

Down-Regulation of CD14 Transcripts in Human Glioblastoma Cell Line U87 MG

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ABSTRACT

Background: Pattern recognition receptors (PRRs) are the main sensors of pathogen and danger signals in innate immunity of which Toll Like Receptors (TLRs) are the most studied ones. The contribution of PRRs in cerebral inflammation induced by microbial infection, tissue damage and cancer has not extensively been addressed so far. Glioma is the most common tumor of the central nervous system and glioblastomas are the most common and most malignant primary brain tumors. **Objective:** The objectives of the present study were to investigate the expression of several PRRs including TLR2, TLR4, MyD88 and CD14 transcripts in human glioblastoma cell line U87 MG and compare their expression level with peripheral blood mononuclear cells (PBMC) obtained from healthy individuals. **Methods:** Touchdown PCR (TD-PCR) and Real-time quantitative PCR (qPCR) were applied to detect and quantify the expression level of TLR2, TLR4, MyD88 and CD14 transcript in U87 MG cell line and (PBMC) of healthy individuals. **Results:** According to our results, human glioblastoma cell line U87 MG expresses TLR2, TLR4, MyD88 and CD14 transcripts in TD-PCR. Moreover, the quantification of the expression of these genes revealed a highly significant down-regulation of CD14 and a slight up-regulation of TLR2 transcripts as compared to PBMC of healthy individuals. **Conclusion:** The lower expression level of CD14 in human glioblastoma cell line, might have a potential implication for CD14 mediated cerebral pathology.

Keywords: CD14, Cell Line, Glioblastoma, Toll-Like Receptor

INTRODUCTION

Central nervous system (CNS) has long been considered as an immune-privileged privileged site. However, several recent studies indicated that the CNS can offer a platform for innate immune responses. This might reflect the ability of the CNS to fight

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infections despite its immune-privileged state. The recognition of infectious non-self is mediated by a limited number of germline-encoded pattern-recognition receptors (PRRs) and triggers inflammatory responses (1). PRRs normally recognize structures conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs) (2). Toll-like receptors (TLRs) are the main family of PRRs which have been found to be expressed by multiple types of immune cells, including CNS microglia and astrocytes (3,4). Similar to peripheral tissues, the expression of PRRs is found in various immune and non-immune cell types of the CNS. Microglia and astrocytes are the main CNS-resident cell types for which consistent data have been published. Microglia are myeloid lineage cells and are regarded as the CNS professional macrophages, owing to their phenotypes and functions are following injury and inflammation (5). For this reason, most of the studies on innate immune responses in the CNS have concentrated on microglia, which express a wide range of PRRs (1). In particular, TLR4 is a transmembrane PRR specialized in the recognition of the bacterial endotoxin, lipopolysaccharide (LPS) (6). It has been well accepted that recognition of bacterial LPS by TLR4 is greatly facilitated by an accessory protein called CD14, which is also a pathogen recognition molecule. CD14, serving as a carrier linking LPS to cell surface TLR4 (6), is expressed by mature myeloid cells (including CNS microglia) and also exists in a secreted form in the circulation (3). Two forms of the CD14 receptor can be found. The membrane bound form (mCD14), which is present at the surface of myeloid cells and acts as a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein. The other form is soluble in the serum (sCD14) and lacks the GPI anchor, although it can bind LPS to activate cells lacking mCD14, such as endothelial, epithelial cells and vascular smooth muscle cells (5). TLR2 plays an important role in the host immune response to gram-positive bacterial infections in the periphery as well as CNS (7). Recently, there is an increasing body of data and evidence for a role of these intensively studied TLRs and CD14 also in neurodegenerative and autoimmune brain diseases (8). TLR stimulation triggers a signal transduction pathway via adaptor protein MyD88 which leads to the secretion of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α through activation of nuclear factor κ B (NF- κ B) (2).

Glioma is the most common tumor of the central nervous system and glioblastomas are the most common and most malignant primary brain tumors (9). Despite combined treatment with surgery, radiotherapy and chemotherapy, the prognosis of glioblastoma remains very poor, with a median survival below 15 months, underlying the need to develop new treatment approaches, such as immunotherapy (10).

Assessing PRRs expression within brain tumors is important to increase the rationale for treatment with drugs in glioblastoma and might allow identification of a subset of target molecules that could be harnessed for therapeutic approaches. It is well recognized that cell line models of human disorders, especially cancers, are important resources for finding new therapeutic as well as biological mechanisms underlying cancer development.

U87 MG cells are in vitro models of human glioblastoma cells for investigating the drug cytotoxicity as well as finding new therapies towards cancer cells. In this study, we chose the human glioblastoma cell line U87 MG as an in vitro model to detect and quantify the expression of PRRs genes in these cells. The focus of our study was on TLR2, TLR4, MyD88 and CD14 genes. We further investigated whether there was a

difference between the expression of these genes in U87 MG cells and PBMC of healthy individuals.

MATERIALS AND METHODS

Cell Culture. Human glioblastoma cell line, U87 MG (A generous gift from Dr. Michel Moenner, University of Bordeaux, France) were grown in DMEM supplemented with 1% L-glutamine (2 mM), 100 µg/ml streptomycin, 100 units/ml penicillin and 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO₂.

Peripheral Blood Mononuclear Cells (PBMC) Isolation. Heparinized venous blood samples were obtained from five healthy individuals and used for isolation of peripheral blood mononuclear cells by density separation over Ficoll-Hypaque (Cedarlane laboratories Ltd, the Netherlands). After two times washing with phosphate buffer saline (PBS), cell pellets were used for RNA isolation.

RNA Extraction and cDNA Synthesis. Total RNA was extracted using the standard Trizol method (Roche Diagnostics, USA). After treatment with DNase I (Fermentas, Lithuania), the quantity of RNA was measured using NanoDrop 3300 (Thermo scientific, Wilmington, DE, USA). 1 µg RNA from each sample was used as template for the reverse transcription reaction. cDNA was synthesized using Oligo-dT primers (Fermentas, Lithuania) and M-MuLV reverse transcriptase (Fermentas, Lithuania). All samples were reverse transcribed under the same conditions (70°C for 5 min, 37°C for 5 min, 42°C for 1 h and 70°C for 10 min).

Reverse Transcription PCR (RT-PCR). Gene specific primers for human TLR2, TLR4, CD14, MyD88 and GAPDH genes were selected based on the information described in Table 1.

The polymerase chain reaction (PCR) mix included 2.5 µl of 10x PCR buffer, 50 mM MgCl₂, 10 pm of each of forward and reverse primers, 250 mM dNTPs, 0.4 unit *Taq* polymerase (Fermentas, Lithuania) and 200 ng cDNA. Total volume of PCR reaction mixture was adjusted to 25 µl with sterile H₂O. Touchdown PCR (TD-PCR) was run under the following thermal cycle conditions: 3 min at 94°C followed by 4 cycles of 20 s at 95°C, 35s at 56°C, and 1 min at 72°C, followed by 4 cycles of 20s at 95°C, 35s at 54°C, and 1 min at 72°C, and 4 cycles of 20s at 95°C, 35s at 56°C, and 1 min at 72°C, followed by 35 cycles of 20s at 95°C, 35s at 52°C, and 1 min at 72°C. Final extension was 10 min at 72°C. PCR products were visualized in a 1.5% agarose gel under UV light.

Real-Time Quantitative PCR (qPCR). qPCR and subsequent data analysis were performed using Swift Spectrum 48 Thermal Cycler (Esco Micro Pte. Ltd, Singapore). The qPCR reactions were performed in total volume of 25 µl: cDNA (150 ng), 12.5 µl absolute qPCR Syber Green master mix (Thermo fisher Scientific Inc, Waltham, MA, USA) and 10 pm of each of forward and reverse primers. The reaction conditions were as follows: 95°C for 15 min, followed by 44 cycles at 95°C for 15s and 51-56°C (depending on the annealing temperature of the primer) for 40s and 72°C for 30s. Fluorescence was measured during the 72°C step for each cycle. All data were calculated by comparative Ct method (13,14). To normalize the amount of sample cDNA, one endogenous control transcripts of 'housekeeping gene' coding for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used.

Statistical Analysis. Statistical significance for differences in gene expression between different groups was determined by One-Way variance analysis method (ANOVA). Also differences in the level of expression between individual genes in each group were determined by Tukey HSD and Duncan statistical method.

Table 1. Product sizes and nucleotide sequence details of primers used for TD-PCR and qPCR analysis of mRNA expression of human TLR2, TLR4, CD14, MyD88 and GAPDH genes.

Gene	Primer	Sequence (5'-3')	Tm	Amplicon Size (bp)	Reference
TLR2	Forward	ATCCTCCAATCAGGCTTCTCT	60.0	163	11
	Reverse	ACACCTCTGTAGGTCAGTGTG	61.3		
TLR4	Forward	ATATTGACAGGAAACCCCATCCA	61.1	300	11
	Reverse	AGAGAGATTGAGTAGGGGCATTT	60.6		
CD14	Forward	ACTTGCACTTTCCAGCTTGC	61.4	202	11
	Reverse	GCCCAGTCCAGGATTGTCAG	62.2		
MyD88	Forward	GACCCCTGGTGCAAGTACC	62.0	197	11
	Reverse	AGTAGCTTACAACGCATGACAG	60.4		
GAPDH	Forward	GAGCCACATCGCTCAGACAC	55.2	150	11
	Reverse	CATGTAGTTGAGGTCAATGAAGG	52.7		

RESULTS

Detection of PRRs Transcripts in PBMC and U87 MG Cell Line by TD-PCR. We used TD-PCR to detect the expression of several innate immune receptors genes in PBMC of healthy human individuals as well as U87 MG cell line. TLR2, TLR4, CD14 and MyD88 mRNA expression was detected in PBMC and U87 MG cell line (Figure 1). To assess if human glioblastoma cells themselves express TLR2, TLR4, MyD88 and CD14 mRNA, we used PBMC as a positive control. By performing TD-PCR experiments it appeared that these innate immune receptor genes are expressed in U87 MG cell line.

Quantification of Expression Level of PRRs Transcripts in U87 MG Cell Line. To quantify the expression level of TLR2, TLR4, MyD88 and CD14 transcripts in U87 MG cell line, we used real-time quantitative PCR (qPCR). According to the results obtained by qPCR, the level of TLR2, TLR4 and MyD88 transcripts were significantly higher than the expression level of CD14 transcripts (Table 2 and Figure 2). No significant difference was observed between the level of TLR2, TLR4 and MyD88 transcripts. Furthermore, a significant down-regulation of CD14 transcript as compared to the other genes was observed (Figure 2 and Table 2).

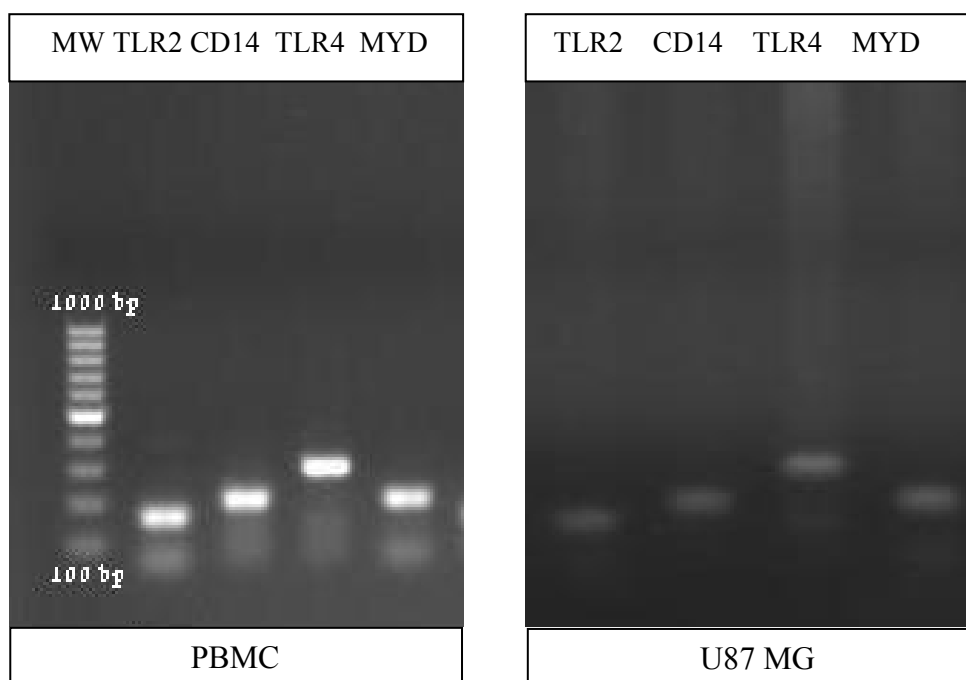


Figure 1. Touchdown PCR (TD-PCR) analysis of TLR2, TLR4, MyD88 and CD14 expression in human PBMC and U87 MG cell line.

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Down-Regulation of CD14 in U87 MG Cell Line. To quantify the expression level of CD14 in U87 MG cell line, we used qPCR. The level of CD14 expression in U87 MG cell line was significantly lower than the expression level of TLR2, TLR4 and MyD88 in U87 MG cell line and PBMC (Figure 2). A 7.580 fold decrease in CD14 mRNA expression level was observed in U87 cell line as compared to PBMC (Table 2). A slight up-regulation of TLR2 was observed in U87 cell line as compared to PBMC which was not significant. The level of expression of TLR4 and MyD88 was not significantly different between U87 MG cell line and PBMC.

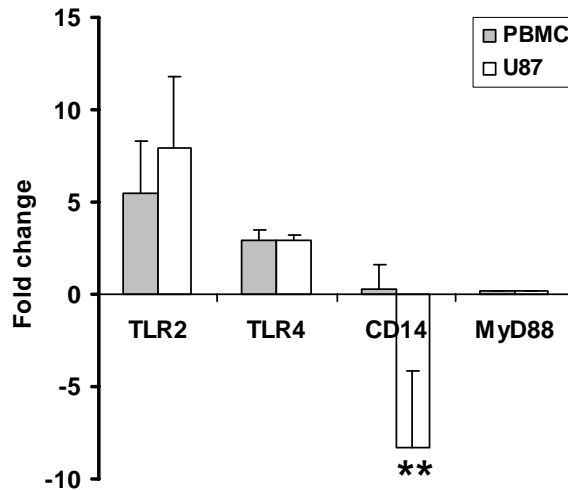


Figure 2. Comparative gene expression analysis of TLR2, TLR4, MyD88 and CD14 in PBMC of human healthy individuals and U87 MG cell line: A highly significant decrease of CD14 expression in U87 cell line is observed (**p<0.01). Fold change was normalized to GAPDH expression level.

Table 2. Real-time quantitative PCR (qPCR) analysis of TLR2, TLR4, CD14 and MyD88 expression in peripheral blood mononuclear cells (PBMC) of healthy individuals and U87 MG cell line: TLR2 is highly expressed in U87 MG cell line as compared to the other genes. CD14 expression in U87 MG cell line is significantly lower than the expression level of other genes in U87 MG cell line and PBMC (p<0.01) Fold change was normalized to GAPDH expression level. Data are presented as means ± S.D.

Gene Cell line	TLR2 (mean ± S D)	TLR4 (mean ± S D)	CD14 (mean ± S D)	MyD88 (mean ± S D)
PBMC	5.486 ± 2.805	2.901 ± 0.564	0.279 ± 1.360	0.205 ± 0.007
U87	7.962 ± 3.860	2.929 ± 0.276	-8.322 ± 4.187	0.210 ± 0.012
U87/PBMC	1.461 ± 0.051	1.053 ± 0.332	-7.580 ± 4.128	1.020 ± 0.141

DISCUSSION

During the past few years it has become evident that the innate immune system and in particular, PRRs such as TLRs play an important role in infectious and non-infectious CNS diseases (15). Recent studies have demonstrated the presence of mRNA and/or protein expression of TLRs and the co-receptor CD14 in microglia and to some extent in astrocytes and such expressions are increased following exposure to bacterial pathogens (16).

The up-regulation of TLRs in the CNS tissues is likely in part due to the infiltration of TLR-expressing inflammatory cells, and in part due to the up-regulation of the receptor

expression on astrocytes and microglia, which happens in response to a variety of inflammatory stimuli (17). In the present study, we demonstrated that several PRRs including TLR2, TLR4, MyD88 and CD14 are expressed in human glioblastoma cell line U87 MG. This finding is perhaps not surprising considering that glioblastoma might share the same myeloid lineage as macrophages and dendritic cells. Although a similar expression of TLR4 transcript in glioblastoma cells and PBMC of healthy individuals was observed, the comparison of gene expression level in glioblastoma cells and PBMC of healthy individuals revealed a highly significant down-regulation of CD14 and a slight up-regulation of TLR2 transcripts in U87 MG cell line.

CD14 is a co-receptor (along with TLR4 and MD-2) for LPS which exists in two forms of membrane bound with GPI anchor or in the form of secreted molecules without GPI anchor (3). It is expressed mainly by dendritic cells and macrophages of different organs (including CNS micoglia). Modulation of immune response by LPS and the role of CD14 as a bacterial pattern recognition receptor are important in bacterial infections.

Expression and release of CD14 by astrocytic brain tumors and several human glioma cell lines have already been reported (18). In the study by Deininger et al., CD14 mRNA was detected in all glioma cell lines analyzed, but CD14 expression and release was observed in four out of six analyzed glioma cell lines (18). In U87 MG cell line however, it was demonstrated that CD14 presents in the form of mRNA and not protein. They concluded that expression and release of CD14 from a subset of human glioma cells and infiltrating macrophages/microglial cells in CNS may contribute to immunodepletion observed in these patients. Although we did not perform western blotting in order to detect CD14 protein in the present study, our results regarding the presence of CD14 mRNA in U87 MG cell line is consistent with these data.

The significant decrease in CD14 mRNA expression observed in our study could be an *in vitro* effect of cell culture and does not correspond to an *in vivo* function. Camphausen et al. have shown the differential gene expression profile of two human glioma cell lines (U251 and U87) by microarray analysis when grown *in vitro* and *in vivo* as subcutaneous (s.c.) or as intracerebral (i.c.) xenografts (19). For each cell line, the gene expression profile generated from tissue culture was significantly different from that generated from the s.c. tumor, which was also significantly different from those grown i.c. Therefore *in vitro* and *in vivo* growth environment and conditions modulate gene expression. Our results might also be affected by these finding as blood samples of healthy individuals used in our study were subjected to RNA isolation and cDNA synthesis immediately after venipuncture. If we can assume that this method corresponds to an *in vivo* origin of PBMC, therefore we have compared an *in vitro* cultured U87 MG cell line with an *in vivo* obtained PBMC which are primary cells. However, as we did not compare the glioblastoma cells from *in vivo* and *in vitro* origins, it is remained to be seen if the lower expression of CD14 mRNA in U87 MG cell line is a result of cell culture growth and whether this is related to membrane bound or secreted form of CD14.

TLR4 acts as co-receptor together with CD14 in sensing LPS of gram negative bacterial infections. Although we cannot conclude on the differential role of TLR4 and CD14 in peripheral immunity and central nervous system, our results on similar expression level of TLR4 in PBMC and U87 MG cells and highly significant down-regulation of CD14 might have an indication on the functional differences of CD14 in human glioblastoma cell line.

TLR2 is a transmembrane PRR which recognizes various components from bacteria, mycoplasma, fungi, and viruses. These ligands include the lipoproteins of bacteria and mycoplasma. A heterodimer with either TLR1 or TLR6 will be formed upon recognizing a ligand by TLR2. The resulting TLR1/TLR2 and TLR6/TLR2 complexes recognize discrete ligands (triacyl and diacyl lipoproteins, respectively) (2). Another important role for TLR2 is the recognition of the gram-positive CNS pathogens such as *Streptococcus pneumoniae* and *Staphylococcus aureus* (16). Although these studies revealed a role for TLR2 in CNS antibacterial immune responses, collectively they suggest the involvement of another, as yet unidentified receptor in bacterial recognition and elimination in the CNS (16). Another important feature of TLR2 is its ability to cooperate with CD14 and myeloid differentiation protein (MD)-2 on the host cell surface. TLR1/2 and TLR2/6 heterodimers cooperate with CD14 to recognize Gram-positive bacterial PAMPs (4). Therefore the up-regulation of TLR2 observed in our study might act as a compensation mechanism for CD14 down-regulation.

Taken together the results presented in this study show that several PRRs of the innate immunity are expressed in human glioblastoma cell line U87 MG. The lower expression level of CD14 in these cells in comparison to PBMC needs to be confirmed in further studies and might have a potential implication for CD14 mediated cerebral pathology. Further in vitro and in vivo experiments to clarify in more detail the mechanisms behind the immunobiological roles of PRRs in cerebral versus peripheral immunity will help us to better understand the innate immune response in cerebral tissue and to develop specific strategies to stimulate these innate immune cells in a selective manner towards neuroprotective functions.

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