes in the hippocampus following TBI compared to sham animals was determined by $2^{(-\Delta\Delta Ct)}$ method [52]. Due to low concentration of RNA in one sham samples, it was not processed for cDNA and was excluded from the study.

2.3. Analysis of cytokine proteins: enzyme-linked immunosorbent assay (ELISA)

Ipsilateral and contralateral hippocampi from each animal (six rats per group) were lysed in chilled 0.1 M, PBS (pH 7.4) containing protease inhibitors. Tissue homogenates were centrifuged at 15,000 *g* for 10 min

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