IL4 Reduces Epileptogenesis Susceptibility Acutely After TBI: The Role of Macrophage/Microglia Polarization

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Abstract

Traumatic brain injury (TBI) is responsible for 5% of all epilepsy cases, which are known as post-traumatic epilepsy. Macrophage/microglia are key players in TBI pathogenesis. They are activated after TBI, transform to inflammatory phenotype (M1) and trigger neuroinflammation, which provokes epileptogenesis. Interleukin-4 (IL-4) is a well-known polarizer of macrophage/microglia to the anti-inflammatory phenotype (M2). We tested the effect of IL-4 on the rate of epileptogenesis, brain expression of inflammatory and anti-inflammatory cytokines, and the lesion size in traumatic rats. Trauma was exerted to temporo-parietal cortex of rats by *Controlled Cortical Impact*. Thereafter, rats received a single dose (100ng/rat) of IL-4 through intracerebroventricular injection. After 24h, pentylenetetrazole (PTZ) kindling started and development of generalized seizures was recorded. Level of TNF- α , TGF- β , IL-10, and arginase-1 (*Arg-1*) was measured in the brain by immunoblotting at 6h, 12h, 24h, 48h, and 5 days after TBI. The lesion size and cell survival were determined by staining. Traumatic rats were kindled by 5 ± 1 PTZ injections (significantly less than 11 ± 2 injections of control and sham-operated rats, $p < 0.001$). IL-4 did not change kindling rate in sham-operated rats but inhibited acceleration of kindling rate in traumatic rats (13 ± 1 PTZ injections, $p < 0.001$). IL-4 decreased post-TBI overexpression of TNF- α (6h, $p < 0.001$) whereas upregulated post-TBI expression of TGF- β (48h, $p < 0.001$), IL-10 (24h, $p < 0.05$; 48h, $p < 0.01$), and *Arg-1* (24h, $p < 0.001$). IL-4 decreased lesion volume and number of dead neurons. IL-4 suppresses TBI-induced acceleration of epileptogenesis in rats by directing macrophage/microglia to the anti-inflammatory M2 phenotype and inhibition of neuronal death.

Introduction

Traumatic brain injury (TBI) is an injury to the brain caused by an external force such as mechanical forces and supersonic blast waves. The global incidence of all-cause, all-severity TBI is estimated to be about 1%; of which 0.74% is related to mild TBI, and 0.073% associated with severe TBI (Dewan et al., 2019). Sixty-nine million people are estimated to suffer TBI from all causes each year, with the Southeast Asian and Western Pacific regions experiencing the greatest overall burden of disease (Dewan et al., 2019).

TBI prompts an array of cellular processes that result in neuroinflammation (Woodcock and Morganti-Kossmann, 2013; Hunt et al., 2013; Turtzo et al., 2014; Xiong et al., 2018). Neuroinflammation, in turn, triggers a series of pathological cascades and underlies neurological diseases such as Alzheimer's disease, Parkinson's disease, stroke, chronic traumatic encephalopathy, and epilepsy (Cruz-Haces, 2017; Webster et al., 2017; Xiong et al., 2018).

Epilepsy is a neurologic disease that has around 1% international incidence. Post-traumatic epilepsy (PTE) is a form of acquired epilepsy. TBI is responsible of 5% of all epilepsy cases; and over than 20% of cases of acquired epilepsy (Brady et al., 2019). No strategy is yet available to avoid PTE. Neuroinflammation has a central role in PTE (Mukherjee et al., 2020; Sun et al., 2021; Sharma et al., 2021).

Interleukin-4 (IL-4) is a cytokine serves as a potent regulator of immunity. It often acts as an anti-inflammatory agent by regulating inflammation cascade toward down-regulation of the inflammatory cytokines such as TNF- α (Woodward et al., 2010) and upregulation of anti-inflammatory cytokines such as IL-10 (Liu et al., 2016; Mitchell et al., 2017). IL-4 knockout mice display increased brain injury and worsened neurological outcome after transient focal cerebral ischemia (Xiong et al., 2011) and traumatic spinal injury (Lee et al., 2010). IL-4 is able to polarize macrophage/microglia to the anti-inflammatory phenotype (Girard et al., 2013). It is found that IL-4 is produced in neurons after stroke (Li et al., 2001) and focal cerebral ischemia (Zhao et al., 2015), and modulates macrophage/microglia function by polarizing them to the M2 “healing” phenotype. *In vivo* alternative activating M2 macrophage/microglia by IL-4 has been of interest as a therapeutic strategy for treatment of the injury-associated neurologic diseases. In this regard, administration of IL-4 to animals with TBI and traumatic spinal cord injury has been accompanied by beneficial behavioral improvements (Lima et al., 2017; Francos-Quijorna et al., 2016; Pu et al., 2020). Meanwhile, IL-4 has been shown antiepileptic activity in the animal study. It is found that early treatment of mice by IL-4 inhibits rate of recurrent seizures in pilocarpine model of epilepsy by inhibiting early increase of M1 macrophage/microglia, and promoting the increase of M2 phenotype (Li et al., 2017).

Nevertheless, the possible inhibitory effect of IL-4 on development of PTE has not yet been examined. It is shown that TBI accelerates development of epileptogenesis in animal models (Eslami et al., 2016, Hesam et al., 2018). Accordingly, we aimed to examine impact of IL-4 on the accelerated rate of epileptogenesis in traumatic rats. We injected IL-4 to brain of rats shortly after TBI. Then, development of generalized seizures was assessed using kindling model of epileptogenesis. Given the essential role of macrophage/microglia in TBI pathogenesis, and IL-4 as the transformer of macrophage/microglia to the M2 phenotype, functional expression of arginase-1 (*Arg-1*) as the typical marker of M2 macrophage/microglia, as well as distinctive anti-inflammatory cytokines produced by the M2 phenotype were measured in the brain of the IL-4-treated traumatic rats.

Materials And Methods

Animals

Adult male Wistar rats (9-weeks old and 240-270 g weight, n=269) were obtained from Pasteur Institute of Iran. The animals were housed in standard polypropylene cages in a room with controlled temperature ($23 \pm 2.0^{\circ}\text{C}$) and 12h light/dark cycle (08:00–20:00). They were fed ad libitum with rodent’s chow and free access to drinking water. Animals were randomly divided to different experimental groups. All animal experiments were carried out in accordance with the Review Board and Ethics Committee of Pasteur Institute of Iran, and Council Directive 2010/63EU of the European Parliament, and the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Induction of TBI

Rats were anaesthetized with intraperitoneal (*i.p.*) injection of 60 mg/kg ketamine and 10 mg/kg xylazine (Alfasan, The Netherlands). A 5-mm burr hole was drilled at left parieto-temporal cortex at coordinates; A, -4 mm from bregma; L, -4 mm from bregma; according to atlas of rat brain (Paxinos and Watson, 2007). The bone was removed and TBI was induced by a Controlled Cortical Impact (CCI) device (AmScien Instruments, Model AMS 201, USA) with 5 mm round tip, 4.5 mm/s velocity, 150 ms duration and 2 mm depth of deformation. Then, the dissected bone was brought back to its position on the skull, and fixed with dental acrylic. The skin was then closed. Sham-operated animals underwent the whole procedure except that they did not received CCI injury.

Intracerebroventricular injection of IL-4

IL-4 (eBioscience, USA) was dissolved in phosphate-buffered saline (PBS). It is injected (100 ng/5µl/rat) into the right lateral ventricle (*i.c.v.*; AP, -0.9 mm from bregma; L, 1.7 mm from bregma; V, 3.5 mm from dura) of the anesthetized rats through a 27-gauge needle that was fitted to a Hamilton microsyringe. The dose of IL-4 was selected based on the effective doses used in trauma and stroke studies (Cherry et al., 2014; Liu et al., 2016)

Pentylene-tetrazole kindling

Twenty-four h after TBI or sham operation, rats received 35 mg/kg pentylene-tetrazole, (PTZ, Sigma-Aldrich, Canada) through *i.p.* route. PTZ injections were repeated every 48 h until generalized seizures were developed in rats in 3 sequential PTZ injections. The seizure behaviors were scored according to Racine's classification (Racine, 1972). Forelimbs clonic seizures associated with rearing (score 4), and generalized convulsions with loss of balance (score 5) were considered as generalized seizures.

Histology

2, 3, 5-Triphenyl tetrazolium chloride staining

The depth and volume of brain contusion was evaluated after CCI by 2, 3, 5-Triphenyl tetrazolium chloride (TTC; Sigma-Aldrich, Canada) staining according to previously described method (Eslami et al., 2015). The animals were euthanized by CO₂ asphyxiation. Brains were quickly removed and cut freshly into 2 mm coronal sections by a rat brain matrix. The slices were instantly immersed in 2% (w/v in 0.9% NaCl) TTC at 37 °C for 10 min. The area with no staining was determined as the damaged area. The non-stained area as well as the whole area of each section was scanned by Image J software, version 1.8 (National Institute of Health). The depth of lesion was determined. Volume of section and volume of lesion area of that section were calculated by multiplying their area by the thickness (2 mm). The whole lesion volume was determined by summing lesion volume of the sections. Data are presented as mean ratio of whole lesion volume to the whole volume of the relevant sections.

Fluorescent staining

The viable cells in the traumatic area were assessed by hematoxylin & eosin (H&E), and propidium iodide (PI) staining. The animals (3 rats in each group) were euthanized by CO₂ asphyxiation and immediately

perfused transcardially with 4% paraformaldehyde in 0.1 M PBS. The brains were harvested, kept in 4% paraformaldehyde in 0.1 M PBS and processed for embedding in paraffin blocks. The brains were then cut horizontally to 8µm thickness. The sections were deparaffinized, rehydrated in a descending alcohol series and stained with PI (1:1000 in Tris-buffered saline, Sigmaaldrich Germany) for 30 s, and or H&E. The sections were cover-slipped with 90% glycerol mounting buffer. The H&E-stained sections were visualized with light microscope (Nikon, Japan). The PI-stained sections were visualized in the dark place with fluorescent microscope (Nikon, Japan) equipped with specific filter cube for fluorescence channels, and connected to a digital camera. Digital photographs were taken using 4x and 10x objective lenses. The number of PI-stained cells were counted in the traumatic area of every 10 sections (5 mm width and 2.2 mm depth from cerebral cortex) by computer-assisted imaging program (Image J, version 1.8). Data are presented as mean number of viable cells per section.

Western blotting

A 5-mm³ piece of the left parieto-temporal cortex at the site of trauma injury was cut up and protein concentration of the tissue homogenate was determined by Bradford assay (Bradford, 1976). Twenty µg of total protein was electrophoresed in 15% SDS-PAGE gel, then transferred to polyvinylidene difluoride membrane and probed with the primary monoclonal antibodies of TNF-α, *Arg-1*, IL-10 (1:1000 dilution; Santa Cruz Biotechnology, USA), TGF-β (1:1000 dilution; Sigmaaldrich, Germany), and β-actin (1:2000 dilution; Invitrogen, USA) as internal control. After washing, the membrane was incubated with peroxidase conjugated rabbit anti-rat IgG (1:50000; Invitrogen, USA). Immunoreactive polypeptides were detected by chemiluminescence using electrochemiluminescence reagents (Amersham Bioscience, UK) and subsequent autoradiography. Quantification of results was performed by densitometry scan of the blots using Image J software, version 1.8. The relative level of the assessed polypeptide was expressed as ratio of the polypeptide blot density to the β-actin blot density.

Experimental design

Five experimental groups with 9 rats in each group were assigned for kindling procedure. Group 1 (as control) included intact rats which underwent PTZ kindling. Group 2 (as sham) consisted of rats which underwent craniotomy with no TBI induction. Then they received PBS (5µl/rat, *i.c.v.*, up to 30 min after craniotomy), and chemical kindling started 24 h thereafter. In group 3 (IL-4) rats received IL-4 (100ng/5µl/rat, *i.c.v.*), and after 24 h kindling procedure started. Group 4 (Trauma) included rats which underwent TBI, and after 24 h kindling procedure started. In group 5 (IL-4 + Trauma) rats underwent TBI. Then they received IL-4 (100ng/5µl/rat, *i.c.v.*, up to 30 min after TBI), and chemical kindling started 24 h thereafter.

In order to determine the time course of the polypeptides expression after TBI, 13 experimental groups with 8 rats in each group were allocated for western blotting. These groups consist of control (intact rats), sham (including 6 subgroups), and trauma (including 6 subgroups). In sham and trauma subgroups, brains were harvested at 3, 6, 12, 24, and 48 h as well as 5 days after sham operation or induction of TBI.

To determine effect of IL-4 on polypeptides expression, 6 experimental groups with 8 rats in each group were assigned. There were 3 “Sham + IL-4” groups in which animals underwent craniotomy with no trauma induction. Then, they received IL-4 (100ng/5µl/rat, *i.c.v.*) after craniotomy. The brain of rats was harvested at 6, 24, and 48 h after craniotomy. The 3 other groups were “Trauma + IL-4” groups in which rats underwent TBI. Then they received IL-4 (100ng/5µl /rat, *i.c.v.*, after trauma induction), and brain was harvested 6, 24, and 48 h thereafter.

In order to determine time course of lesion development after TBI and effect of IL-4 on lesion volume, 8 experimental groups with 8 rats in each group were allocated. Three groups were considered as sham, and 3 groups were assigned as trauma. Brain of rats was harvested at 24, and 48 h as well as 5 days after sham operation or induction of TBI. In the last two groups effect of IL-4 on lesion volume was assessed. Rats underwent TBI. Then they received IL-4 (100ng/5µl /rat, *i.c.v.*, after trauma induction), and brain was harvested 24, and 48 h thereafter. All the brains were then stained by TTC and injury volume was measured.

In order to determine the effect of IL-4 on cell survival after trauma, 6 experimental groups with 3 rats in each group were allocated. Two groups were considered as sham, and 2 groups were assigned as trauma. The brain was harvested at 24, and 48 h after sham operation or induction of TBI. In the remaining two groups effect of IL-4 on cell viability was assessed. Rats underwent TBI. Then they received IL-4 (100ng/5µl /rat, *i.c.v.*, after trauma induction), and the brain was harvested 24, and 48 h thereafter. All the brains were then stained by H&E and PI and number of viable cells was determined.

Statistical analysis

All statistical analyses were performed using Graph Pad Prism 8 Software. Kaplan-Meier survival analysis was performed for PTZ kindling experiments. One-way ANOVA with Tukey-Kramer multiple comparisons test was used to analyze number of PTZ injections required to kindle rats, and the lesion volume. In order to determine significant interaction between treatment and time factors in the PI staining, and Western blot data, two-way ANOVA and multiple comparisons test were used. The results were expressed as mean $\pm$ SEM (standard error of mean) and the differences with *p* values less than 0.05 were considered statistically significant.

Results

IL-4 inhibited the accelerated rate of kindling in traumatic rats

During kindling experiment 3 rats of the trauma group were excluded due to death after trauma. The data of one rat from control and one rat from sham groups were excluded from results due to out of range values. A Kaplan-Meier survival analysis showed that traumatic rats became kindled significantly (*p* < 0.05; Log-rank Mantel-Cox test) earlier than the sham or control rats (Fig. 1A). Treatment of traumatic rats with IL-4 reversed the accelerated rate of kindling epileptogenesis. Animals in control and sham groups

became kindled with the similar rate with the mean number of 11 PTZ injections (Fig. 1B). Non-traumatic rats that received IL-4 were kindled after around 9 PTZ injections. However, there was no significant difference between IL-4, control and sham groups in number of injections. TBI significantly accelerated development of kindling as traumatic rats were kindled after just 5 injections ($p < 0.001$ compared to control and sham groups). Rats which received IL-4 after trauma became kindled after mean number of 13 PTZ injections, which was more than that of sham and control groups. However, it was not significant compared to sham and control groups.

Effect of IL-4 on brain lesion volume and cell viability in the traumatic rats

With the severity applied in our study, CCI induced 2 ± 0.5 mm depth of deformation from dura 24h and 48h after CCI (Fig. 2). A lesion with 0.21 mm^3 mean volume developed 24h after CCI and preserved till 48h. One way ANOVA revealed a significant difference between groups [$F(7, 34) = 37.99, p < 0.0001$]. Then the mean lesion volume decreased to 0.14 mm^3 5 days after trauma but it still had significant difference with the corresponding sham group ($p < 0.01$, Fig. 2). Treatment of traumatic rats with IL-4 decreased lesion volume. IL-4 significantly decreased injury volume 48 h after TBI ($p < 0.05$).

Effect of TBI and IL-4 on the number of PI-positive cells in the traumatic region is demonstrated in Fig. 3. PI positive cells were significantly decreased 24h ($p < 0.01$) and 48h ($p < 0.001$) after TBI. Two-way ANOVA revealed a significant interaction [$F(3, 16) = 3.88, p < 0.05$] between treatment ([$F(3, 16) = 33.86, p < 0.0001$]) and time ([$F(1, 16) = 7.67, p = 0.013$]) factors among groups. Administration of IL-4 to traumatic rats did not significantly change the number of PI-positive cells at 24h after TBI. However, at the 48h post-TBI period, number of PI-positive cells in the IL-4-treated traumatic rats was significantly higher than that of the traumatic rats ($p < 0.05$).

IL-4 prevented overexpression of TNF- α 6h after TBI

Figure 4A demonstrates time course of TNF- α protein expression from 3h to 5 days after TBI. TNF- α significantly increased at early times (3h and 6h) after TBI, and returned to control level 24h thereafter. Two-way ANOVA revealed a significant interaction [$F(3, 16) = 42.86, p < 0.0001$] between treatment ([$F(1, 16) = 63.35, p < 0.0001$]) and time ([$F(3, 16) = 73.77, p < 0.0001$]) factors among groups. The 6h post-TBI period was selected to evaluate the effect of IL-4 on TNF- α expression.

As shown in Fig. 4B, One way ANOVA revealed a significant difference between groups [$F(3, 8) = 280.1, p < 0.0001$]. Administration of IL-4 to sham-operated rats (sham + IL-4 group) increased TNF- α expression after 6h ($p < 0.01$, compared to sham group). However, administration of IL-4 to traumatic rats inhibited overexpression of TNF- α at this time point. A significant difference was found between traumatic rats and IL-4-treated traumatic rats ($p < 0.0001$) in TNF- α level.

IL-4 augmented overexpression of IL-10 after TBI

The time course of IL-10 protein expression after TBI is demonstrated in Fig. 5A. IL-10 significantly increased 6h after CCI. This increase lasted up to 24h after TBI. Two-way ANOVA revealed a significant interaction [$F(6, 60) = 16.37, p < 0.0001$] between treatment [$F(2, 60) = 230.6, p < 0.0001$] and time [$F(3, 60) = 20.7, p < 0.0001$] factors among groups.

The 24h and 48h periods after TBI were selected to assess the effect of IL-4 on IL-10 expression. Two-way ANOVA revealed a significant interaction [$F(3, 40) = 111.3, p < 0.0001$] between treatment [$F(3, 40) = 168.3, p < 0.0001$] and time [$F(1, 40) = 28.12, p < 0.0001$] factors among groups (Fig. 5B).

Administration of IL-4 to sham-operated rats (sham + IL-4 group) significantly increased IL-10 expression after 48h ($p < 0.0001$ compared to the corresponding sham group). The extent of IL-10 expression at 48h was significantly higher than that at 24h after sham operation ($p < 0.0001$). Administration of IL-4 to traumatic rats significantly amplified overexpression of IL-10 at both 24h and 48h after TBI ($p < 0.0001$ compared to the corresponding Trauma group).

IL-4 amplified overexpression of TGF- β 48h after TBI

The time course of TGF- β protein expression after TBI is demonstrated in Fig. 6A. TGF- β significantly increased 6h after CCI, persisted for 24h and then decreased to a level less than control at 48h after TBI ($p < 0.0001$). Two-way ANOVA revealed a significant interaction [$F(4, 18) = 241.7, p < 0.0001$] between treatment [$F(2, 18) = 206.5, p < 0.0001$] and time [$F(2, 18) = 297.5, p < 0.0001$] factors among groups.

The 24h and 48h periods after TBI were selected to evaluate effect of IL-4 on TGF- β expression. Two-way ANOVA revealed a significant interaction [$F(3, 16) = 28.59, p < 0.0001$] between treatment [$F(3, 16) = 54.62, p < 0.0001$] and time [$F(1, 16) = 25.34, p < 0.001$] factors among groups (Fig. 6B). Administration of IL-4 to sham-operated rats (sham + IL-4 group) did not change TGF- β expression after both 24h and 48h. Administration of IL-4 to traumatic rats significantly amplified overexpression of IL-10 at 48h after TBI ($p < 0.001$). There was no significant difference between the 24h and 48h trauma groups receiving IL-4.

IL-4 increased expression of Arg-1 in the traumatic brain

As seen in Fig. 7, expression of *Arg-1* significantly increased 24h ($p < 0.0001$), but not 48h after TBI. Two-way ANOVA revealed a significant interaction [$F(3, 24) = 22.79, p < 0.0001$] between treatment [$F(3, 24) = 108.2, p < 0.0001$] and time [$F(1, 24) = 58.83, p < 0.0001$] factors among groups. Administration of IL-4 to sham-operated rats increased *Arg-1* expression after 24h ($p < 0.0001$) but not 48h. Administration of IL-4 to traumatic rats significantly amplified overexpression of *Arg-1* at 48h after TBI ($p < 0.0001$). There was significant difference between the 24h and 48h trauma groups receiving IL-4 ($p < 0.01$).

Discussion

The present study implies that the accelerating effect of TBI on development of kindled seizures is inhibited by single *i.c.v.* injection of IL-4 shortly after TBI. IL-4 could reduce lesion size, and significantly rescue neurons from death in traumatic rats. IL-4 also suppressed expression of the M1 cytokine TNF- α .

Moreover, IL-4 amplified overexpression of the M2 cytokines IL-10, and TGF- β , and the M2 marker *Arg-1* in the damaged area. Therefore, directing TBI neuroinflammation toward an anti-inflammatory tone seems to be mainly involved in the blunting effect of IL-4 on the accelerated epileptogenesis in TBI context.

It is reported that intraperitoneal administration of IL-4 to mice 5 h before and 4 days after induction of epilepsy by pilocarpine could decrease frequency of seizures and inhibits development of epilepsy (Li et al., 2017). Therefore, we first assessed the ineffective dose of IL-4 on kindling rate in non-traumatic rats. In addition, we intended to exclude potential effect of IL-4 on peripheral immune system. Therefore, the *i.c.v.* route of administration was selected. Given that antiepileptogenic interventions are necessary after the presumed epileptogenic insult, in order to translate the drug-therapy protocol into practical clinical application, we administered IL-4 shortly after TBI. IL-4, 100ng/rat, slightly decreased the rate of kindling epileptogenesis. However, this effect was not statistically significant. Therefore, we examined the effect of this dose on development of kindled seizures in traumatic rats. IL-4 could successfully inhibit acceleration of kindling development in TBI state. The present study is the first report indicating that IL-4 if is administered for a while after trauma is able to prevent acceleration of epileptogenesis in traumatic rats. There are indirect studies that support finding of the present study. It is reported that the bacterial endotoxin LPS and its derivative monophosphoryl lipid A inhibit the increased potential for acquisition of seizures in traumatic rats (Eslami et al., 2015; Hesam et al., 2018). LPS can stimulate de novo IL-4 gene expression in murine microglia both *in vitro* and *in vivo* (Mukherjee et al., 2009; Lively and Schlichter, 2018). Moreover, LPS is able to induce M2 polarization through a time-dependent (Zhang et al., 2019) and species-dependent manner (Orecchioni et al., 2019). However, LPS is too toxic to be introduced in human clinical trials. In contrast to LPS, clinical studies suggest that IL-4 has acceptable safety. Clinical trials state recombinant IL-4 is safe and well tolerated in humans at the doses of 0.25–5 μ g/kg/day and up to 10 μ g/kg once administered 3 times/week (Leach et al., 1997; Vokes et al., 1998). Therefore, IL-4 may have significant clinical relevance for prevention of PTE.

There is strong evidence on the beneficial impacts of IL-4 on tissue repair following brain injury (Mantovani et al., 2013; Salmon-Her et al., 2000; Xiong et al., 2011). It is shown that administration of IL-4 48 h after spinal cord injury (SCI) markedly improves functional outcomes and reduces tissue damage after contusion injury (Francos-Quijorna et al., 2016). Therefore, we first examined the possible impact of IL-4 on TBI size as the primary mechanism of anti-epileptogenic effect of IL-4 in the TBI state. In our study, TTC staining showed a remarkable cortical injury 24 h after CCI. The size of the injured tissue did not expand at 48h post-TBI period. Then, 5 days after TBI, the injured area showed a size reduction. Our results are in agreement with the study by Başkaya et al., (2000). They showed that CCI with moderate severity (2 mm deformation depth) induces maximum cortical injury in rats after 1 and 2 days. Similar to our results, they found a gradual decrease in the lesion volume starting from day 4 and then a constant injury volume was observed from day 5 till 7. In our study, treatment of traumatic rats with IL-4 caused significant reduction in injured size at 48h post-TBI period. This finding is in line with the beneficial preventive effects of IL-4 on lesion development after SCI (Francos-Quijorna et al., 2016). Some research groups suggested that TTC staining has low power and cannot detect the tissue viability below a limit because the cells that are between death and survival cannot be detected by this method (Benedek et al.,

2006). Moreover, this technique is only able to detect irreversible brain damage (Liszczak et al., 1984). At the same time, it is reported that despite protective effect on both neuronal and oligodendrocyte populations, IL-4 could not reduce the size of the injured tissue in SCI (Lima et al., 2017). In order to further verify the potential protective effect of IL-4 against TBI-induced tissue injury, we examined cell viability in the trauma area by PI staining. Administration of IL-4 (100 ng/rat, *i.c.v.*) after TBI significantly prevented development of neuronal death 48h after TBI. This finding is correlated with reduction of the injured size by IL-4 at 48h post-TBI period. In contrast to our results, intrahippocampal injection of mesenchymal stromal cells transiently expressing 100 ng IL-4, 5 days after TBI could not reduce hippocampal and cortical neurodegeneration and improve functional outcomes in mice (Enam et al., 2020). The early times after TBI are critical in subsiding development of TBI pathogenesis. Regardless of the tool used to deliver IL-4 into the CNS, we injected IL-4 acutely, up to 15 min, after TBI. This period is quite early compared to the 5 days post-TBI period of IL-4 injection used by Enam et al. This might be the main reason of positive results obtained in our study. It is known that IL-4 inhibits cell death and apoptosis (Zamorano et al., 1996). IL-4 also enhances survival of murine basophils (Reinhart and Kaufmann, 2018) and mast cells (Burton et al., 2013). Various mechanisms are suggested for this attribute of IL-4 (Zamorano et al., 1996; Reinhart and Kaufmann, 2018; Chen et al., 2020). In cerebral ischemic-reperfusion injury, which is associated with hyperexcitability of neurons and cell death, supplemental IL-4 has been able to increase viability of cortical neurons by reducing spontaneous neuronal firing and network burst activity (Chen et al., 2020). This mechanism seems to be also involved in the preserving viability of neurons by IL-4 in our study. This suggestion is supported by the behavioral finding of our study as IL-4 could decrease brain hyperexcitability and the accelerated rate of kindling epileptogenesis induced by TBI.

TBI is obviously associated with acute neuroinflammation and release of inflammatory cytokines. Neuroinflammation is the main causative factor in PTE (Mukherjee et al., 2020; Sun et al., 2021). Therefore, modulation of neuroinflammation would have major role in preventing PTE. Hence, we measured impact of IL-4 on the TBI-induced brain expression of TNF- α , as the typical inflammatory cytokine, and IL-10 as well as TGF- β , as the classic anti-inflammatory cytokines. Our results showed that TNF- α protein level increased in the traumatic brain to a level more than twice of the sham level at 3 and 6 h after TBI. Then the TNF- α level returned to sham level 24 h after TBI. This finding is consistent with previous studies demonstrating the increase of TNF- α protein level in rat brain during a 3-12 h period after CCI (Eslami et al., 2015) and fluid percussion injury (Taupin et al., 1993; Clausen et al., 2019). We selected the 6 h post-CCI interval, and measured TNF- α brain expression after treatment of traumatic rats with IL-4. IL-4 by itself increased TNF- α level in the sham-operated rats. This is the first report indicating increase in TNF- α level in sham (control) group by IL-4. This finding is in contrast to the previous report indicating that IL4 does not change TNF- α level in control conditions such as unstimulated monocytes (Hart et al., 1988). The difference in doses of IL-4 and conditions of the two studies (*in vivo* versus *in vitro*) might be the underlying reason for the different findings. IL-4 is predominantly regarded as an anti-inflammatory cytokine. However, there are *in vitro* and *in vivo* evidence suggesting that IL-4 by itself can also promote the inflammatory response including TNF- α and IL-1 β expression in parallel (Latta et al., 2015; Ratthé et

al., 2009; Van Kampen et al., 2005). The target cell, the time of application, and concentration during various phases of immune responses are determinant of pro- or anti-inflammatory effects of IL-4. The TNF- α increase by IL-4 in the sham-operated rats, is in line with the kindling data of the present study. IL-4 increased the rate of epileptogenesis in sham-operated rats, which was marginally significant ($p=0.05$). Given the proconvulsant effect of TNF- α (Chong et al., 2018; Lagarde et al., 2016; Riazi et al., 2008), the increased expression of TNF- α (and other inflammatory cytokines such as IL-1 β) seems to be mainly involved in the acceleration of kindling development by IL-4 in non-traumatic rats. On the other hand, IL-4 blocked the increase in TNF- α level after TBI. Anti-inflammatory activity of IL-4 is attributed to suppression of TNF- α and interleukin 1- β production and activity (Hart et al., 1989; Leving et al., 1999). Considering the positive impact of TNF- α on seizure threshold and epileptogenesis (Chong et al., 2018; Patel et al., 2017; Rana and Musto, 2018) the suppressing effect of IL-4 on the acceleration of kindling development by TBI is mediated (in part) via inhibiting TNF- α increase in the traumatic brain area.

In order to verify impact of IL-4 on expression of the classic anti-inflammatory cytokines IL-10 and TGF- β after TBI, the time course of expression of these cytokines was first determined. IL-10 level increased 12 h after TBI, and remained higher than basal level till 48 h. TGF- β showed different pattern of expression. The level increased 6 and 24h after TBI but then decreased to the level less than control at 48h after TBI. IL-10 is shown modest elevation in mice 24 h after CCI with the peak 3 days after CCI (Lagraoui et al., 2012). However, in a weight drop model of TBI in rats, IL-10 acutely raised in brain from 2 h after trauma followed by a progressive increase over 24 h (Kamm et al., 2006). These findings demonstrate variability in the degree of cytokine response based on the mechanism and severity of injury. Then, we determined the effect of IL-4 on brain expression of IL-10 and TGF- β at 24 h (the time of rise in both cytokines) and 48h (the time of decline in both cytokines) after TBI. At both 24 h and 48 h after sham operation, IL-4 had no effect on TGF- β brain level but increased IL-10 brain level. IL-4 has induced IL-10 expression from stimulated T helper 1 cells (Mitchell et al., 2017) as well as endotoxin-stimulated monocytes/microglia (Kambayashi et al., 1996; Cao et al., 2005) but not from unstimulated (control) cells. We report for the first time that IL-4 is able to induce IL-10 production in control condition *in vivo*. Administration of IL-4 to traumatic rats significantly intensified IL-10 expression at both 24 h and 48 h post-CCI periods. IL-10 is a well-known anti-inflammatory cytokine which is expressed following TBI. IL-10 inhibits development of epileptiform activity evoked by transient episodes of hypoxia in rat hippocampal slices (Levin and Godukhin, 2007), and focal convulsions in electrical kindling (Godukhin et al., 2009). IL-10 can also increase threshold of temperature-induced seizures in rats (Ishizaki et al., 2009). Thus, amplification of IL-10 expression by IL-4 plays a role in suppressing the TBI-induced acceleration of kindling development. With regard to TGF- β , IL-4 did not affect the increased TGF- β expression in traumatic rats 24 h after TBI but could significantly raise TGF- β expression at 48 h (the time of fall in the cytokine level after TBI) post-TBI period. TGF- β possesses both pro- and anti-inflammatory functions depending on the context. TGF- β is involved in epilepsy and PTE. Over-expression of cerebral TGF- β in transgenic mice causes development of a set of neuropathological complications including seizures (Wyss-Coray et al., 1995). Moreover, TGF- β and TGF- β signaling pathway are demonstrated among the main elements in development of epileptiform activity in rats after the brain injury induced by blood brain barrier disruption

(Cacheaux et al., 2009). TGF- β is overexpressed 3 days after CCI in mice, and *i.p.* injection of TGF- β blocker is associated with a decrease in seizure behavior and EEG power spectrum (Wang et al., 2017). On the other hand, TGF- β has shown neuroprotective action against glutamate neurotoxicity and ischemic brain injury (Prehn et al., 1993). The genetic defects in the TGF- β pathway is accompanied by epilepsy in humans (Kotlarz et al., 2018). Moreover, inhibition of TGF- β signaling blocks the anti-seizure effects of the oligonucleotide miRNA inhibitors (antagomirs) in three different rodent models of temporal lobe epilepsy (Venø et al., 2020). Thus, strengthening the TGF- β expression by IL-4 also seems to play a role in suppression of TBI-induced acceleration of kindling development. Given the central role of neuroinflammation in pathogenesis of PTE (Mukherjee et al., 2020; Sun et al., 2021), amplification of anti-inflammatory response greatly contributes in the suppressing impact of IL-4 on TBI-induced acceleration of kindling development.

We found that administration of IL-4 shortly after TBI causes overexpression of IL-10 and TGF- β , and impedes expression of TNF- α . TNF- α is the typical proinflammatory cytokine released from M1 macrophage/microglia, whereas IL-10/TGF- β are the representative cytokines of M2 macrophage/microglia (Zhou et al., 2012; Laffer et al., 2019; Yao et al., 2019). Therefore, we assessed potential polarization of macrophage/microglia to M2 phenotype by measuring brain expression of the classic marker of M2 macrophage/microglia *Arg-1*. In our study, TBI itself was associated with *Arg-1* overexpression in 24 h but not 48 h after TBI. Overexpression of *Arg-1* 24 h after TBI is comparable with the period of overexpression of M2 cytokines IL-10 and TGF- β after TBI as they also enhanced in our study during first 24 h after TBI. It is reported that in addition to M2 phenotype, *Arg-1* is also upregulated in a fraction of murine M1 macrophage as well (Amici et al., 2017). Therefore, in addition to M2 phenotype, M1 inflammatory macrophage/microglia might be another source of *Arg-1* expression in the brain of traumatic rats. In a study similar to ours, Turtzo et al., utilized CCI model of trauma and measured the cytokine expression at post-transcription level (Turtzo et al., 2014). However, they found a 5-7 days post-TBI period for microglia mixed M1 and M2 response in rats (Turtzo et al., 2014). In contrast to our study, they used female rats. Macrophage/microglia response *in vivo* is highly complex depending on many factors, of which gender differences is critical and seems to be the key reason for different macrophage/microglia time response between the two studies.

In our study, administration of IL-4 to sham-operated (non-traumatic) rats significantly increased expression of *Arg-1* after 24h. This stimulatory effect on *Arg-1* was not seen at 48h after administration, which is most likely due to short half-life of IL-4 (Conlon et al., 1989). Our finding is in line with previous study indicating temporal upregulation of *Arg-1* in frontal cortex and striatum microglia of naïve control rats 8-16h after direct injection of IL-4 into the third ventricle (Pepe et al., 2014). In addition, lentiviral delivery of IL-4 into the fourth ventricle of naïve control mice has been associated with overexpression of *Arg-1* of spinal cord microglia at posttranscriptional level (Rossi et al., 2018). In contrast to the time effect of IL-4 in control non-traumatic rats, we observed IL-4 administration to traumatic rats could increase *Arg-1* expression 48 h but not 24 h after TBI. It is recently reported that injection of IL-4 to the hippocampus of mice 5 days after head-closed injury could not significantly increase M2-like macrophages after 48h (Enam et al., 2020). In our study overexpression of *Arg-1* in traumatic rats 48h after IL-4 administration is

associated with increased TGF- β expression at this time point. However, IL-10 was overexpressed both 24 h and 48 h after IL-4 administration to traumatic rats. There is a range of activation states for microglial cells that span from the M1 to the M2 phenotypes, and the phenotype of the activated microglia will fall somewhere along this spectrum depending on the signal encountered. Moreover, the *in vivo* effects of IL-4 are also complex, and the final response depends on the local environment, pathological state, and the doses used. These factors might be responsible for different pattern of expression of *Arg-1*, IL-10 and TGF- β in the traumatic rats after IL-4 administration.

Macrophages (both resident and the invaded blood borne) as well as microglia have critical role in TBI (Hirschberg and Schwartz, 1995; Kumar and Loane, 2012). Meanwhile, IL-4 directly or indirectly attracts monocytes/macrophages from the hematopoietic system to infiltrate the CNS (Rossi et al., 2018). Although macrophage may differ from microglia for their capability to respond to IL-4 signaling, they share common markers and secreted cytokines (Rossi et al., 2018). Therefore, we cannot discriminate between the extent of contribution from microglia and from macrophages to the finding of the present study. Though many methods have been developed for distinguishing between macrophages and microglia, they are challenging and inconclusive. Hence, we used the term macrophage/microglia throughout the text.

We conclude that a single *i.c.v.* administration of IL-4 suppresses accelerated rate of kindling epileptogenesis in traumatic rats. Various mechanisms are involved in this finding. IL-4 capability to polarize macrophage/microglia to the M2 anti-inflammatory phenotype, blocking inflammation cascade, initiating anti-inflammation processes, and protecting cells from death are supported by our study.

Declarations

Author contribution

Study concept and design: MS. Acquisition of data: MR, BK, and TA. Analysis and interpretation of data: MS, HGP, and MR. Drafting the manuscript: MS. Experimental design and statistical analysis: MS, HGP, and MR. Obtained funding: MS. Administrative, technical, and material support: MS. Study supervision: MS. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

All animal experiments were carried out in accordance with the Review Board and Ethics Committee of Pasteur Institute of Iran, and Council Directive 2010/63EU of the European Parliament, and the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

Figure 1

Effect of IL-4 on development of generalized seizures in traumatic rats. A: Kaplan-Meier survival analysis for development of PTZ kindling in traumatic rats. Traumatic rats became kindled significantly ($p < 0.05$; Log-rank Mantel-Cox test) earlier than the sham-operated rats. IL-4 restored the accelerated rate of kindling in traumatic rats to control level. B: Number of PTZ injections needed for acquisition of generalized seizures. Each plot represents Mean $\pm$ SEM. ***: $p < 0.001$ compared with control and sham

groups, ###: $p < 0.001$ compared with trauma group (analyzed by one way ANOVA with Tukey's post-hoc multiple comparisons test).

Figure 2

Effect of intracerebroventricular administration of interleukin 4 on size of temporoparietal cortical tissue injury induced by controlled cortical impact in rats. The viable tissue (red stained) was distinguished from the dead tissue (white unstained) by 2,3,5-Triphenyltetrazolium staining. Data are presented as mean± SEM. **: $p < 0.01$, and ****: $p < 0.0001$ compared to corresponding sham group (analyzed by one way ANOVA with Tukey's post-hoc multiple comparisons test).

Figure 3

Effect of interleukin 4 on the cell viability in the traumatic brain region of rats. The number of propidium iodide (PI)-stained cells were counted in the traumatic area (5 mm width, and 2.2 mm depth from cerebral cortex) of every 10 sections (8 μ m thickness of each section). Data are presented as mean $\pm$ SEM number of PI-positive cells per section. *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ analyzed by two-way ANOVA with multiple comparisons test.

Figure 4

Effect of interleukin 4 on TNF- α up-regulation after traumatic brain injury in rats. A: Time course of TNF- α protein expression after Controlled Cortical Impact injury. B: Effect of IL-4 on TNF- α overexpression 6 h after traumatic brain injury. Upper part: The representative blots of brain TNF- α and β -actin (internal control) obtained by Western blot technique. Lower part: The mean ratio of TNF- α to β -actin. *: $p < 0.05$,

** $: p < 0.01$, *** $: p < 0.001$, and **** $: p < 0.0001$ analyzed by two-way ANOVA with multiple comparisons test.

Figure 5

Effect of interleukin 4 on Interleukin 10 up-regulation after traumatic brain injury in rats. A: Time course of IL-10 protein expression after Controlled Cortical Impact injury. B: Effect of IL-4 on IL-10 overexpression 24 and 48 h after traumatic brain injury. Upper part: The representative blots of brain IL-10 and β -actin

(internal control) obtained by Western blot technique. Lower part: The mean ratio of IL-10 to β -actin. *: $p < 0.05$, **: $p < 0.01$, and ****: $p < 0.0001$ analyzed by two-way ANOVA with multiple comparisons test.

Figure 6

Effect of interleukin 4 on Transforming growth factor- β expression after traumatic brain injury in rats. A: Time course of TGF- β protein expression after Controlled Cortical Impact injury. B: Effect of IL-4 on TGF- β overexpression 24 and 48 h after traumatic brain injury. Upper part: The representative blots of brain TGF-

β and β -actin (internal control) obtained by Western blot technique. Lower part: The mean ratio of IL-10 to β -actin. **: $p < 0.01$, ***: $p < 0.001$, and ****: $p < 0.0001$ analyzed by two-way ANOVA with multiple comparisons test.

Figure 7

Effect of IL-4 on brain expression of arginase-1 in traumatic rats. Upper part: The representative Western blots of brain arginase-1 and β -actin (internal control) obtained 24 and 48 h after Controlled Cortical

Impact injury. Lower part: The mean ratio of arginase-1 to β -actin. *: $p < 0.05$, ***: $p < 0.001$, and ****: $p < 0.0001$ analyzed by two-way ANOVA with multiple comparisons test.