

Quantification of Expression of Pattern Recognition Receptors (PRRs) in Human Peripheral Blood Mononuclear Cells (PBMC) by Real-Time Quantitative PCR

¹Maryam Heidari Kharaji and ^{1,2}Alireza Haghparast

¹Department of Pathobiology, Faculty of Veterinary Medicine,
Immunology and Biotechnology Sections, Iran

²Institute of Biotechnology, Ferdowsi University of Mashhad, Iran

Abstract: Pattern recognition receptors (PRRs) are the main sensors of pathogen and danger signals in innate immunity. They are mainly expressed by macrophages and dendritic cells of different organs. Toll like receptors (TLRs) are the most studied and best characterized PPRs which are responsible for sensing pathogen associated molecular patterns (PAMP). The contribution of PRRs in inflammation induced by microbial infection, tissue damage and cancer are a major topic in innate as well as adaptive immunity. In the present study, peripheral blood mononuclear cells (PBMC) of healthy individuals were used to investigate the expression level of TLR2, TLR4, MyD88 and CD14 transcript in these cells. Real-time quantitative PCR was applied to quantify the expression level of these genes in PBMC of human healthy individuals. According to our results, TLR2, TLR4, MyD88 and CD14 transcripts are expressed in PBMC of healthy individuals but the level of expression of TLR2 and TLR4 are significantly higher than the expression level of MyD88 and CD14 transcript. Detection of expression level of PPRs in PBMC might have a potential implication for diagnosis of inflammatory and immune mediated disorders.

Key words: Human peripheral blood mononuclear cells % Pattern recognition receptors % Toll like receptors
% Real-time quantitative PCR

INTRODUCTION

The innate immunity is the major arm of immune system and the main contributor to acute inflammation induced by microbial infection or tissue damage. Furthermore, innate immunity is also important for the activation of acquired immunity. Innate immune cells including macrophages and dendritic cells (DCs) and also non-professional cells such as epithelial cells, endothelial cells and fibroblasts through their germ line-encoded pattern recognition receptors (PRRs) are responsible for sensing the presence of microorganisms [1, 2]. They do this by recognizing structures conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs). Recent evidence indicates that PRRs are also responsible for recognizing endogenous molecules released from damaged cells, termed damage associated molecular patterns (DAMPs) [3]. Currently, four different classes of PRR families have been identified. These families include transmembrane proteins such as

Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). These PRRs are expressed not only in macrophages and DCs but also in various non-professional immune cells. With the exception of some NLRs, the sensing of PAMPs or DAMPs by PRRs up-regulates the transcription of genes involved in inflammatory responses. These genes encode pro-inflammatory cytokines, type I interferons (IFNs), chemokines and antimicrobial proteins, proteins involved in the modulation of PRR signaling and many uncharacterized proteins. The expression patterns of the inducible genes differ among activated PRRs [1-4].

The TLR family is one of the best-characterized PRR families and is responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes. TLR stimulation initiates 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) [1-6].

TLRs sense broad spectrum of PAMPs. TLR2 is a plasma membrane bound PRR which senses various components from bacteria, mycoplasma, fungi and viruses. These components include the lipoproteins of bacteria and mycoplasma. TLR2 recognizes its ligands by forming a heterodimer with either TLR1 or TLR6. The resulting TLR1/TLR2 and TLR6/TLR2 complexes recognize distinct ligands (triacyl and diacyl lipoproteins, respectively) [1-6]. TLR2 has been shown to play an important role in the host immune response to Gram-positive bacterial infections in the periphery and also central nervous system (CNS) [7, 8].

TLR4 is a transmembrane protein specialized in the recognition of the bacterial endotoxin, lipopolysaccharide (LPS) [1]. It has been well accepted that recognition of bacterial LPS by TLR4 is greatly amplified by an accessory protein, CD14, which is also a pathogen recognition molecule. CD14, serving as a carrier linking LPS to cell surface TLR4 [9], is expressed by mature myeloid cells and also exists in a secreted form in the circulation [10]. Two forms of the CD14 receptor can be found. The first one is present at the surface of myeloid cells (mCD14) and acts as a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein. The other form is soluble in the serum (sCD14) and lacks the GPI properties, although it can bind LPS to activate cells devoid of mCD14, such as endothelial, epithelial cells and vascular smooth muscle cells [11]. Recently, there is increasing evidence for a role of these intensively studied TLRs and CD14 also in molecular pathogenesis of several diseases [12-15].

Assessing PRRs expression within cells is important to increase the rationale for biological functions, diagnostic as well as potential treatment approaches. These might allow identification of a subset of target molecules that could be harnessed for biological or therapeutic approaches. It is well recognized that primary cells or cell line models of human disorders, are important resources for finding new therapeutic as well as biological mechanisms underlying disease development. A reverse transcription-polymerase chain reaction (RT-PCR) approach using total RNA isolated from cells allows the analysis of TLR expression at the transcriptional level. RT-PCR analysis using TLR-specific oligonucleotides allows for the specific amplification of TLRs [16]. Real-time quantitative PCR can quantify furthermore the expression patterns of PRRs and their signaling molecules.

In this study, human peripheral blood mononuclear cells (PBMC) were chosen as a model to detect and quantify the expression of PRRs transcripts in these cells by real-time quantitative PCR. In our study we developed an assay to detect and quantify several PRRs transcripts including TLR2, TLR4, CD14 and adaptor molecule MyD88 as signaling molecules of most TLRs.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells (PBMC) Isolation:

Heparinized venous blood samples from human healthy individuals were used for isolation of peripheral blood mononuclear cells (PBMC) 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), RNA was quantified using NanoDrop 3300 (Thermo scientific, Wilmington, DE, USA). Total 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 5 min, 42°C for 1 h and 70°C 10 min).

Quantitative real-time RT-PCR (qPCR): Gene specific primers for human TLR2, TLR4, CD14, MyD88 and GAPDH genes were selected based on the information described in Table 1. qPCR and subsequent data analysis were performed using Swift Spectrum 48 Thermal Cycler (Esco Micro Pte. Ltd, Singapore). The qPCR reactions were performed in 25 μ l of cDNA (148 ng), 12.5 μ l 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 [19, 20]. To normalize the amount of sample cDNA, one endogenous control transcripts of 'housekeeping gene' coding for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used.

Table 1: Product sizes and nucleotide sequence details of primers used for Real time quantitative PCR 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 | 17 |
| | Reverse | ACACCTCTGTAGGTCAGTGTG | 61.3 | | |
| TLR4 | Forward | ATATTGACAGGAAACCCCATCCA | 61.1 | 300 | 17 |
| | Reverse | AGAGAGATTGAGTAGGGGCATTT | 60.6 | | |
| CD14 | Forward | ACTTGCACTTTCCAGCTTGC | 61.4 | 202 | 17 |
| | Reverse | GCCCAGTCCAGGATTGTCAG | 62.2 | | |
| MyD88 | Forward | GACCCCTGGTGCAAGTACC | 62 | 197 | 17 |
| | Reverse | AGTAGCTTACAACGCATGACAG | 60.4 | | |
| GAPDH | Forward | GAGCCACATCGCTCAGACAC | 55.2 | 150 | 18 |
| | Reverse | CATGTAGTTGAGGTCAATGAAGG | 52.7 | | |

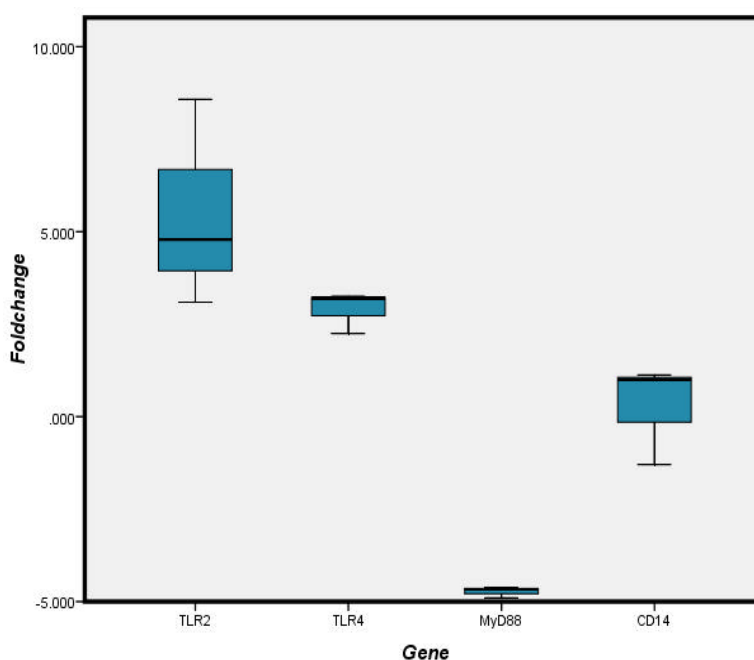


Fig. 1: Quantitative real-time PCR (qPCR) analysis of TLR2, TLR4, MyD88 and CD14 expression in peripheral blood mononuclear cells (PBMC) of human healthy individuals. TLR2 and TLR4 expression are significantly higher than MyD88 and CD14. CD14 expression is significantly higher than the expression level of MyD88. No significant differences observed between the level of expression of TLR4 and TLR2 transcripts. Fold change was normalized to GAPDH expression levels. Data are presented as means \pm S.D.

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.

RESULTS

Analysis of expression level of PRRs transcripts in PBMC by real-time quantitative RT-PCR: To quantify

the expression level of TLR2, TLR4, CD14 and MyD88 transcripts in PBMC of healthy human donors, we used real-time quantitative RT-PCR. According to the results obtained by real-time quantitative RT-PCR, the level of TLR2 and TLR4 transcripts were significantly higher than the expression level of CD14 and MyD88 transcripts (Figure 1). No significant difference was observed between the level of TLR2 and TLR4 transcripts ($P < 0.01$). Furthermore, a significant down-regulation of MyD88 transcript as compared to the other genes was observed (Figure 1).

DISCUSSION

In the past few years, it has become evident that the innate immune system and in particular, PRRs play a major role in infectious but also in non-infectious diseases [6]. Recent studies have demonstrated the presence of mRNA and/or protein expression of TLRs and the co-receptor CD14 in various immune as well as non-immune cells and such expression are increased following inflammations and exposure to pathogens [13, 15].

The up or down-regulation of TLRs in the periphery is likely in part due to the infiltration or diminishing of TLR-expressing inflammatory cells and in part due to the up or down-regulation of receptor expression on these cells which occurs in response to a variety of stimuli. In the present study, we measured, quantified and compared the expression of several PRRs including TLR2, TLR4, CD14 and adaptor protein MyD88 in PBMC of healthy individuals by real-time quantitative PCR. Our findings revealed that these genes are expressed in various quantities in PBMC of healthy human individuals. By using an endogenous house keeping gene as internal control to normalize the amount of cDNA we were able to quantify and compare the level of gene expression of TLR2, TLR4, CD14 and adaptor protein MyD88 by real-time quantitative PCR. This finding is perhaps not surprising considering that PBMC is composed of B and T lymphocytes and monocytes which share the same PRRs expression patterns as other immune cells present in secondary lymphoid organs. Although a somewhat similar expression level of TLR2 and TLR4 transcripts were observed in our study, their expression levels were significantly higher than CD14 and MyD88. The lowest PRRs transcript level in our study was adaptor protein MyD88.

Toll-like receptors (TLRs) are innate immune receptors critical in the innate immune defense against invading pathogens. Recent advances also reveal a crucial role for TLRs in shaping adaptive immune responses, conferring a potential therapeutic value to their modulation in the treatment of diseases. Extensive analysis of TLRs, however, has revealed specificity in terms of ligand recognition, expression in different cell types and tissues and, importantly, a role for TLRs in the pathogenesis of multiple diseases involving both the innate and adaptive immune systems. Therefore, comparison of PRRs gene expression level in human PBMC in various circumstances of health and disease gives more insight on the immunoregulatory as well

pathophysiologic mechanisms underlying disease development and potential therapeutic interventions. There is also a growing body of evidence to indicate that certain TLRs also sense products of damaged tissue. Both pathogen-derived factors and also damaged tissue will provoke inflammation; it has therefore been hypothesized that TLRs initiate the inflammatory response in both cases. Also of interest are the different signaling pathways activated by TLRs. Different adapter proteins are recruited in different combinations to different TLRs, allowing for tailored responses to each pathogen [21]. Among these, adaptor protein MyD88 is recruited by all TLRs except TLR3, therefore its role is crucial in TLR signaling pathways [5]. Once activated by their respective ligands, TLRs recruit their specific repertoire of the TIR adapters like MyD88 resulting in the recruitment and activation of downstream protein kinases which eventually leads to the activation of NF- κ B pathway and secretion of pro inflammatory cytokines [21].

A remarkable feature of TLR2 and TLR4 is its ability to cooperate with CD14 and myeloid differentiation protein (MD)-2 on the host cell surface in sensing LPS of gram negative bacterial infection [9]. Modulation of immune response by LPS and the role of CD14 as a bacterial pattern recognition receptor are important in bacterial infections. Although we can not speculate on the differential role of TLR4 and CD14 in peripheral immunity, our results on higher expression level of TLR2 and TLR4 in PBMC as compared to CD14 might have an indication on the functional association of these molecules in innate immunity.

Although the roles of TLRs in human disease are still not fully understood, there are significant *in vitro* and animal model data to support roles for particular TLRs in disease initiation and progression. There is a growing interest in the targeting of Toll-like receptors (TLRs) for the prevention and treatment of cancer, rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus (SLE) and inflammatory diseases [21, 22]. Taken together the results presented in this study show that detection and quantitation of PRRs in PBMC by real-time quantitative PCR is feasible, reliable and easy to perform. The data obtained by this method could help to further experiment on revealing the requirement for developing TLR modulation strategies and the significant role they play in pathophysiology of diseases. Understanding of TLR involvement in such processes may enable the invention of novel TLR-based therapies.

ACKNOWLEDGEMENTS

This work was supported by a grant from Ferdowsi University of Mashhad.

REFERENCES

1. Takeuchi, O. and S. Akira, 2010. Pattern recognition receptors and inflammation, *Cell*, 140: 805-20.
2. Kumagai, Y. and S. Akira, 2010. Identification and functions of pattern-recognition receptors. *J. Allergy Clin. Immunol.*, 125: 985-92.
3. Kawai, T. And S. Akira, 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.*, 11: 373-84.
4. Kumar, H., T. Kawai and S. Akira, 2009. Toll-like receptors and innate immunity. *Biochem. Biophys. Res. Commun.*, 388: 621-5.
5. Akira, S., 2006. TLR signaling. *Curr. Top. Microbiol. Immunol.*, 311: 1-16.
6. Kaisho, T. and S. Akira, 2006. Toll-like receptor function and signaling, *J. Allergy Clin. Immunol.*, 117: 979-87.
7. Konat, G.W., T. Kielian and I. Marriott, 2006. The role of Toll-like receptors in CNS response to microbial challenge. *J. Neurochem.*, 99: 1-12.
8. Kielian, T., 2006. Toll-like receptors in central nervous system glial inflammation and homeostasis. *J. Neurosci. Res.*, 83: 711-30.
9. Miyake, K., 2004. Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol.*, 12: 186-92.
10. Cao, L., F.Y. Tanga and J.A. Deleo, 2009. The contributing role of CD14 in toll-like receptor 4 dependent neuropathic pain. *Neurosci.*, 158: 896-903.
11. Nadeau, S. And S. Rivest, 2000. Role of Microglial-Derived Tumor Necrosis Factor in Mediating CD14 Transcription and Nuclear Factor B Activity in the Brain during Endotoxemia. *J. Neurosci.*, 20: 3456-68.
12. Chen, Z., Y. Cheng, Y. Xu, J. Liao, X. Zhang, Hu Y, *et al.*, 2008. Expression profiles and function of Toll-like receptors 2 and 4 in peripheral blood mononuclear cells of chronic hepatitis B patients. *Clin. Immunol.*, 128: 400-8.
13. Komatsuda, A., H. Wakui, K. Iwamoto, M. Ozawa, M. Togashi, R. Masai, *et al.*, 2008. Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Clin. Exp. Immunol.*, 152: 482-7.
14. Webb, R.N., J.M. Cruse and R.E. Lewis, 2009. Decreased TLR4 gene expression in leukemic leukocyte populations. *Exp. Mol. Pathol.*, 87: 117-26.
15. Salomao, R., M.K. Brunialti, N.E. Gomes, M.E. Mendes, R.S. Diaz, S. Komninakis, *et al.*, 2009. Toll-like receptor pathway signaling is differently regulated in neutrophils and peripheral mononuclear cells of patients with sepsis, severe sepsis and septic shock. *Crit. Care Med.*, 37: 132-9.
16. Siednienko, J. And S.M. Miggin, 2009. Expression analysis of the Toll-like receptors in human peripheral blood mononuclear cells. *Methods Mol. Biol.*, 517: 3-14.
17. Spandidos, A., X. Wang, H. Wang and B. Seed, 2010. Primer Bank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Research*, 38: 792-9.
18. Pombo-Suarez, M., M. Calaza, J.J. Gomez-Reino and A. Gonzalez, 2008. Reference genes for normalization of gene expression studies in human osteoarthritic articular cartilage. *BMC. Mol. Biol.*, 29: 9-17.
19. Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25: 402-8.
20. Schmittgen, T.D. and K.J. Livak, 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*, 3: 1101-8.
21. O'Neill, L.A., C.E. Bryant and S.L. Doyle, 2009. Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer. *Pharmacol. Rev.*, 61: 177-97.
22. Hennessy, E.J., A.E. Parker and L.A. O'Neill, 2010. Targeting Toll-like receptors: emerging therapeutics? *Nat. Rev. Drug. Discov.*, 9: 293-307.