

Human endogenous RNAs: Implications for the immunomodulation of Toll-like receptor 3

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Abstract. Toll-like receptors (TLRs), a family of mammalian receptors, are able to recognize nucleic acids. TLR3 recognizes double-stranded (ds)RNA, a product of the replication of certain viruses. Polyinosinic-polycytidylic acid, referred to as poly(I:C), an analog of viral dsRNA, interacts with TLR3 thereby eliciting immunoinflammatory responses characteristic of viral infection or down-regulating the expression of chemokine receptor CXCR4. It is known that dsRNA also directly activates interferon (IFN)-induced enzymes, such as the RNA-dependent protein kinase (PKR). In the present study, the mRNA expression of TLR3, CXCR4, IFN γ and PKR was investigated in a culture of peripheral blood mononuclear cells (PBMCs) stimulated with poly(I:C) and endogenous RNA from human PBMCs. No cytotoxic effect on the cells or on the proliferation of CD3⁺, CD4⁺ and CD8⁺ cells was observed. TLR3 expression in the PBMCs in the presence of poly(I:C) was up-regulated 9.5-fold, and TLR3 expression in the PBMCs treated with endogenous RNA was down-regulated 1.8-fold ($p=0.002$). The same trend was observed for IFN γ where in the presence of poly(I:C) an 8.7-fold increase was noted and in the presence of endogenous RNA a 3.1-fold decrease was observed. In the culture activated with poly(I:C), mRNA expression of CXCR4 increased 8.0-fold and expression of PKR increased 33.0-fold. Expression of these genes decreased in the culture treated with endogenous RNA when compared to the culture without stimulus. Thus, high expression of mRNA for TLR3, IFN γ , CXCR4 and PKR was observed in the presence of poly(I:C) and low expression was observed in the cells cultured with endogenous RNA. In conclusion, TLR3 may play major physiological roles

that are not in the context of viral infection. It is possible that RNA released from cells could contain enough double-stranded structures to regulate cell activation. The involvement of endogenous RNA in endogenous gene expression and its implications in the regulation thereof, are still being studied, and will have significant implications in the future.

Introduction

The innate immune system is the first line of defense against invading pathogens (1). This system uses Toll-like receptors (TLRs) to recognize conserved pathogen-associated molecular patterns and to orchestrate the initiation of immune responses. Various TLRs recognize and respond to nucleic acids. Double-stranded (ds)RNA, a frequent viral constituent, has been shown to activate TLR3 (2,3). Data indicate that TLR3 regulates amplification events during inflammation mediated by nonviral mechanisms (4). It is known that dsRNA-activated dendritic cells induce an increase in Th1 and a decrease in Th2 differentiation, resulting in extremely polarized responses relative to those induced by unstimulated and other TLR ligand-activated dendritic cells (5).

In recent years, diverse combinations of drugs targeting multiple pathways have been used in cancer treatment. One of the agents for tumor chemotherapy assessed with favorable outcome in clinical trials is synthetic dsRNA (6,7). The ability of dsRNA to directly stimulate TLR3 and produce type I interferons (IFNs) was primarily the rationale for its clinical use in cancer patients (8). More recently, several studies have demonstrated that the activation of TLR3 by dsRNA directly inhibits cell proliferation and induces apoptosis in tumor cells (9-11). In view of these promising effects, the use of dsRNA-derived compounds in combination with anticancer agents for chemotherapeutic warrants robust investigation (12).

TLR3 mediates antiviral immune response by activating the innate immunity and cross-priming CD8⁺ T cells. TLR3 agonists have the capacity to directly trigger apoptosis in human cancer cells and have been used as adjuvants to treat cancer patients with the aim of inducing an IFN-dependent immune response. Chemokine receptors such as CXCR4 have been implicated in organ-specific metastasis of various types

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of cancers, and the results from functional chemotaxis assays demonstrated that the treatment of nasopharyngeal carcinoma cells with polyinosinic-polycytidylic acid [poly(I:C)] reduced CXCR4 expression in a dose-dependent manner (13).

Double-stranded RNA-activated protein kinase (PKR) has a key role in the innate immune response to viral infection in higher eukaryotes. PKR contains an N-terminal dsRNA-binding domain and a C-terminal kinase domain. In the prevalent auto-inhibition model for PKR activation, dsRNA binding induces a conformational change that leads to the release of the dsRNA-binding domain from the kinase, thus relieving the inhibition of the latent enzyme.

Further discovery and characterization of RNAs will improve our understanding of innate immunity and thereby provide the opportunity to develop new and improved RNA-based therapeutics (14). The dsRNA, an intermediate virus replication and a signature of infection and dsRNA were recognized in the cytoplasm via PKR, RIG-I and MDA-5 (15). These trigger the release of inflammatory cytokines, that is, they activate innate immunity which shapes the adaptive immune response (16,17).

The purpose of the present study was to analyze the expression of mRNA for TLR3, CXCR4, IFN γ and PKR in PBMCs stimulated with poly(I:C) and human endogenous RNA.

Materials and methods

The Human Ethics Committee of the State University of Londrina approved the present study, and voluntary written consent was obtained from each of the patients enrolled. Peripheral blood mononuclear cells (PBMCs) were collected from healthy blood donors with negative serology for HIV, HBV and HCV.

Cell cultures. Peripheral blood cells were directly collected in EDTA (ethylenediaminetetraacetic acid) vacutainers. The PBMC samples were extracted by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, USA). The PBMCs were maintained in RPMI-1640 plus 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) in 24-well plates (Costar, Austria) at 1×10^6 cells/well. The media were supplemented with 2 mM glutamine, 100 units/ml penicillin G and 100 units/ml streptomycin. The cells were maintained at 37°C in a humidified incubator containing 5% CO $_2$ in the presence or absence of stimuli for 24 h. Poly(I:C) (Sigma-Aldrich) was used at a concentration of 50 μ g/ml. Endogenous total RNA was obtained from the PBMCs of donors in this study, in the same manner as described in the 'RNA isolation and reverse transcriptase reaction' section and used at a concentration of 500 μ g/ml in the cell cultures.

Quantification of lactate dehydrogenase (LDH) activity. The cytoplasmatic enzyme LDH was quantified in all of the samples for analysis of cytotoxicity. The Dimension[®] (DADE Behring, Newark, DE USA) clinical chemistry system was used to determine the LDH activity. The lactic dehydrogenase method is a modification of the enzymatic lactate to pyruvate procedure modified by Gay *et al* (18).

Flow cytometric analysis. A total of $1-3 \times 10^6$ cells were saturated with purified normal mouse Ig (Becton Dickinson) at room

temperature for 10 min. The cells were then incubated for 30 min at 4°C with mouse monoclonal antibodies anti-CD3 labeled with fluorescein isothiocyanate (FITC), anti-CD8 labeled with phycoerythrin (PE) and anti-CD4 labeled with allophycocyanin (APC). The cells were counted by flow cytometry performed with a FACSCalibur[™] flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) equipped with 635 nm and 488 nm lasers that are capable of detecting light scatter (forward and side) and four-color fluorescence with emission detectable in four ranges: 515-545 nm, 562-607 nm, >650 nm and 652-668 nm.

RNA isolation and reverse transcriptase reaction. Total cellular RNA was extracted from the cell cultures with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified total RNA was measured and assessed for purity by determining the absorbance at 260 and 280 nm and was then stored at -80°C until testing. Reverse transcriptase reaction was performed using 500 ng RNA, 20 units cloned Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen), 4 units recombinant ribonuclease inhibitor (RNaseOUT[™]; Invitrogen) under the following conditions: 2.5 μ M oligo dT, 50 mM Tris HCl pH 8.3, 75 mM KCl, 1.5 mM MgCl $_2$, 1.25 mM dNTP, at 42°C for 60 min in a Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada).

Molecular analysis of β -actin mRNA. PCR for β -actin was determined as described by Amarante *et al* (19). Briefly, cDNA synthesis was carried out as previously described, and the PCR conditions were: 94°C for 1 min followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and finally, 72°C for 10 min in a Biocycler (Biosystems). PCR products were analyzed by electrophoresis on acrylamide gel (10%) and detected by a nonradioisotopic technique using a commercially available silver staining method.

Quantitative real-time PCR conditions. Real-time PCR using SYBR Green fluorescence was performed with 80 ng cDNA. Each real-time PCR reaction consisted of 2.5 μ l RT product, 10 μ l Platinum[®] SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.25 μ M of each sense and antisense primer. The amount of cDNA was estimated by the quantitative polymerase chain reaction (qPCR) and amplified using the sense and the antisense primer according to Table I. The PCR reaction was performed for 40 cycles, and the conditions for each cycle were as follows: 95°C for 30 sec, annealing temperature for 30 sec and 72°C for 30 sec using a Chromo4[™] real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The reported Ct values (the cycle number at which emitted fluorescence exceeds an automatically determined threshold) are the mean fold change \pm SEM for three independent determinations. Data from the control PBMCs and PBMCs activated with poly(I:C) or RNA are shown as the mean of Ct values, adjusted for Ct values for CXCR4, TLR3 and IFN γ and corrected according to Ct values for GAPDH. PKR was corrected according to Ct values for 18S from control samples, considering efficiency values, according to the Pfaffl method (20). Subsequently, a melting curve was recorded between 50 and 98°C with a hold every 2 sec.

Statistical analysis. Statistical analysis was carried out using the SPSS Statistics 17.0 program (SPSS inc., Chicago, IL,

Table I. Primers used in the qPCR reactions.

Gene mRNA	GenBank accession no.	Primers	Sequence 5'-3'	Annealing temperature (°C)
GAPDH	NM_002046	Forward	GAA GGT GAA GGT CGG A	54.0
		Reverse	GGG TCA TTG ATG GCA AC	
TLR3	NM_003265	Forward	AAA TAG ACA GAC AGA CAG AACAGT	54.0
		Reverse	AAA AAC ACC CGC CTC AAA	
CXCR4	AF025375	Forward	TCTACTCCATCATCTTCTTTA	54.0
		Reverse	ACGTTGGCAAAGATGAAGGTC	
IFN γ	NM_000619	Forward	AAT TGT CTC CTT TTA CTT CA	54.0
		Reverse	GTCATC TCG TTT CTT TTT GT	
PKR	M85294	Forward	ACA GCA AAA ATA GTT CAA GGT CA	57.0
		Reverse	AAA GAG TTC CAA AGC CAA AA	
18S	NR_003286	Forward	GTA ACC CGT TGA ACC CCA TT	57.0
		Reverse	CCA TCC AAT CGG TAG TAG CG	

USA). $P \leq 0.05$ was considered statistically significant. The paired sample correlations for relative expression were tested using the Student's t-test.

Results

Culture was performed with PBMCs from 6 healthy blood donors in the presence and absence of dsRNA poly(I:C) and endogenous RNA. Poly(I:C) was used for cell activation at a concentration of 50 $\mu\text{g/ml}$. Endogenous RNA was obtained from the same PBMC blood donors and each PBMC culture was sensitized with the endogenous RNA.

In the first step, after 24 h, PBMC toxicity was analyzed by quantifying LDH activity. The concentration of LDH found in the cultures was 70.33 ± 17.39 U/l for control PBMCs, 47.0 ± 12.29 U/l for PBMCs activated with poly(I:C) and 60.00 ± 5.29 U/l for PBMCs stimulated with RNA. No statistical significance was observed between the control PBMCs and PBMCs stimulated with poly(I:C) and RNA ($p > 0.05$), and no toxicity was observed in any culture.

The next step was to determine the number of CD4, CD8 and CD3 antibody binding sites (ABSs) in the unstimulated and stimulated culture cells. The differences in ABSs between the unstimulated culture cells and cells stimulated with poly(I:C) and endogenous RNA were not statistically significant (Fig. 1).

RNA was extracted from the cell cultures in the presence or absence of poly(I:C) and endogenous RNA for 24 h at 37°C and 5% CO₂. The viability and integrity of the RNA samples and cDNA quality were analyzed by conventional PCR for β -actin, performed with specific primers. All RNA samples presented detectable quantities of β -actin mRNA and acceptable integrity during amplification. No contamination with genomic DNA was verified, since all amplified products presented a fragment corresponding to 353 bp.

Quantitative PCR was used to investigate the expression of mRNA for TLR3, IFN γ , CXCR4 and PKR in the human blood cells activated with poly(I:C) and endogenous RNA. The level of TLR3 expression in the PBMCs in the presence of poly(I:C) was up-regulated 9.5-fold, while the level of TLR3

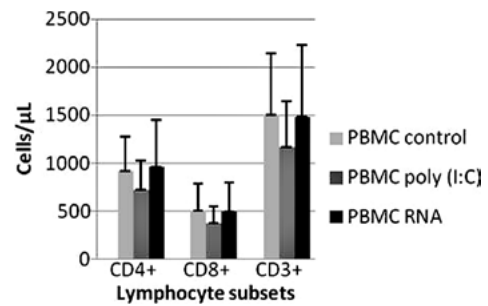


Figure 1. Quantification of CD4⁺, CD8⁺ and CD3⁺ antibody binding sites by flow cytometry. CD4⁺: PBMC control, 918.25±352.19 μl ; PBMC poly(I:C), 723.00±304.63 μl ; PBMC RNA, 958.75±492.89 μl . CD8⁺: PBMC control, 507.87±283.46 μl ; PBMC poly(I:C), 372.33±174.72 μl ; PBMC RNA, 494.75±299.95 μl . CD3⁺: PBMC control, 1501.87±644.04 μl ; PBMC poly(I:C), 1169.67±473.65 μl ; PBMC RNA, 1486.50±747.30 μl .

expression in the PBMCs treated with endogenous RNA was down-regulated 1.8-fold ($p = 0.002$). The same trend was noted for IFN γ . IFN γ expression in the PBMCs in the presence of poly(I:C) was increased 8.7-fold increase while in the PBMCs in the presence of endogenous RNA a 3.1-fold decreased IFN γ expression was noted. mRNA expression of CXCR4 increased 8.0-fold and expression of PKR was increased 33.0-fold, while expression of these genes decreased in the culture stimulated with endogenous RNA when compared to the culture without stimulus (1.2- and 2.01-fold, respectively). This relationship was statistically significant ($p = 0.001$). Thus, high mRNA expression for TLR3, IFN γ , CXCR4 and PKR was confirmed in the presence of poly(I:C) and low expression in the culture cells was verified with endogenous RNA stimulus, as shown in Fig. 2.

Discussion

Pathogen recognition is largely assigned to an evolutionarily conserved family of receptors, the toll-like receptors, which function in innate immunity and subsequent acquired immunity against microbial infection or tissue injury.

In the present study, CD4 and CD8 levels were uniform among the lymphocyte subsets of CD3⁺ cells, and stimula-

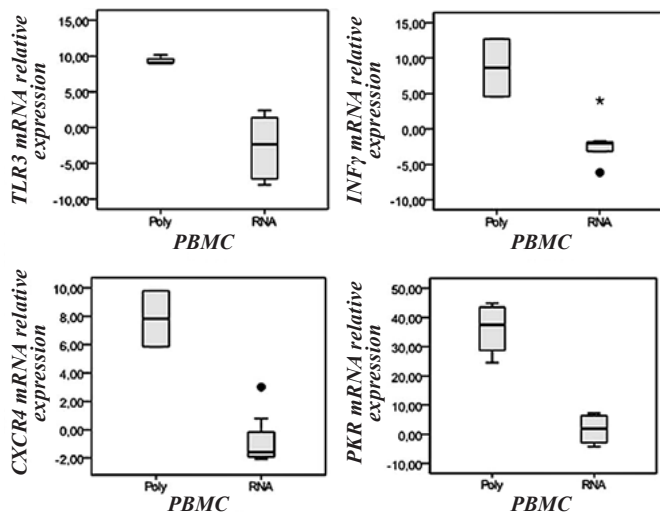


Figure 2. Relative expression of TLR3, IFN γ , CXCR4 and PKR mRNA in culture cells in the presence of poly(I:C) and endogenous RNA. Pfafl values were compared between values from the culture in the presence of poly(I:C) and the culture stimulated with endogenous RNA.

tion with poly(I:C) and with endogenous RNA for 24 h did not produce toxic and lytic activity as demonstrated by LDH assay. All RNA used in this study was tested for integrity through β -actin expression evaluation.

It has been described that the RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via TLR3. This study by Brentano *et al* (21) indicated that the RNA released from necrotic cells may act as an endogenous TLR3 ligand for the stimulation of pro-inflammatory gene expression in rheumatoid arthritis synovial fibroblasts. In the present study, we verified that stimulation with endogenous RNA obtained from healthy PBMCs, in contrast to poly(I:C), reduced the expression of TLR3, IFN γ , CXCR4 and PKR in PBMCs when compared with control PBMCs, and the difference achieved statistical significance.

Ben-Asouli *et al* (22) revealed that human IFN γ mRNA activates the PKR kinase. IFN γ mRNA activates PKR through a pseudoknot in its 5'UTR. This 5'UTR creates a stem-loop with a remote sequence in the RNA molecule. Thus, IFN γ mRNA regulates its own translation by an RNA pseudoknot. Several reports have described messenger RNA-like transcripts as polyadenylated but with no defined open reading frames, indicating that they lack protein coding capacity and therefore allow regulatory RNAs to exert their action at the RNA level (23,24).

Research by Tabiasco *et al* (25) demonstrated that TLR3 is also present in cells that participate directly in the adaptive immune response in which human effector CD8⁺ T lymphocytes express TLR3 as a functional co-receptor. In this context, TLR3 ligation was shown to directly increase IFN γ production by CD8⁺ T cells. This evidence suggests that TLR3 is a 'danger' receptor with pleiotropic potential in innate and adaptive immunity (26). These authors demonstrated that TLR3 contributes to the elimination of specific viruses, although others have demonstrated that certain viruses benefit from TLR3 stimulation. The general outcome is probably dependent on several factors, including the type of virus, the

viral load, its mode of infection (endoplasmic versus cytoplasmic), the type of cell infected and the stage of infection.

To date, attention has been primarily focused on the role of TLR3 in eliciting cellular responses to virus infection, due to the antimicrobial functions of other TLRs and the fact that dsRNA, the ligand of TLR3, is produced during the replicative cycle of certain viruses. The physiological roles of TLR3 remain to be clearly defined. It is possible that TLR3 has major physiological roles that are not in the context of viral infection. In this case, the origin of the dsRNA is unclear. It is possible that extracellular RNA released from cells, due to their apoptotic or necrotic death, contains enough double-stranded structures to activate or inhibit the receptor. *In vitro*-transcribed mRNA or endogenous RNA released from necrotic cells, when added extracellularly, has been shown to activate experimental or natural cells expressing TLR3 (27).

PKR plays a significant role in mediating the antiviral effects of IFNs, but in uninfected cells PKR is also implicated in the regulation of cell proliferation under normal conditions (28). Several studies have demonstrated that the activation of TLR3 by dsRNA directly inhibits cell proliferation and induces apoptosis in tumor cells (9-11,29).

Karikó *et al* (30) reported that a variety of natural RNAs have various capacities to activate immune cells. The most potent RNAs were those that had the least number of modified nucleosides. Therefore, it was hypothesized that nucleoside modification suppresses the immune-stimulatory effect of RNA. In a quest to prove this, several novel lines of evidence were discovered in support of RNA-mediated immune activation.

Cells may employ various mechanisms to regulate RNA synthesis. In this context, it is noteworthy that the expression of non-coding T cell RNA may be implicated in the T lymphocyte response (19). In the context of adaptive immunity response, it was demonstrated that TLR3 is also present in cells that participate directly in the adaptive immune response in which the human effector T lymphocytes express TLR3 as a functional co-receptor. If there is an endogenous non-coding short or long human RNA, these molecules could be a candidate for TLR3 or other intracellular receptors.

TLR3 has gained recognition as a novel molecular target for cancer therapy as TLR3 activation by its synthetic ligand poly(I:C) directly causes tumor cell death (12). TLR3 agonists have the capacity to directly trigger apoptosis in human cancer cells and have been used as adjuvants to treat cancer patients with the aim of inducing an IFN-dependent immune response. Zhang *et al* (13) verified that TLR3 activation down-regulated the expression of the chemokine receptor CXCR4 in a dose-dependent manner and inhibited cell migration in response to the CXCR4 ligand. In this study, although poly(I:C) increased expression of CXCR4 in PBMCs, the contrary effect was verified with endogenous RNA. Although poly(I:C) was found to be a potent IFN γ inducer, it is possible that endogenous RNA promotes regulation of the expression of CXCR4, IFN γ and PKR, and there is no involvement with TLR3.

The involvement of RNA and its receptors and the release of several cytokines and chemokines in the pathogenesis of immune disorders and other diseases, including cancer, are still being studied and will have significant implications in the future. Insights gained from the study of endogenous RNA as non-coding regulatory RNAs may advance our understanding

of various diseases where nucleic acids play a prominent role in the pathogenesis or regulation of gene expression. Furthermore, these insights may determine a role for nucleoside modifications on RNA, and provide future direction in the design of therapeutic RNAs (31).

The investigation of RNA-based immunology has been reinvigorated with the observation that TLR3 interacts with RNA. mRNA therefore joins the list of endogenous ligands for TLRs and is the first endogenous ligand described for TLR3. The presence of these host ligands during inflammation activates the immune system. The further finding that nucleoside modification alters RNA-mediated TLR signaling presents a mechanism for the long-observed differences in immunogenicity between bacterial, viral and mammalian RNAs. This is one of the first studies to comprehensively compare not only the effects of TLR ligands such as double-stranded RNA on human PBMC activation, but more importantly, the inhibitory effect of endogenous RNA on PBMCs. The involvement of RNA modification in the pathogenesis of immune disorders and other diseases, and its implications in therapeutics, are still being studied, and should have significant implications in the future.

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