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Simultaneous Detection of Pattern Recognition Receptors (PRRs) in Human Peripheral Blood Mononuclear Cells (PBMC) by Touchdown PCR

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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 of TLR2, TLR4, MyD88 and CD14 transcript in these cells. Touchdown PCR (TD-PCR) was applied to simultaneously detect the expression of these genes in PBMC of healthy human individuals. According to our results, it is possible to detect TLR2, TLR4, MyD88 and CD14 transcripts in PBMC of healthy individuals by TD-PCR. Detection of expression of PPRs in PBMC by this technique is simple and straightforward and has a potential application in diagnosis of inflammatory and immune mediated disorders.

Key words: Human peripheral blood mononuclear cells · Pattern recognition receptors · Toll like receptors · Touchdown 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

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signal transduction pathway via adaptor protein MyD88 which leads to the secretion of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α through activation of nuclear factor κB (NF-κB) [1-6].

TLRs sense a broad spectrum of PAPMs. 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 PPRs 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].

Touchdown PCR (TD-PCR) is another modification of conventional PCR that may result in a reduction of nonspecific amplification. It involves the use of an annealing temperature that is higher than the target optimum in early PCR cycles. The annealing temperature is decreased by 1°C every cycle or every second cycle until a specified or 'touchdown' annealing temperature is reached. The touchdown temperature is then used for the remaining number of cycles. This allows for the enrichment of the correct product over any non-specific product [18].

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

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells (PBMC) Isolation: Heparinized venous blood samples from 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 for 5 min, 42°C for 1 h and 70°C for 10 min).

Reverse Transcription TD-PCR (RT-TD-PCR): Gene specific primers for human TLR2, TLR4, CD14 and MyD88 genes were selected based on the information described in Table 1. The polymerase chain reaction (PCR) mix included 2.5µl of 10x PCR buffer, 50 mM $MgCl_2$, 10 pm of each of forward and reverse primer, 250 mM dNTPs,

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

Sgene	Primer	Sequence (5' - 3')	Tm	Amplicon Size (bp)	Reference
TLR2	Forward	ATCCTCCAATCAGGCTTCTCT	60.0	163	17
	Reverse	ACACCTCTGTAGGTCACTGTTG	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	62.0	197	17
	Reverse	AGTAGCTTACAACGCATGACAG	60.4		

0.4 unit *Taq* polymerase (Fermentas, Lituania) and 200 ng cDNA. Total volume of PCR reaction mixture was adjusted to 25 µl with sterile H_2O. TD-PCRs were run under the following thermal cycle conditions: 3 min at 94°C followed by 4 cycles of 20s 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.

RESULTS

Detection of Expression of PRRs mRNA in PBMC by TD-PCR: We used TD-PCR to detect the expression of several innate immune receptors genes in PBMC of healthy human individuals. TLR2, TLR4, CD14 and MyD88 mRNA expression was detected in PBMC (Figure 1). By performing TD-PCR experiment it was appeared that it is possible to simultaneously detect these innate immune receptor genes in human PBMC.

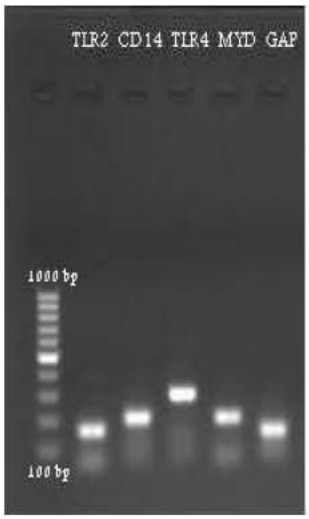

Fig. 1: TLR2, CD14, TLR4 and MyD88 expression detection in human PBMC by TD-PCR

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 demonstrated the expression of several PRRs including TLR2, TLR4, CD14 and adaptor protein MyD88 in PBMC of healthy individuals by TD-PCR. Our findings revealed that these genes are expressed in PBMC of healthy human individuals. This finding is perhaps not surprising considering that PBMC is composed of B and T lymphocytes and monocytes which share the same PPRs expression patterns as other immune cells present in secondary lymphoid organs. But in our study we offered a simple and rapid means to simultaneously detect PPRs by TD-PCR with increased specificity, sensitivity and yield, without the need for lengthy optimizations and/or the redesigning of primers. The only inherent limitation of TD-PCR is that it is not suitable for quantitative assessment of target concentration, which is ideally performed by real-time PCR approaches under optimized condition [23].

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, detection of PRRs gene expression properties in human PBMC in various circumstances of health and disease gives more insight on the immuoregulatory 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-κB pathway and secretion of pro inflammatory cytokines [21].

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 [21, 22]. Taken together the results presented in this study show that detection of PRRs in PBMC by TD-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 diagnosis strategies and the significant role they play in pathophysiology of diseases.

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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. Neuroscience, 158: 896-903.
11. Nadeau, S. and S. Rivest, 2000. Role of Microglial-Derived Tumor Necrosis Factor in Mediating CD14 Transcription and Nuclear Factor k B Activity in the Brain during Endotoxemia. J. Neurosci, 20: 3456-68.
12. Chen, Z., Y. Cheng, Y. Xu, J. Liao, X. Zhang, Y. Hu, *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 Res., 38: 792-9.

18. Roux, K.H., 2009. Optimization and troubleshooting in PCR. Cold Spring Harb Protoc., pp: 4.
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.
23. Korbie, D.J. and J.S. Mattick, 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nat Protoc., 3(9): 1452-6.