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Original Article

Co Expression of *GMFβ*, *IL33*, *CCL2* and *SDF1* Genes in the Acute Stage of Toxoplasmosis in Mice Model and Relation for Neuronal Impairment

Mehri Najafi [1], Razieh Amini [2], Amir Hossein Maghsood [1], Mohammad Fallah [1], *Faeze Foroughi-Parvar [1]

1. *Department of Medical Parasitology and Mycology, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran*
2. *Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran*

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***Correspondence**
Email:
f.foroughi@umsha.ac.ir

Abstract

Background: *Toxoplasma gondii* is an obligate intracellular parasite that migrates through macrophages or dendritic cells to neurons and nerve cells. Glia Maturation Factor (GMF) is a pre-inflammatory protein that is expressed in the central nervous system (CNS). *GMFβ* expression is related to *IL33* and *CCL2* and *SDF1* in some neurodegenerative diseases. According to the importance of *GMFβ* in neurodegenerative diseases and its association with *IL33*, *CCL2* and *SDF1* genes, this study was designed to determine the level of expression of these genes in the brains of mice with acute toxoplasmosis.

Methods: Tachyzoites of *T. gondii* RH strains were injected to 5 Swiss Albino mice. At the same time, healthy mice were inoculated with the Phosphate-buffered saline (PBS). Their brains were removed and kept at -70 ºC in order to RNA extraction, cDNA syntheses and Real Time PCR performance. The level of gene expression was investigated with SYBR Green Quantitative Real-Time PCR.

Results: *GMFβ* gene expression increased significantly (P=0.003) 3.26 fold in *Toxoplasma* infected mice in comparison to the control. *GMFβ* gene expression was associated with increased expression level of *IL33*, *CCL2*, and *SDF1* genes.

Conclusion: Considering the prominent role of *GMFβ* in CNS as well as the immune system, the elevation of *GMFβ*, *IL33*, *CCL2* and *SDF1* genes expression in the early stage of toxoplasmosis is associated with the occurrence of *neuropathological* alterations. Detection of these genes as an indication of brain damage in the early stages of *Toxoplasma* infection can prevent neurodegenerative disorders following acquired toxoplasmosis.

Introduction

Toxoplasma gondii is a compulsive intracellular parasite which can infect, survive and replicate in nearly all mammalian cells (1, 2). In the complicated life cycle of this parasite, warm blooded animals as well as human are regarded as intermediate hosts but cats as definitive hosts. Transmission may occur by oral ingestion of tissue cysts containing raw or under-cooked meat or infective oocysts excreted in cat feces (3). During infection the parasite disseminates via lymph or blood circulation and the infection is established in different organs including the brain. Based on in-vitro studies, neurotropic parasites affect the central nervous system, although the astrocytes, microglia and neurons are considered as a preference for parasite localization (1).

Glia maturation factor (GMF) is a potent proinflammatory factor that plays a key role in regulation, progression and pathogenesis of neurodegenerative and autoimmune diseases of the central nervous system (CNS). GMF is implicated in proinflammatory mediator release from glial cells (2). Interleukin33 (IL33) as a member of IL1 family is extremely expressed in glia and astrocytes of CNS (3). IL33 has a critical effect on the pathophysiology of the CNS diseases such as Alzheimer; meanwhile the expression of *GMFβ* is associated with stimulation of *IL33* production (4).

Stromal cell derived factor 1 (SDF1) also known as CXCL12 appears to be associated with the various physiological and pathological processes, including inflammatory processes, neurogenesis and angiogenesis in the CNS. It is widely expressed in neurons, glial cells, endothelial cells, and meningeal cells (5, 6).

The chemokine C-C motif ligand 2 (also called monocyte chemotactic protein-1 (MCP1)) released by neurons and microglia in CNS (7). Glial cells as perivascular astrocytes produce CCL2 in various neuroinflammatory disorders (8). Increased expression of CCL2 during neuropathological complications was reported in various studies (9-10). Neural CCL2 expression is attributed to different types of degeneration such as ischemia, Alzheimer`s disease, multiple sclerosis (MS), axonal injury, amyotrophic lateral sclerosis (ALS) or peripheral nerve injuries (7). For example up-regulation of CCL2 in the brain might be a new target for disease progression (8). *T. gondii* parasites affected on the dopamine signaling pathway and disrupted the regulation of dopamine and amyloid pathways and resulted in pathological effects during the early stages of acquired or congenital toxoplasmosis; however, *T. gondii* behavioral effects are observed in the form of neurodegenerative disorders or eye complications in the years after the infection (11). *GMFβ* prominently involved in the regulation, progression and pathogenesis of the central nervous system diseases, as well as apoptosis, by interfering with the production of cytokines, chemokines and neurotransmitters.

As the simultaneous increase of *GMFβ* gene along with other genes (IL33, CCL2, and SDF1) seemed to be contributed to apoptosis, cell death and oxidative stress which played an important role in neuropathy because of toxoplasmic encephalitis so, we aimed to determine the expression level of these genes in the brain of *Toxoplasma* infected mice.

Materials and Methods

Six Swiss albino mice were injected intraperitoneally by 10^3 *T. gondii* RH strain tachyzoites (maintained continuously by intraperitoneal injection of mice in the Department of Parasitology, Hamadan University of Medical Sciences, Iran). Six mice were inoculated by the same volume (0.2 ml) of PBS as control. After the confirmation of infection by peritoneal aspiration and observation of tachyzoites under the light microscopy, mice were sacrificed

on the 5th day of infection by an overdose of ketamine/xylazine (12) and their brains were removed.

Brain samples were transferred to -70 ºC and kept until the performance of RNA extraction, cDNA synthesis and Real Time PCR. Total RNA was extracted based on guanidine and thiocyanate-phenol: chloroform method (13). RNA concentration and purity were determined by optical density measurement using a NanoDrop spectrophotometer (Bio-TeK, USA). Electrophoresis was performed on 1% agarose gel to assess the quality of RNA samples.

The first-strand cDNA synthesis was conducted using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's protocol (BIOFACT Co, South Korea) by incubation at 25 °C for 5 min, 42 °C for 1 h and 70 °C for 5 min. Quantification of gene expression was followed by adding a volume of 20 µl containing 1 µl cDNA, 7 µl H2O, 10 µl SYBR Premix Ex Taq II Kits (Takara, Japan), and 1 µl of 10 pmol/ml specific primer (GMF, IL-33, SDF1 and CCL2) (Table 1).

All protocols were performed in terms of the principles and ethical considerations of working with laboratory animals as confirmed by the ethics committee of Hamadan University of Medical Sciences (Ethics committee code: IR.UMSHA.REC.1398.271).

Table 1: Specific primer sequences for analysis of *GMFβ*, *IL33*, *SDF1* and *CCL2* mRNA expression

Gene	***Sequence***
GMFβ	Forward: 5"-GCC ACA CACAGG ACT CTT AG-3"
	Reverse: 5"-ACC TTA CAA ACG ACC CAT CTA C-3"
IL33	Forward: 5"-TGG CTC TAG TGG AGG AGA AAG ATG-3"
	Reverse: 5"-TCA TTC AAC CCA GAA CGC ACA G-3"
SDF1	Forward: 5"-GCT CTG CAT CAG TGA CGG TAA AC-3"
	Reverse: 5"-AGG GCA CAG TTT GGA GTG TTG-3"
CCL2	Forward: 5"-AAA CCT GGA TCG GAA CCA AAT GAG-3"
	Reverse: 5"-TAC GGG TCA ACT TCA CAT TCA AAG G-3"

The Allele ID primers software was designed to analyze the binding site and the desired features. The specificity of primers pairs was checked by NCBI BLAST. Each PCR cycle included an initial denaturation at 95 °C for 10 min, 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s (14). The values were normalized using 18S rRNA as an endogenous internal standard (15). Melting curve analysis was used to confirm the specificity of the real-time PCR reaction. The real-time PCR instrument programmed to include a melting profile immediately following the thermal cycling protocol to give complete melting curve data. Mean CT values and standard deviations of the gene expression were used in the ΔΔCT calculations. Fold-differences calculated using the ΔΔCT method (16). The final result of this method was presented as the fold change of target gene expression relative to a reference sample, normalized to a reference gene. The relative gene expression is usually set to 1 for reference samples because $\Delta\Delta CT$ is equal to 0 and therefore 2^0 is equal to 1 (15).

All statistical analysis was performed using SPSS V.16. (IBM Corp., Armonk, NY.USA). Variables were analyzed by Mann-Whitney and Kruskal-Wallis tests. All data was expressed as mean ± standard deviation (SD). The statistical level of significance was set at $P < 0.05$.

Results

The PCR experiment produced a discreet amplicon corresponding to the expected size, which confirmed the specificity of the primers. Melt curve analysis was used to assess qPCR assay and single melting curve verified a single PCR product for *GMFβ*, *IL33*, *CCL2* and *SDF1* genes and 18SrRNA Non-pattern control (NTC) was used to control any contamination (fluorescence or PCR products) or presence (Genomic DNA). Follow up analysis gel electrophoresis revealed that curves generated a single amplicon, verifying a single PCR product (Fig. 1).

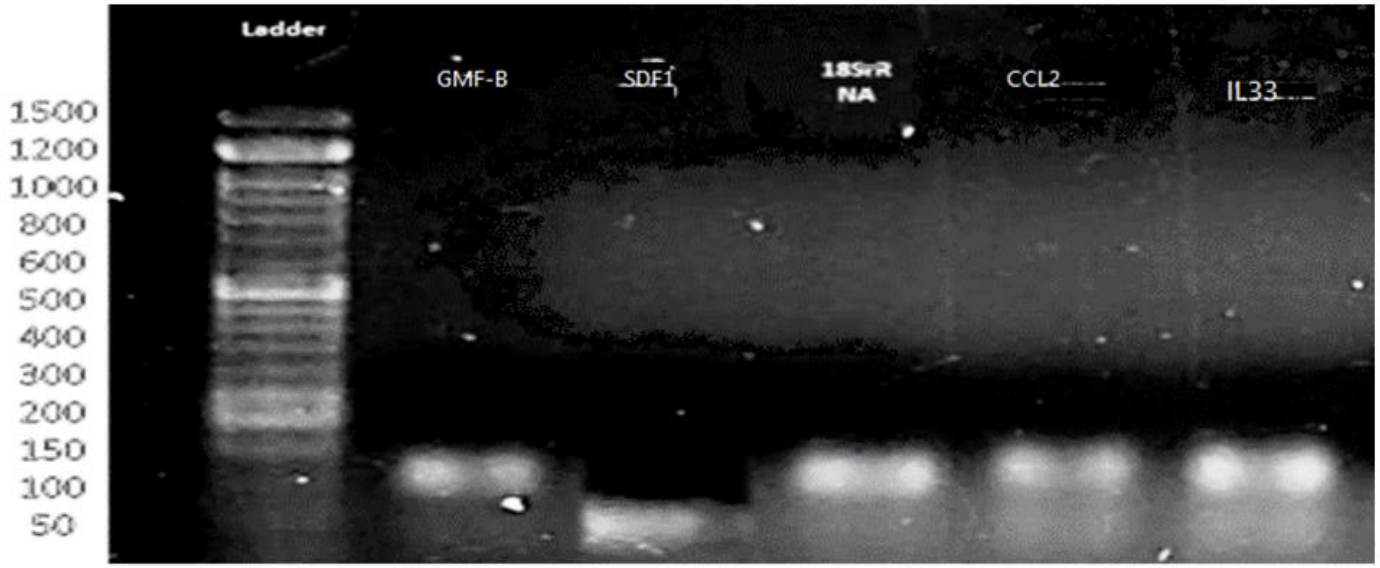

Fig. 1: Gel Electrophoresis showing the expression of *GMFβ* (109 bp), *IL33* (100 bp), *SDF1* (126 bp), *CCL2* (101bp) and 18s rRNA genes in brain of mice. The visible bands correspond to base pairs of amplicons with different molecular weights along the 100 bp ladder (Mbiotech, Korea)

Gene expression level of cytokines: The relative expression of the genes was calculated by measuring the delta threshold cycle (ACT) value for each sample. The relative fold change in the expression was then calculated using the comparative 2–ΔΔCT formula. According to the results, the expression level of GMF was significantly increased up to 3.2-fold in the infected group compared to the healthy group (P <0.001). It was found that *SDF1*, *CCL2* and *IL33* expression up-regulated 5.05 (P = 0.002), 3.74 (P<0.001) and 4.24 (P<0.001) respectively in the infected group compared to the healthy group, which were statistically significant, Fig. 2.

Discussion

In the present study, the expression of *GMFβ*, *SDF1*, *CCL2* and *IL33* genes in the brains of *Toxoplasma* infected mice was investigated. Regarding the prominent role of *GMFβ* in the onset of pathological damage in the brain and its relationship with associated genes with inflammation, namely *IL33*, *CCL2* and *SDF1*, a significant increase in the expression of these genes was also observed.

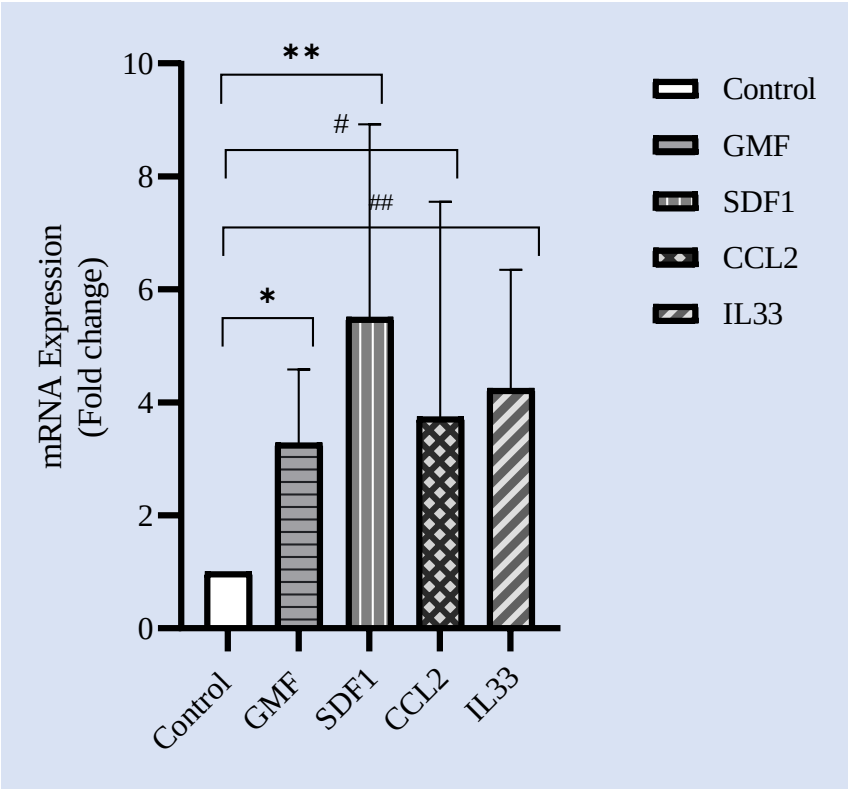

Fig. 2: Comparison of *GMF-β* gene expression, *IL 33, CCL2* and *SDF1* in *Toxoplasma* infected and healthy control group. The Real Time PCR reaction was performed twice on each genes and the mean ± SEM of gene folds were documented. Significant changes were found in genes expression folds in infected and control groups. **P*=0.003, ***P*=0.002, #*P*=0.0002, ##*P*<0.0001

In accordance with the present study, Dinsel using immunohistochemistry test showed that the level of *GMFβ* in the brains of *Toxoplasma*-infected rats was significantly higher than the control ones. An increase of *GMFβ* level was observed on days 10 and 30 of toxoplasmosis in infected rats (17). *GMFβ* has a high level of oxidative activity and plays an active role in the production of reactive oxygen species (ROS) (18-20). *GMFβ* has also been shown to play an important role in inflammation and oxidation, leading to apoptosis and cell death (18); for example, using GMF-null astrocytes increased the cell's antioxidant activity, meanwhile the production of reactive oxygen species (ROS) and lipid peroxidation reduced (20). Therefore, it seems that GMF plays a key role in causing tissue damage. In the light of the fact that one of the causative agents of brain damage in toxoplasmic encephalitis is oxygen-reactive species, it can be concluded that elevated GMF levels provide the basis for toxoplasmic encephalitis. Various studies have found that *T. gondii* is associated with neurodegenerative diseases, including schizophrenia. The pathogenesis of schizophrenia is very similar to that of *Toxoplasma* encephalitis (21, 22), it is observed in both the increase of oxygen radicals, the production of nitric oxide (23, 24) and, the decreases in enzymes such as superoxide dismutase (25, 26). In Other neurodegenerative diseases, such as Alzheimer's, elevated levels of *GMFβ* in the brain was also detected (27). Experimental models of Alzheimer's have shown an increase in GMFβ expression as well as other cytokines such as interferon-gamma (IFNγ) tumor necrosis factor-alpha (TNFα) and interleukin-1 beta

(IL1β). Therefore, increasing the expression of *GMFβ* seems to be involved in neuropathy of neurodegenerative diseases, including Alzheimer's. Although its exact mechanism is not known, it seems that *GMFβ* reduces the number of neurons and creates neurofibrillary tangles (28, 29).

In the present study, increased level of *GMFβ* expression was also associated with increased level of IL33 expression. According to Kempuraj et al, GMF induced the IL33 release and IL33 enhancement as a new inducer of nerve cells led to GMF mediated TNF-α release from mouse astrocytes which resulted in neurodegeneration (16). IL33 induces glial cells to produce inflammatory mediators that have neuroprotective effects in small amounts and neurotoxic in high quantities (16, 30).

Following the induction of toxoplasmosis infection, an increase in *CCL2* expression was observed along with an increase in *GMFβ*. This finding is consistent with the results of other studies which *GMFβ* induced the release of CCL2; in fact, IL33 is released from degraded and necrotic cells and caused the release of CCL2 from mouse astrocytes (16, 31). Farfara et al (30) found high levels of CCL2 in the cerebrospinal fluid of people with Alzheimer's. Considering the release of CCL2 had been observed in the early stages of Alzheimer's, it could be used as a biomarker to monitor the progression of the disease (32). CCL2 is also released from brain glial cells in patients with multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) (33). In the present study, the increase in gene expression in the infected group compared to the control group indicates the onset of pathological damage in the acute phase of the disease.

After focal cerebral ischemia, SDF1 rises within 24 h by astrocytes, microglia, and vascular endothelial cells in damaged areas (34). It is perivasculary expressed in the injured area, up to 30 days after the injury suggesting that it could be a therapeutic target (35). SDF1 is released from astrocytes and microglia in hypoxia and ischemia and causes neurogenesis and angiogenesis by invoking EPCs and increasing their proliferation (34, 36). Azin et al (37) by comparing the serum of 100 healthy individuals with MS patients, declared that the serum level of SDF1 in the patient group is higher than the healthy group. It has been shown in experimental models that increased expression of SDF1 (CXCL12) stimulated leukocytes migration and inflammation in the central nervous system. It appears that SDF1 production is under control of TNFα; for instance in hepatitis patients, an increase in SDF1 and other CXC class chemokines such as IP10 was due to up-regulation of TNFα (38, 39).

It will be helpful in the early diagnosis of neurodegenerative diseases. All changes in the mentioned genes can be a documented reason for using them to predict the response to treatment. Targeting chemokine and cytokines or their receptors is a growing therapeutic line for neuroinflammatory disorders. By understanding the genetic and molecular mechanism of neuroinflammation, gene inhibitors can be designed in vitro and used for therapeutic purposes (40). Blockage of proinflammatory cytokines activity decreases inflammation. Anti-cytokine therapies by deactivation of specific proinflammatory cytokines has changed the lives of millions of patients with autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, multiple sclerosis, and others (41). Astrocytes which are implicated in the pathogenesis of AD are activated by proinflammatory cytokines. Cytokine inhibitors may have more effectiveness in the early phase of AD, when integrity of the CNS is relatively preserved (41). Based on Shamim et al (42) results inhibition of TNFα potentially slow down the AD complications and result in clearance of PAPP and Aβ plaques. Treatment of animals with cytokines inhibitors reduced neuroinflammation and degeneration of neurons. In general, studies on the *GMFβ* gene and its association with *IL33*, *CCL2*, and *SDF1* genes pointed to a key role in patholog-

ical damage in the brain. Molecular biomarkers, such as GMF, play a significant compartment in disease progression, screening, early detection and prognosis of infections that involve CNS (43).

Based on this study *GMFβ*, *IL33*, *CCL2* and *SDF1* can be used as sensitive indicators for brain damage in acute toxoplasmosis infection and also as a drug target for preventing brain damage or follow-up treatment (if expression is reduced).

Conclusion

GMFβ gene expression is increased in the acute phase of toxoplasmosis. *GMFβ* gene expression was also shown to be associated with raised levels of *IL33*, *CCL2*, and *SDF1* gene expression. Onset expression of *GMFβ*, *IL33*, *CCL2*, and *SDF1* genes in the infected group compared to the control group indicates the occurrence of pathological damage in the acute phase of toxoplasmosis. Considering the notable existence of GMFβ in glia cells and neurons, it can be concluded that the mentioned biomarkers will be influential in the early detection of neurodegenerative complications in toxoplasmosis. Anti-cytokine therapies by targeting cytokine or chemokine genes and CNS biomarkers is a new therapeutic line in neuroinflammatory disorders, so detection of these genes, as an indication of brain damage in the early stages of *Toxoplasma* infection can prevent neurodegenerative disorders following acquired toxoplasmosis.

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Conflict of interest

The authors declare that there is no conflict of interest.

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