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Original Research Article

An Investigation of Innate Immune Response of Human Blood Macrophage to Sense and Antisense dsRNA

Nasrin Mohajeri[a], Zarrin Sharifnia[b], Mojgan Bandehpour[b], Fatemeh Yarian[b], Amaneh Koochaki[c], Nariman Mosaffa[d], Kazem Parivar[a] and Bahram Kazemi[b & c*]

[a] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.*
[b] *Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
[c] *Department of Biotechnology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
[d] *Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

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HIGHLIGHTS

- siRNA (small interfering RNA) is powerful approach to study the functional roles of mammalian genes.
- dsRNA induced antiviral response by induction of different cytokines including TNF-α, IL-12 and IL-8.
- dsRNA showed promising results as a vaccine adjuvant for both antiviral and antitumor prophylaxis.
- The strong response of IL-8 chemokine indicated the linkage between innate immunity and adaptive immunity in progressive malignances.

ABSTRACT

Silencing of gene expression by siRNA (small interfering RNA) is a powerful approach used to study the genetic analysis and functional roles of mammalian genes. There is at present no report about the effects of mammalian two-hybrid system plasmids delivery of sense and antisense strands. The leishmania pteridine reductase 1 (PTR1) gene was cloned as sense and antisense strands into mammalian two hybrid system plasmids. The constructs were transfected into human blood macrophages on the basis of eight experimental groups. (Antisense strand ± LPS, sense strand ± LPS, dsRNA ± LPS, negative control ± LPS). After 24 hours, cytokines production was assessed with ELISA. Transfection of sense and antisense strand RNA into monocyte-derived macrophages (MDM) was confirmed by RT-PCR. Single strands RNA expressed IL-8, IL-12, IL-1β inflammatory cytokines and dsRNA induced IL-8, IL-12 and TNF-α production in MDM. In contrast, random uptake from a mixture of two plasmids was downregulated IL-8, IL-12, IFN-γ cytokines, with a significant difference of $p<0.05$ in macrophage.With respect to the increased level of IL-8 in macrophage detected in single strand groups, the chemokine production—as a major feature of innate immunity—is a powerful tool for evaluation of sense/antisense in experimental and therapeutic gene vaccine delivery. siRNA–based gene therapy could have great potential in cancer treatment.

Introduction

RNA interference (RNAi) was first discovered in transgenic plants (Ecker and Davis, 1986) and has rapidly become the fastest expanding field in molecular biology. The enzyme dicer, which cleaves long dsRNA molecules

* *Corresponding Author*:
Email: kazemi@sbmu.ac.ir (B. Kazemi).

into siRNA fragments of 21–23 nucleotides (Sui et al., 2002; Khaliq et al., 2010), initiates RNAi pathway.

It was believed that multiple dsRNAs uptake inducts several sources into the cell and uses different methods to trigger RNAi pathway, such as transposon element, RNA sequences of virus (Agrawal et al., 2003), inverted repeat transgene (Skårn et al, 2006), synthetic siRNA and dsRNA transfection (Judge et al., 2005), secondary siRNA and transitive RNAi (Nishikura, 2001). The presence of dsRNA longer than 29–30 bp—intracellular or extracellular—can induce innate inflammatory cytokines in mammalian cells. The long dsRNAs bind to cytoplasmic sensors and a chain of events activates the promotion of inhibition of protein synthesis, transcriptional induction of interferon and other cytokines, and finally, apoptosis (Leung and Whittaker, 2005; Gantier and Williams, 2007). Researchers suggested that dsRNA induces antiviral response and is involved in stimulating protective responses, leading to the activation of dicer-related antiviral pathway and induction of different cytokines, including TNF-α, IL-12 and IL-8 (Robbins et al., 2009; Karpala et al., 2005).

In the present study, vector-based dsRNAs were used to evaluate IL-8, IL-12, IL-1β, TNF-α and IFN-γ cytokine expression in monocyte cells based on sense/antisense transfect,

Materials and Methods

Leishmania major cultivation

L. major was cultivated in $RPMI_{1640}$ (Roswell Park Memorial Institute) medium, supplemented with 10% foetal bovine serum (Mirzaahmadi et al., 2011) (Gibco, UK), 100 IU/ml penicillin (Gibco, UK), 100 µg/ml streptomycin (Gibco, UK) and, at 25ºC, 15 µg/ml haemin (Sigma, USA).

Cloning and identifying sense and antisense vectors

PTR1 gene amplified with a set of primers (Gene Bank Accession No. L01699). Forward 5′- GGA TCC ATG ACT GCT CCG ACC -3' and reverse 5′- GGT ACC TCA GGC CCG GGT AAG -3'. Gene amplification performed by *pfu* DNA polymerase enzyme (Fermentas, Lithuania) (Kherandish et al., 2013). The PCR product ligated into pACT and pBIND mammalian two-hybrid system plasmids in EcoRI site. Then recombinant pBIND/PTR1 and pACT /PTR1 constructs were analysed by PCR.

The PCR program consisted of an initial denaturation at 94ºC for 5 min. Thirty cycles were carried out, comprising the following steps: 94ºC for 30s, 60ºC for 40s, followed by 72ºC for 1 min as a final extension. The sequence of universal primers was as follows: pACT Forward: 5′- GGTCCGGGATCG CAG -3′ and pACT Reverse: 5′-GCGGCCTTAGTTATTCA -3′, pBIND Forward: 5′- GTATCGCCGGAA TTCCCGG-3′ and pBIND Reverse: 5′- GAAGCGGTTAGTTATTC-3′. The following constructs included PTR1 sense strand into pACT and PTR1 antisense strand into pBIND (Sambrook and Rossell, 2001).

Isolation and culture of primary macrophage derived monocyte (MDM)

The samples were obtained from healthy persons from the Cellular and Molecular Biology Research Center. The peripheral blood mononuclear cells (PBMCs) collected, using heparin sodium 5000 IU/ml (Aburaihan, Iran) as anti-coagulant, was diluted with an equal volume of $RPMI_{1640}$, layered onto a cushion of Ficoll-Hypaque (1.074 g/ml) (Baharafshan, Iran) and centrifuged at 18ºC for 20 min at 2000 rpm. The mononuclear cells layer separated by centrifugation as a white mass at the interface between the $RPMI_{1640}$ and Ficoll-Hypaque. The cells, washed once with $RPMI_{1640}$ and counted on a hemacytometer, were then seeded at $1x10^3$ cells per well. The mononuclear cells were incubated in the 24-well plates at 37ºC in 5% CO_2 in moist air in $RPMI_{1640}$ medium supplemented, 10% foetal bovine serum (Gibco, UK), 100IU/ml penicillin (Gibco, UK), 100µg/ml streptomycin (Gibco, UK). After 6 h in culture, the non-adherent cells were removed by washing with warm medium. A night later, the MDM was used for cell transfection.

Cell transfection and RT-PCR

Before constructs transfected to MDM and MΦ cells (CalPhos Mammalian Transfection kit, Japan), the subjects were evaluated by Limulus Amebocyte Lysate (LAL) test (Morris et al., 2010) (Cambr, USA).

After the incubation period (24 hours), the cells were prepared for RNA extraction by the RNeasy Mini Isolation kit (Qiagen, USA), and cDNA synthesis was carried out using MMLV reverse transcriptase enzyme (Fermentas, Lithuania). RT reaction was carried out at 42ºC for 60 min. Specific PCR primers amplified L. major PTR1 cDNA.

Cytokine secretion assay and statistical analysis

After 24 hours, supernatants were collected for IL-1β, IL-8, IL-12, TNF-α and IFN-γ analysis by the enzyme-linked immune sorbent assay (ELISA) Development System R&D kit (DuoSet®, Germany). Data was analysed by SPSS software 16.0.2. The significant results were evaluated using an independent two-tailed student's *t*-test ($P<0.05$).

Results

Transfection and dsRNA synthesis

We used expression mammalian two hybrid system plasmids—pACT and pBIND—respectively for the

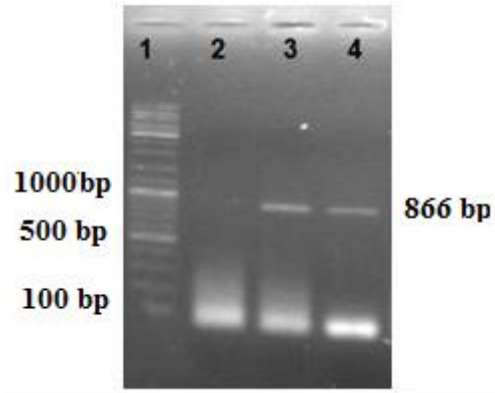

Figure 1. Electrophoresis of leishmania major PTR1 enzyme PCR product on 15% agaros gel.
Lane 1, 100 bp DNA ladder marker
Lane 2, negative control
Lane 3, 866 bp PCR product of sense strand
Lane 4, 866 bp PCR producr of antisense strand

transcription of sense and antisense strands. Cells were transfected, based on calcium phosphate with 200ng/ml plasmid, and incubated for 24 hours. Accordingly, PTR1 sense and antisense RNA single strands were confirmed with RT-PCR (Figure 1). Four groups were considered for transfected cells with antisense plasmid, sense plasmid, double stranded RNA and negative control. To generate dsRNA ex vivo, cells were transfected by random uptake of a mixture of two plasmids—100 ng/ml sense-pACT and 100ng/ml antisense-pBIND.

Cytokine release determination by ELISA assay

To test the effect of antisense RNA strand, sense RNA strand and dsRNA on the MDM cell, four groups of MDM cells were considered: untreated cells, stimulated cells by antisense strand, sense strand, and both of the sense and antisense strands. An analysis of the ELISA results confirmed that there was a significant induction of IL-8, IL-12 and TNF-α production in the immunostimulatory dsRNA compared with other groups.In addition, the sense and antisense single strands induced the IL-8, IL-12 and IL-1β cytokines with significant differences. The pattern of release of cytokines is summarized in Figure 2.

The MDM cells were differentiated into macrophages, using 10 ng/ml LPS treated with 200 ng/ml antisense RNA strand, sense RNA strand and dsRNA in four groups. The amount of IL-8, IL-12 and IFN-γ cytokines secreted in

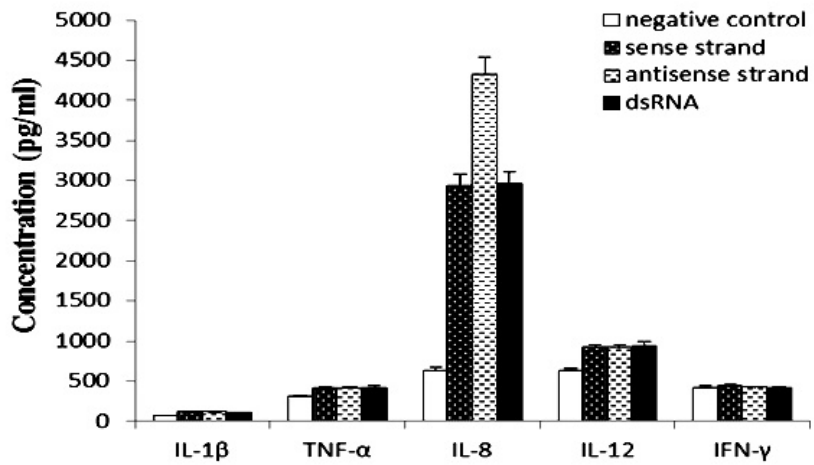

Figure 2. Cytikine profiles of transfected monocytes in different groups after 24h induction

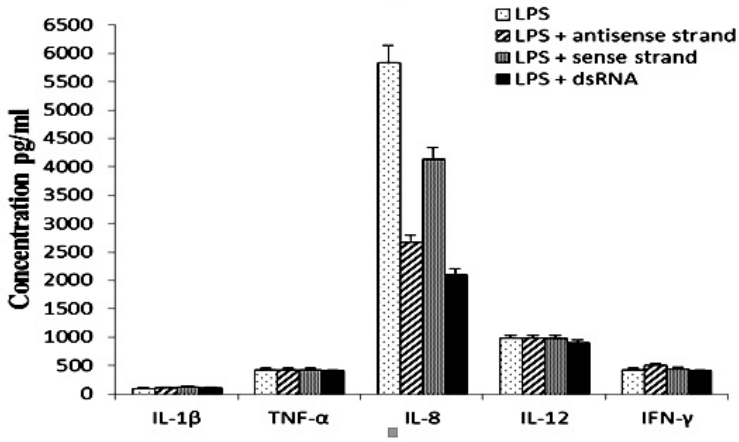

Figure 3. Cytikine profiles of transfected macrophages after 24h

dsRNA group significantly decreased. In single strand groups, IL-8 production was upregulated. These released cytokines patterns are summarized in Figure 3.

Discussion

Although antisense technique and RNA interference are often used to eliminate or suppress target gene expression in the past few decades, RNAi was shown to be a most powerful approach for analysing gene function, especially in immune cells. Cellular delivery of single strand RNA and dsRNA, based on RNAi by plasmid, was used to study the role of immediate early expression of genes and the function of immune cells. We described cytokines' response to sense strand RNA, antisense strand RNA and full-length dsRNA by evaluating the cytokines production of MDM and MΦ cells.

The double strand RNA is an immune-stimulatory agent that interacts with Toll-like receptors (TLR) or cytosolic pattern recognition receptors (e.g. RIG-I or MDA-5 helicases). TLR3, the serine-threonine protein kinase R (PKR), and cytoplasmic helicase proteins like RIG-I and MDA-5, are shown to identify long dsRNA in the cytoplasm and to sustain pro-inflammatory responses (Wiese et al., 2010; Ranjan et al., 2009).

Innate immunity is the first line of defence against pathogen-associated molecular patterns (PAMP). One of the famous stimulations of this system is dsRNA. Immunologically important due to dsRNA, it has been transported by viruses, endogenous and exogenous gene production, leading to modulate cellular responses. dsRNA fragments longer than 30 bases stimulate macrophages to produce pro-inflammatory cytokines, such as IL-12 and TNF-α (Gantier and Williams, 2007; Karpala et al., 2005).

An important function of macrophage is early production of cytokines after PAMP recognition to engage effector cells. Cytoplasmic sensors identified dsRNA for transcription of immediate early genes, such as TNF-α, IL-8, IL-12 (Loving et al., 2006). Some of the reports show that siRNA are able to induce IL-8 production, but cannot express IL-1β cytokine. Recent studies show that long dsRNA (>700 bp) induced a strong secretion of IL-8 (Pauls et al., 2007; Gern et al., 2003).

The macrophage populations responded differentially to the same stimulation. IL-1β cytokine induced after PAMP recognition in tissue-derived macrophages—alveolar macrophage. IL-1β production has been expressed at a very low level in human blood monocyte-derived macrophage (Loving et al., 2006).

The only known member of the Type II IFN family of inflammatory cytokines is an important cytokine to activate macrophages, making up early and delayed responses (Chesler et al., 2002). IFN-γ is mainly expressed by activated T lymphocytes and natural killer cells in response to intracellular pathogens. In adaptive immunity, IFN-γ cytokine may express strongly. IL-12 is a potent regulator and activator of IFN-γ production (Schoenborn and Wilson, 2007).

The capacity of RNA to induce inflammatory responses depends on dsRNA concentration (Persengiev et al., 2004), position of UG dinucleotide within the molecule, size and structure of dsRNA (Marques and Williams, 2005), cell type and transfection reagents (Yoo et al., 2006). Researchers reported that immuno stimulatory siRNA induce cytokine production in a dose-dependent manner in PBMC cells. In siRNA, various concentrations were varied cytokines expression level (Sioud, 2005). Unlike our study, Limmon et al. used poly (I: C) as a dsRNA that induct the TNF-α, IL-6, IL-8 cytokines in epithelial cells (Limmon et al., 2008).

Table 1. Summary of changes of cytokine expression in MDM and MQ cells after dsRNA uptake

Cytokine expression			
	Up regulated	No changes	Down regulated
dsRNA/MDM cell	IL8,IL12, TNFα	INFγ, IL1β	
LPS & dsRNA/MQ cell		INFγ, IL1β	IL8,IL12, INFγ

The current data indicated that dsRNA introduced IL-8, IL-12 and TNF-α production and IL-8, IL-12 and IL-1β cytokines increased in single strand RNA groups in MDM cells. It is interesting to note that dsRNA inhibited LPS-induced cytokine production in MΦ. IL-8 cytokine and IL-12, IFN-γ production levels in transfected cells were decreased 65% and 5% respectively when dsRNA was formed by sense and antisense strands. These altered cytokine expression patterns are summarized in Table 1.

Thus, the main focus of this study performed in our laboratory was to investigate innate immune responses to dsRNA in the MDM. We suggested that dsRNA formation based on RNAi technology shows promise as a vaccine adjuvant for both antiviral and antitumor prophylaxis on the basis of apoptosis induction as well as antiviral drug therapy (DeWitte-Orr and Mossman, 2010; Jin et al., 2010). The strong response of IL-8 chemokine is an effective linkage between innate immunity and adaptive immunity in progressive malignances. Advances in the learning of the role of cytokines in immunity have led to the improvement of cytokine rules in the effector mechanism of immune response (Cutler and Brombacher, 2005; Kapoor and Hotte, 2007).

Competing interests

The authors declared no competing interests.

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