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Original article

pUC18-CpG stimulates RAW 264.7 via TBK1-mediated pathway and presents adjuvanticity in mice

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Abstract

Phosphorothioate CpG oligodeoxynucleotides (ODN) are reported to be recognized by the membrane-bound TLR9 and trigger the MyD88-dependent up-regulation of Type I interferons and pro-inflammatory cytokines. Whether plasmids containing multiple CpG motifs stimulate the same signaling pathway is yet to be determined. The present results show that the CpG motifs enrich plasmid pUC18-CpG stimulates RAW 264.7 in vitro, mainly through the TBK1-mediated signaling pathway, causing the up-regulation of IFN-β, and pro-inflammatory cytokines TNF-α and IL-6. When pUC18-CpG is co-administered with the recombinant *Echinococcus granulosus* antigen, the antigen-specific antibody titers are markedly increased compared to the Quil-A adjuvanted group. Antigen specific cytokine quantification shows that cytokine profiles from the pUC18-CpG adjuvanted-group are switched to a Th1-biased immune response.

Key words: adjuvant, CpG DNA, Echinococcus granulosus, TBK1, type I interferons

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Introduction

Mammalian cells are equipped with pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) from invading pathogens (Janeway et al. 2002, Medzhitov et al. 2009, Diana et al. 2018). As an important PAMP, microbial DNA is reported to be recognized by various PRRs, including TLR9, DAI, IFI16, DDX41, AIM2 and cGAS, etc (Wu et al. 2014, Dempsey et al. 2015). The sensing of microbial DNA then triggers signaling cascades that result in the up-regulation of Type I interferons and pro-inflammatory cytokines expression, contributing to the activation and regulation of following adaptive immune response (Takeuchi et al. 2010, Paludan et al. 2015).

Previous reports suggest that CpG DNA is recognized by the membrane-bound TLR9, triggers the MyD88-dependent signaling pathway and proves to be a potent adjuvant towards a broad range of antigens (Hemmi et al. 2000, Scheiermann et al. 2014, Shirota et al. 2014). However, the recent discovery of the central cGAS-STING-TBK1 axis in the sensing of cytosolic DNA leads to the hypothesis that cytosolic DNA sensors may also participate in the recognition of DNA plasmid containing multiple CpG motifs (Hemmi et al. 2000, Sun et al. 2013, Tao et al. 2016). In the present study, the immunostimulatory effects of pUC18-CpG containing mouse-specific CpG motifs were evaluated in Raw 264.7 cells and its adjuvanticity to the recombinant *Echinococcus granulosus* antigen was tested *in vivo*.

Materials and Methods

Ethical approval

BALB/c mice were purchased from Weitong Lihua Co., Ltd, Beijing, China. All animals used in this research were treated with care and with the approval of the Animal Ethics Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences, China.

Cells and reagents

The mouse macrophage cell line, RAW 264.7, was purchased from the American Type Culture Collection (Manassas, VA, USA). The pUC18-CpG plasmid containing 10 copies of mouse-specific CpG motifs (CpG motif sequence: 5′-TCCATGACGTTCCTGACGTT-3′) was constructed and preserved in the Department of Veterinary Medicine, Institute of Animal Sciences. CpG ODN 1826 (5′-TCCATGACGTTCCTGACGTT-3′, phosphorothioate backbone), TLR9 antagonist ODN 2088 (5′-TCCATGACGTTCCTGACGTT-3′, phosphorothioate backbone), TBK1 inhibitor BX795 and Quli-A were purchased from Dakewei Co., Ltd, Beijing, China. MiniBEST Universal RNA Extraction Kit, PrimeScript™ II 1st Strand cDNA Synthesis Kit and SYBR® Premix Ex Taq TM II (Tli RNaseH Plus) were purchased from TaKaRa, Dalian, China. Recombinant *Echinococcus granulosus* protein (Eg95 antigen) for immunization was purified and stored in the the Department of Veterinary Medicine, Institute of Animal Sciences. Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in goat was from Sigma-Aldrich, St. Louis, MO, USA.

Profile of DNA PRRs expression in RAW 264.7 cells

Specific primers were synthesized based on the gene sequences encoding mouse DNA pattern recognition receptors cGAS, IFI204, AIM2, DDX41, TLR9, STING, and DAI, respectively (Table 1). mRNA was extracted from cultured RAW 264.7 cells and reverse-transcribed into cDNA by use of the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). PCR amplification was carried out following an initial denaturation step at 94°C for 2 min, then 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified products were analyzed by electrophoresis in a 1.2% agarose gel.

Stimulation of RAW 264.7 with pUC18-CpG/ CpG ODN 1826

RAW 264.7 cells were cultured in 6-well plates (Nunc, Roskilde, Denmark) until reached ~80 % confluence. Cells were firstly treated with culture medium, TLR9 antagonist ODN 2088 (5 μM) or TBK1 inhibitor BX795 (5 μM), according to the manufacturer's instructions. After incubation, the cells were stimulated with pUC18-CpG (5 μg/well) or CpG ODN 1826 (5 μg/well), respectively. After 6 hours post-stimulation, mRNA was extracted from cultured RAW 264.7 cells and reverse-transcribed into cDNA. The differential expression of selected genes was assessed by SYBR Green based RT-qPCR using an Applied Biosystems 7900 Real-time PCR System (Applied Biosystems, Forster City, CA, U.S.A.). Each RNA sample was assayed in triplicates. The housekeeping gene (β-actin) and target genes from each sample were run in parallel on the same plate. The analysis of relative change (relative to the mock-treated control) in mRNA expression of target genes was based on the $2^{-\Delta\Delta Ct}$ method (Livak et al. 2001). The primer sequences for target genes as well as β-actin are listed in Table 1.

Table 1. The primers used in this study for PCR and real-time quantitative PCR assay.

Genes	Primer sequence (5′-3′)	Genbank NO.
β-actin	F: AAGGGTGTAAAACGCAGCTCA	NM_007393.5
	R: CACGCACGATTTCCCTCTCA	
cGAS	F: AGAAGGACTACCTATTCAAGGCT	NM_173386.5
	R: GGGTACGAGATAAAACGGCTC	
IFI204	F: TGGTCCCAAACAAGTGATGGTGC	NM_008329.2
	R: TCAGTTTCAGTAGCCACGGTAGCA	
AIM2	F: AATCTAACCACGAAGTCCC	NM_134059.2
	R: CATTGTCCCTGTTTTATCAT	
DDX41	F: TTGAGGGTGACATTCGTA	NM_134059.2
	R: TGATGGTGACAGGCTTTA	
TLR9	F: ACGGGAACTGCTACTACAAGA	NM_031178.2
	R: CCCAGCTTGACAATGAGGTTAT	
STING	F: AAATAACTGCCGCCTCATTG	NM_028261.1
	R: TGGGAGAGGCTGATCCATAC	
DAI	F: GACGGCGGCCCTGTGAAGAT	NM_021394.2
	R: TTGGCAATGGAGATGTGGCTGTT	
IFN-α	F: ATTTTGGATTCCCCTTGGAG	NM_010504.2
	R: TATGTCCTCACAGCCAGCAG	
IFN-β	F: AGGACGAACATTCGGAAATGT	NM_010510.1
	R: TCTTGGATGGCAAAGGCAG	
IFN-γ	F:GTGGCATAGATGTGGAAGAAAAGA	NM_008337.4
	R: CTGGACCTGTGGGTTGTTGAC	
IL-6	F: CCAGAAACCGCTATGAAGTTCC	NM_031168.2
	R: TTGTCACCAGCATCAGTCCC	
TNF-α	F: ACAGAAAGCATGATCCGCG	NM_013693.3
	R: GCCCCCCATCTTTTGGG	

F: Forward primer, R: Reverse primer.

Immunization of mice with Eg95 in combination of different adjuvants

24 6-week-old BALB/c mice were randomly divided into 4 groups, 6 per group. Group A was immunized with 25 μg Eg95 antigen adjuvanted by 15 μg Quli-A; Group B was immunized with 25 μg Eg95 antigen adjuvanted by 10 μg pUC18-CpG; Group C was immunized with 25 μg Eg95 antigen adjuvanted by 10 μg CpG ODN 1826; Group D was immunized with PBS and set as the Control Group. All the formulas were administered intramuscularly with a total volume of 100 μL. After 28 days of prime immunization, animals in each group were given a boost injection with the same formula. All procedures involving animals conformed to the policies of the local animal care committee.

Antibody titration

Blood samples from each group were collected on day 14, 28 and 42 after the prime immunization, respectively. Sera were separated and stored at -80°C until use. 96-well plates were coated with recombinant Eg95 antigen (5 μg/ mL) at 4°C for 16 hr. The plates were washed with PBST 3 times and blocked with 5% skim milk powder in washing buffer at 37°C for 2 hr. After removing the blocking buffer, the plates were washed 3 times with PBST. Sera were diluted at 1:200 and added to each well (100 μL /well). The plates were incubated at 37°C for 1 hr, washed 6 times, and Goat anti-mouse IgG-HRP (1:8000) was added to each well (100 μL /well). After incubation at 37°C for 1 hr, the plates were washed 6 times, and 100 μL TMB was added to each well. Plates were incubated in the dark for 10 min. The reaction was stopped by the addition of 50 μL of 1 M sulphuric acid to each well. Plates were then read at room temperature at 450 nm.

Antigen specific cytokines measurement

Spleens from each group were crushed in DMEM Basic and pipetted with sterile syringe to obtain homo-

Table 2. Profile of major DNA PRRs in Raw 264.7 cells.

	Genes	Size of amplicons (bp)	Genbank NO.	Results
1	β-actin	216	NM_007393.5	+
2	cGAS	81	NM_173386.5	+
3	IFI204	102	NM_008329.2	+
4	AIM2	176	NM_134059.2	+
5	DDX41	129	NM_134059.2	+
6	TLR9	184	NM_031178.2	+
7	STING	202	NM_028261.1	+
8	DAI	491	NM_021394.2	+

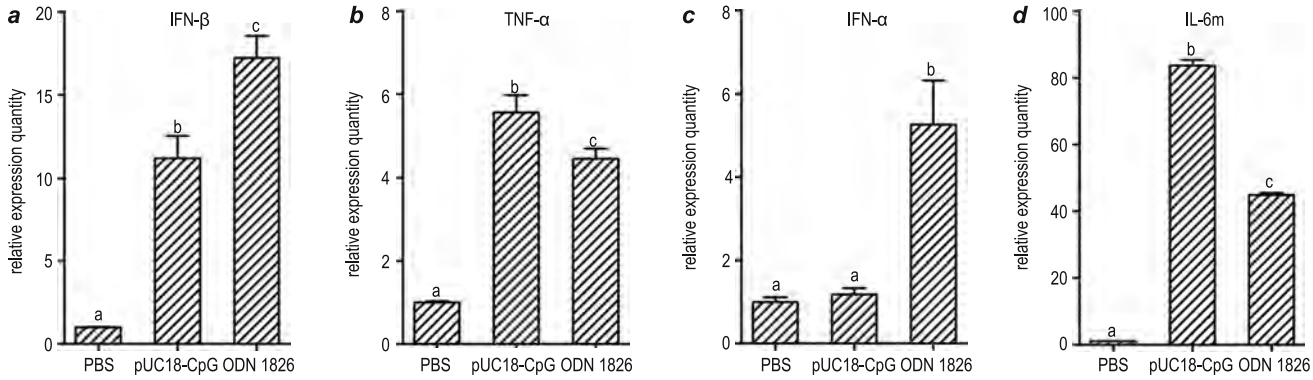

Fig. 1. RAW 264.7 cells were cultured in 6-well plates and stimulated by pUC18-CpG (5 μg/well) or ODN 1826 (5 μg/well), respectively. After stimulation, mRNA was extracted and reverse-transcribed into cDNA. The differential expression of selected genes (IFN-β, Fig. 1a; TNF-α, Fig. 1b; IFN-α, Fig. 1c; IL-6, Fig. 1d) was assessed by SYBR Green based RT-qPCR.

geneous cell suspension. Mononuclear cells were obtained by density gradient centrifugation over Lymphocyte Separation Medium. After counting, cells were allocated and cultured in 24-well plates (2.5×10^5 cells/well). The cells from each group were stimulated *in vitro* with Eg95 antigen (10 μg/ml) for 8 hr at 37°C, then total RNA was extracted from cultured cells and reverse-transcribed into cDNA by use of the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). SYBR Green real-time quantitative PCR was employed for the assessment of antigen-specific cytokines (IFN-β, IFN-γ and TNF-α) expression. Cells mock-treated by medium were used for each group as an internal negative control. The housekeeping gene (β-actin) and target genes from each sample were run in parallel on the same plate. The analysis of relative change (relative to non-treated control) in mRNA expression of target genes was based on the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data analysis was performed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Comparison was made by using the Student's t-test. All values are presented as the mean ± SD. A p-value <0.05 is considered statistically significant.

Results

DNA PRRs in RAW 264.7 cells

As RAW 264.7 is a mouse macrophage cell line, it possesses many traits of innate immune cells. In our study, the expression of major DNA PRRs in RAW 264.7 was verified on the transcriptional level. As presented in Table 2, RAW 264.7 cells are equipped with multiple DNA sensors, including TLR9, cGAS, and STING, etc., which enable them to recognize CpG DNA derived inside the endosome, as well as micro-bial/endogenous DNAs that appear in the cytosolic environment.

pUC18-CpG signals through a TBK1-dependent pathway

As shown in Fig. 1a and 1b, both pUC18-CpG and CpG ODN 1826 induced a Th1 type cytokine profile that was characterized by the up-regulation of IFN-β and TNF-α. CpG ODN 1826 triggered a significant up-regulation of IFN-α, while no effect of pUC18-CpG on IFN-α expression was observed (Fig. 1c).

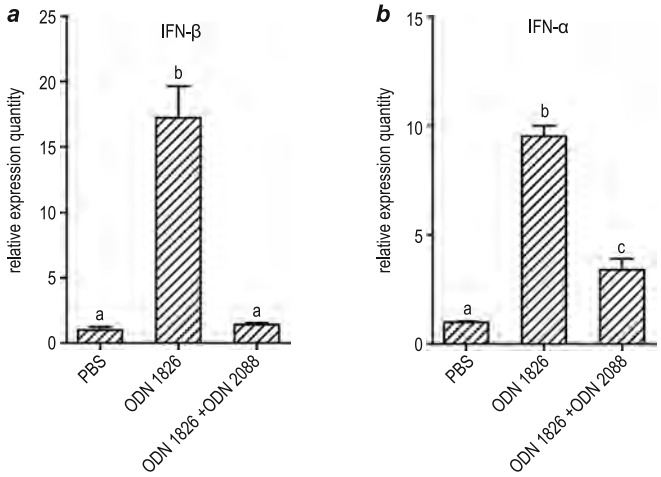

Fig. 2. RAW 264.7 cells were cultured in 6-well plates and firstly treated TLR9 antagonist ODN 2088. After incubation, the cells were stimulated with ODN 1826. After stimulation, mRNA was extracted and reverse-transcribed into cDNA. The differential expression of selected genes (IFN-β, Fig. 2a; TNF-α, Fig. 2b) was assessed by SYBR Green based RT-qPCR.

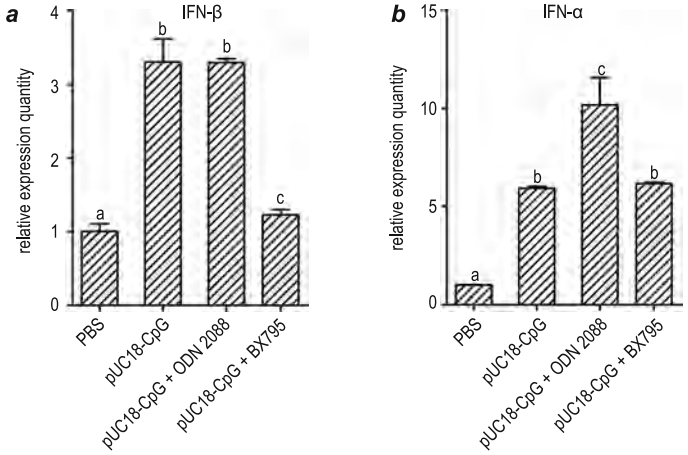

Fig. 3. RAW 264.7 cells were cultured and firstly treated with BX795 or ODN 2088. After incubation, the cells were stimulated with pUC18-CpG. After stimulation, mRNA was extracted and reverse-transcribed into cDNA. The differential expression of selected genes (IFN-β, Fig. 2a; TNF-α, Fig. 2b) was assessed by SYBR Green based RT-qPCR.

In terms of IL-6, which is marked as a Th 2-type cytokine, treatment of RAW 264.7 with pUC18-CpG induced a much higher level of IL-6 than CpG ODN 1826 (Fig. 1d).

When RAW 264.7 were pretreated with the TLR9 antagonist ODN 2088, a paramount inhibitory effect was observed against ODN 1826 as the expression of IFN-β and TNF-α were significantly reduced after stimulation (Fig. 2a, 2b). On the other hand, ODN 2088 proved to have little inhibitory effect on the pUC18-CpG-triggered IFN-β or TNF-α expression (Fig. 3a, 3b). Indeed, pretreatment of RAW 264.7 with ODN 2088 seemed to augment the pUC18-CpG-triggered TNF-α expression (Fig. 3b). Treatment of RAW 264.7 with TBK1 inhibitor BX795 greatly decreased pUC18-CpG-stimumalated IFN-β expression (Fig. 3a). Yet no inhibitory effect was observed in the expression of TNF-α by BX795 after pUC18-CpG stimulation (Fig. 3b).

Comparison of humoral immune responses among different Eg95 immunization groups

Blood samples from each Eg95-immunized group were collected at day 14, 28, 42 after the prime immunization. The average levels of IgG antibody titers in the sera were determined. As shown in Fig. 4, specific humoral immune response could be elicited in each immunization group except the Control Group. Anti-Eg95 IgG antibody levels from the Quli-A-adjuvanted group increased overtime and showed significant difference at each time point when compared to the Control Group ($p<0.05$). The anti-Eg95 IgG antibody levels from either pUC18-CpG or CpG ODN 1826 adjuvanted groups were higher than that of the Quli-A-adjuvanted group at each time point and showed significant difference on Days 14 and 28 ($p<0.05$). No significant difference was observed in the antibody levels between the pUC18-CpG and CpG

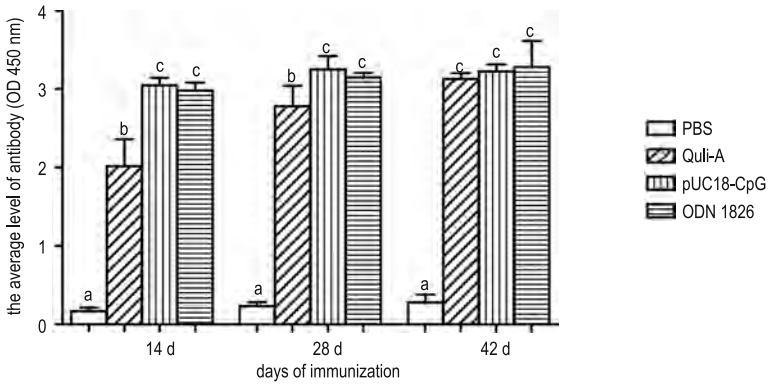

Fig. 4. Blood samples from each vaccinated group were collected on Days 14, 28 and 42, respectively. Antibody titers were determined by reading the adsorption of plates at 450 nm. For each group, antibody titers were expressed as mean ± SD (n = 6). The difference between groups with different letters at each time period is significant ($p<0.05$).

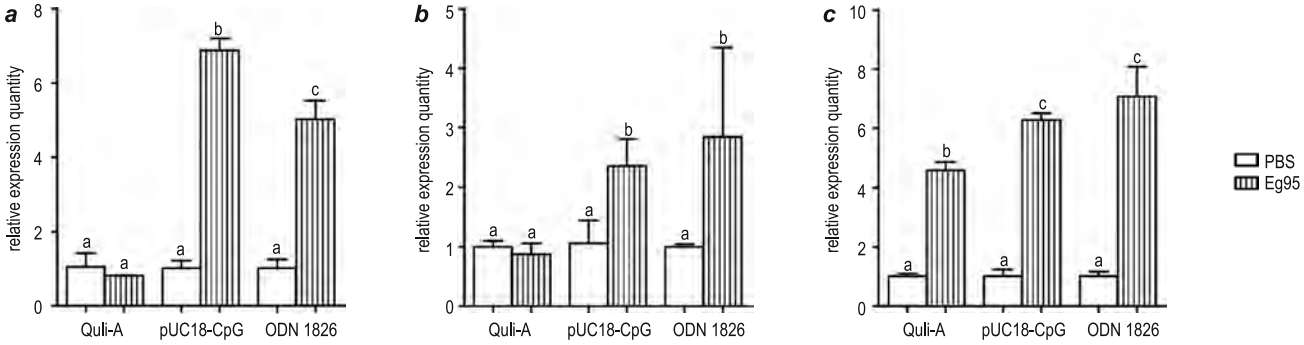

Fig. 5. Mouse spleen mononuclear cells were cultured in 24-well plates and stimulated *in intro* with Eg95 antigen. Total RNA was extracted from cultured cells and reverse-transcribed into cDNA. SYBR Green real-time quantitative PCR was employed for the assessment of antigen- specific cytokines (IFN-β, IFN-γ and TNF-α) expression.

ODN 1826 adjuvanted groups during the experiment, although the latter exhibited higher antibody levels at each time point ($p\geq0.05$).

Quantification of Eg95-specific cytokines upon splenocytes stimulation

As shown in Fig. 5a and 5b, splenocytes from the Quli-A-adjuvanted group showed no significant up-regulation in IFN-β or IFN-γ expression after *in vitro* stimulation with Eg95 antigen, while splenocytes from either pUC18-CpG or CpG ODN 1826 treated groups showed much higher IFN-β and IFN-γ expression when treated with Eg95 antigen ($p<0.05$). On the other hand, Quli-A did significantly up-regulated the expression of TNF-α during splenocyte stimulation ($p<0.05$), while the TNF-α levels were much higher in both the pUC18-CpG and CpG ODN 1826 vaccinated groups after *in vitro* stimulation (Fig. 5c) ($p<0.05$).

Discussion

Plasmid containing multiple CpG motifs have been reported to induce a Th 1 dominant cytokine profile and enhance antigen specific humoral immune response to viral antigens (Guo et al. 2011, Guo et al. 2012, Luo et al. 2012, He et al. 2016). It has been reported that phosphorothioate CpG ODN triggers innate immune activation through the TLR9-mediated signaling pathway (Hemmi et al. 2000, Kumagai et al. 2008, Lange et al. 2018), yet whether CpG motif enriched plasmid also stimulates the TLR9 signaling cascade is currently unknown. On the other hand, TBK1 was previously reported to play crucial roles in the adjuvant effect of DNA vaccines and is a key factor in the cGAS-mediated cytosolic DNA sensing (Ishii et al. 2008, Li et al. 2013, Sun et al. 2013). Hence, the role of TBK1 in pUC18-CpG-triggered immune activation was investigated in the present study. While the use of TLR9 antagonist ODN 2088 did not affect the pUC18-CpG stimulated up-regulation of IFN-β expression in RAW 264.7, the TBK1 inhibitor BX795 treatment of RAW 264.7 strongly down-regulated IFN-β expression, indicating the pivotal role of the TBK1-IRF3 axis in the pUC18-CpG triggered Type I interferons expression. Yet BX795 seemed to have little effect on the pUC18-CpG stimulated TNF-α expression, which needs to be further studied.

Although signaling via different PPRs, both pUC18-CpG and CpG ODN 1826 presented potent adjuvanticity to the recombinant Eg95 antigen, induced higher antibody titers than QuliA, and stimulated a Th 1-type antigen-specific cytokine profile. QuliA was the common adjuvant used in vaccine studies against *Echinococcus granulosus* infection (Heath et al. 2011, Heath et al. 2012). In recent years, other types of adjuvants have been reported for the replacement of Quli-A during Eg95 immunization (Poggio et al. 2016, Umair et al. 2017), albeit few immunostimulators based on PAMPs have been studied. The present study revealed that both CpG ODN and pUC18-CpG enhanced the humoral immune response against Eg95 and induced antigen-specific cytokine expression towards a Th1 biased immune response. The protective efficacy of CpG adjuvanted Eg95 vaccine still needs to be further tested in field experiments using sheep and goats.

Acknowledgments

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