International Immunopharmacology 22 (2014) 366–370

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Short communication

CpG-ODN class C-mediated immunostimulation and its potential against *Trypanosoma evansi* in equines

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ARTICLE INFO

Article history:
Received 15 January 2014
Received in revised form 3 July 2014
Accepted 3 July 2014
Available online 24 July 2014

Keywords:
Trypanosoma evansi
CpG-ODN
Horse
IFN-α
TNF-α
Nitric oxide

ABSTRACT

Trypanosoma evansi is the causative agent of surra, which is the most common and widespread trypansomal disease. The infection is mainly restricted to animals, but it has also been documented in human. Trypanosomes possess the thick immunogenic surface coat known as variant surface glycoprotein (VSG). The parasite modifies the VSG constantly resulting in continuous antigenic variations and thus evades the host immune response. Due to antigenic variations, vaccination against trypanosomosis is not useful. Therefore, alternate strategies to augment the immune response are required. CpG-ODN class-C has combined immune effects of both A and B classes of CpG-ODN. In this study, we observed that CpG-ODN class-C stimulated horse peripheral blood mononuclear cells (PBMC) induce the expression of interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), IL-12 and nitric oxide (NO) indicating enhanced innate immune response. We have for the first time demonstrated that co-culture of CpG-ODN with *T. evansi* antigen induces lymphocyte proliferative responses and result in a synergistic effect in eliciting the immune response.

1. Introduction

Trypanosoma evansi is the causative agent of surra, which is the most common and widespread trypansomal disease leading to significant economic losses. It is transmitted mechanically by biting flies such as *Tabanus* and *Stomoxys* [1]. This trypanosome can infect most mammals, but horses and camels are the principal hosts. Reports also indicate their ability to infect human [2]. Surra is endemic in many parts of Africa, Asia, and South America where thousands of animals die owing to disease outbreaks each year. Trypanosomes are unusual among protozoan parasites because of their unique thick immunogenic surface coat which is known as variant surface glycoprotein (VSG) [3]. These parasites modify their VSG constantly, leading to antigenic variation and thus evade the immune system of the host [4]. Because of antigenic variation, vaccination against trypanosomosis is not useful. Resistance to trypanosomosis was previously thought to be largely conferred by the adaptive immunity, including VSG-specific B and T lymphocyte responses [5,6]. Moreover, the parasite induces immuno-suppression to a variety of antigens in infected hosts, with severe impairment of cell mediated immune responses [7–9].

A strategy to stimulate the innate immunity is useful, where the adaptive immune system is considered immature and the risk of exposure to infectious agents is high. For this, considerable interest has been generated in finding ways to stimulate the innate immune system. One approach to achieve this goal is to use molecules that interact with pattern recognition receptors on immune cells [10]. Non-methylated cytosine-phosphate-guanosine (CpG) motifs present in viral and bacterial DNA are one of the pathogen associated molecular patterns (PAMPs) recognized by the mammalian innate immune system [11]. CpG oligodeoxynucleotides (CpG-ODNs) stimulate immune cells from a wide spectrum of mammalian species, including human, mice, rabbits, horses, cattle, buffalo, sheep, pigs, rats, cats, dogs and non-human primates [12–18].

The CpG-ODNs are recognized by Toll-like receptor 9 (TLR9) and signaling by this receptor triggers a pro-inflammatory cytokine response which in turn, influences both innate and adaptive immune responses. Cells responsive to the CpG DNA are largely cells of the innate immune system, particularly dendritic cells (DCs) and macrophages, but it has also been reported to activate B cells, monocytes and NK cells [19–21]. TLR9 expression has been reported in activated human neutrophils, monocytes and monocyte-derived cells, activated CD4+ T cells, pulmonary epithelial cells, natural killer (NK) cells, and intestinal epithelium [15]. Equine TLR9 expression has been reported in polymorphonuclear cells (PMNs), CD4+ and CD8+ T lymphocytes as well as other leukocytes using a cross-reactive TLR9 mAb [22].

Three distinct classes of synthetic CpG-ODNs that differ in structure and function have been described [12,23]. Class A CpG-ODN is rapidly degraded in vivo with a half-life of nearly 5 min [24]. In contrast, class

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http://dx.doi.org/10.1016/j.intimp.2014.07.016
1567-5769/

B CpG-ODN is much more stable than class A ODN due to nuclease-resistant phosphorothioate backbone. These CpG-ODNs induce marked B cell proliferation in vitro, but are poor at NK cell activation [25,26]. Class C CpG-ODN is relatively stable [29] and has the combined immune effects of both A and B classes of CpG-ODNs as indicated by their capacity to stimulate both B cells and pDCs to induce IFN-α and NK cell activation [25,27–29]. Class A and class C ODNs stimulate plasmacytoid dendritic cells (pDCs) which secrete cytokines involved in the innate immune response, including type I IFNs and tumor necrosis factor-alpha (TNF-α) and also activate natural killer cells. Furthermore, pDCs with activated TLR9 secrete IL-12. Class B and class C ODNs mainly stimulate B cells. Class C ODN 2395 has been shown to strongly stimulate human B cell or NK cell activation and IFN-α production in vitro of human PBMCs [25]. It was also demonstrated that pDCs were responsible for the IFN-α production by comparing pDC and pDC depleted PBMCs [25].

An enhanced immune response has also been observed against *Trypanosoma cruzi* and *Trypanosoma brucei* in mice after treatment with CpG-ODN [30–32]. Unlike *T. cruzi* and *T. brucei*, *T. evansi* is an extracellular parasite and causes immuno-suppression in the infected host. Previously, we have demonstrated that class C CpG-ODN, has immuno-stimulatory properties against *T. evansi* in experimentally infected rabbits [33]. Enhanced humoral immune responses (IgG levels), reduction in parasitic load, delayed onset of clinical signs with reduced severity were observed in CpG treated and *T. evansi* challenged rabbits as compared to that of *T. evansi* infected rabbits. Considering that the class C CpG-ODN can be valuable in boosting the immune response in horses as well, we evaluated the immunostimulatory effects on equine PBMC and its potential against *T. evansi* infection. We also investigated the dose dependent proliferative responses of equine PBMC consequent upon stimulation with CpG-ODN and *T. evansi* antigen. We studied the functional responsiveness of horse PBMC to CpG-ODN class A, C, *T. evansi* antigen or co-culture of CpG ODN with *T. evansi* antigen.

2. Materials and methods

To evaluate activation of the equine innate immune system by CpG-ODN, the expression of cytokines IFN-α, TNF-α, IL-12 and TLR9 by PBMC was quantitated following CpG-ODN class C treatment. PBMCs were isolated from the venous blood of three Marwari horses using density gradient centrifugation on histopaque. All the reagents for isolation and culture of PBMCs were purchased from Sigma-Aldrich (St. Louis, MO, USA). PBMCs at a concentration of 5×10^5 cells/ml in Eagle's minimum essential medium (EMEM) (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 mM 2-mercaptoethnol, 50 mg/ml streptomycin sulfate and 10 mg/ml gentamicin sulfate), were seeded in six well round bottom plates (NUNC) @ 2 ml/well and incubated at 37 °C in an atmosphere of 5% CO_2. After 24 h of rest, endotoxin free CpG-ODN class C (2395) in different concentrations (1–15 μg/ml) was used for the stimulation of PBMCs. The ODN sequences used in these studies are listed in Table 1. After 24 h of stimulation, total RNA was extracted from the PBMC using RNeasy kit (Qiagen, GmBH, Hilden, Germany), and treated with DNAse I (Fermentas, Lithuania) to remove genomic DNA. To transcribe the purified RNA into complementary DNA (cDNA), reverse transcription was carried out using enhanced avian RT first strand synthesis kit (Sigma-Aldrich, St. Louis, MO, USA) as per manufacturer's instructions.

The cytokine gene expression was assessed by real time PCR using gene specific primers (Table 2) and the change in expression was quantitated relatively by comparing the threshold cycles (Ct) of cytokine gene to the Ct generated by a reference sample (unstimulated PBMC) and the calibrator (β-actin, housekeeping gene). The quantitative RT-PCR was performed on cDNA samples from stimulated and unstimulated PBMCs using SYBR® Green *Taq* Ready Mix (Sigma-Aldrich, St. Louis, MO, USA). Amplification and detection were performed in an automated Real time RT-PCR (BIO-RAD, Hercules, California, USA). Fold increase of target gene expression over unstimulated controls was calculated by $2^{-\Delta\Delta Ct}$ method [34].

We also evaluated NO production in supernatants of the unstimulated and CpG-ODN stimulated PBMC cultures by using Griess assay as previously described [35,36]. Briefly, equal amounts of a Griess reagent (1% sulfanilamide, 0.1% N-naphthylene diamine, 5% phosphoric acid) and the test sample were mixed in 96 well culture plates and incubated at 37 °C for 10 min. The absorbance of the test samples was determined at 540 nm using an ELISA reader (Biotek Instruments, powerwave X2, USA). The amount of NO was calculated from a standard curve generated from absorbance of known concentrations of sodium nitrite in culture media.

To determine the proliferative responses induced by CpG-ODN against *T. evansi*, PBMCs at a concentration of 5×10^6 cells/ml were seeded in 100 μl medium per well into 96-well flat-bottomed microtiter plates and incubated for 24 h at 37 °C and 5% CO_2. The trypanosomes from *T. evansi* infected mice's blood (first parasitemic peak) were purified by DEAE-cellulose chromatography, followed by centrifugation [37]. The whole cell lysate antigen of *T. evansi* was prepared using purified trypanosomes. The disruption of purified trypanosomes was performed using an ultrasonic cell disruptor (Branson Sonifier 450, Branson, USA) at 20 kHz using 5 mm tapered tip with 4 disruptions of 15 s each with 30 s interval in ice-bath at 20% duty cycle. The sonicated whole cell lysate was centrifuged at 10,000 × *g* for 15 min at 4 °C and the supernatant was collected. Proteolytic enzyme inhibitors were added to the antigen after ultrasonication. The antigen was aliquoted (1 mg/ml) and stored at −40 °C till further use.

The lymphocyte proliferation was determined using calorimetric technique 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT) assay. Optimal stimulant concentrations and culture times were determined in preliminary kinetic experiments. The PBMCs were treated, in triplicates, with 5 μg/ml CpG-A or 5 μg/ml CpG-C or 20 μg/ml whole cell lysate antigen of *T. evansi* or co-culture of CpGs with *T. evansi* antigen. The unstimulated culture was used as negative control, while Con A at 6 μg/ml was used as positive control. The cell cultures were incubated for 72 h at 37 °C and 5% CO_2. A medium without cells was used as background. After 72 h, 50 μl of MTT (5 mg/ml) was added to each well and incubated for 4 h at the same conditions. Then, a medium with MTT was removed from every well and added 50 μl of DMSO to solubilize formazan crystals. The solution was mixed vigorously and incubated for 15 min. Absorbance was read at 570 nm in ELISA plate reader (Biotek Instruments, powerwave X2, USA). Proliferative response was expressed as stimulation index (SI), representing the ratio of optical density of the stimulated cells to the optical density of unstimulated cells after normalizing the background. The data were analyzed statistically by paired Student's t-test.

Table 1
Structural chemistry of CpG-ODN used in the present study.

ODN name	Class	Sequence	Structural chemistry
2216	A	5′-ggGGACGA:TCGTCgggggg-3′	Phosphodiester central CpG-containing palindromic motif and a phosphorothioate 3′ poly-G string.
2395	C	5′-t**cgt**c**g**ttttt**cgg**c**g**c**g**c**cg**-3′	complete phosphorothioate backbone and a CpG-containing palindromic motif.

CpG motifs are indicated in bold font. Bases shown in capital letters are phosphodiester, and those in lower case are phosphorothioate. Note: Bases are phosphorothioate, palindrome is underlined.