

Vaccination with *Toxoplasma* lysate antigen and CpG oligodeoxynucleotides: comparison of immune responses in intranasal versus intramuscular administrations

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Abstract *Toxoplasma gondii* (*T. gondii*) is one of the most successful intracellular protozoan parasites on earth and highly prevalent in most warm-blooded vertebrates. There are no drugs that target the chronic cyst stage of this infection; therefore, development of an effective vaccine would be an important advance in disease control. Oligodeoxynucleotides (ODN) which contain immunostimulatory CG motifs (CpG ODN) can promote T-helper 1 (Th1) responses, an adjuvant activity that is desirable for vaccination against intracellular pathogen. In this study, we compare the immune responses of *Toxoplasma* susceptible C57BL/6 mice following intranasal and intramuscular vaccination with *Toxoplasma* lysate antigen (TLA) with or without CpG ODN as adjuvant. Immunized and control non-immunized mice were challenged with 85 cyst of the moderately virulent Beverley strain of *T. gondii*. Intranasal vaccination gave significantly a higher protection compared to other groups as indicated by prolonged survival and significantly reduced brain cyst burden ($P < 0.01$). Intranasal vaccination stimulated cellular immunity towards Th1 response characterized by significant INF- γ production

($P < 0.01$). Furthermore, fecal IgA antibody levels as an indicator of mucosal immune responses were significantly higher ($P < 0.05$) in intranasal vaccinated group before the challenge compared to all other groups. Intranasal vaccination was not able to upgrade the Th1 humoral arm. In contrast, intramuscular vaccination enhanced humoral immunity towards a type Th1 pattern characterized by a significant increase of specific IgG and Ig2a. Our results suggest that intranasal administration of CpG/TLA would provide a stable, pronounced, and effective vaccine against toxoplasmosis through stimulation of Th1 cellular immunity and mucosal IgA.

Keywords *Toxoplasma gondii* · CpG ODN · Immunization · Intranasal · IgA

Introduction

Toxoplasma gondii (*T. gondii*) is one of the most successful protozoan parasites on earth and highly prevalent in most warm-blooded vertebrates. After oral ingestion, *T. gondii* crosses the intestinal epithelium, disseminates into the deep tissues, and traverses biological barriers such as the placenta and the blood-brain barrier to reach the sites, where it causes severe pathology (Hunter and Sibley 2012). The following immune mechanisms are widely accepted, following *Toxoplasma* infection; Macrophages infected by *T. gondii* secrete interleukin-12 (IL-12), which activates T cells and NK cells to produce IFN- γ . IFN- γ , in the presence of cofactors such as tumor necrosis factor alpha (TNF- α) in turn, activates macrophage toxoplasmicidal activity. Similarly, stimulated T cells secrete IL-2 and IFN- γ , leading to a Th1 dominant cellular immune response (Gazzinelli et al. 1996).

So far, treatment of this disease is difficult due to the toxic effects of available drugs. In addition, when *T. gondii* encysts in

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the tissues, there are no drug treatments available to eliminate the parasite (Suzuki et al. 2010). Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjuvants in mice, boosting the humoral and cellular response to coadministered antigens (Chu et al. 1997; Cooper et al. 2004). CpG ODN when used as vaccine adjuvants, they increase the speed, magnitude, and duration of vaccine-specific immune responses (Klinman et al. 2010). CpG ODN has been used as an adjuvant for the clearance of a wide range of parasitic, bacterial, and viral pathogens (Corral and Petray 2000; Costa et al. 2012; Daifalla et al. 2012; Freidag et al. 2000; Harandi 2004; Ramirez et al. 2013; Shargh et al. 2012; Srivastava et al. 2013; Teixeira de Melo et al. 2013). Over the last decade, dozens of human clinical trials have been conducted with different CpG ODN for applications ranging from vaccine adjuvant to immunotherapies for allergy, cancer, and infectious diseases with many positive results (Krieg 2012).

In order to elicit maximal levels of Ag-specific immune responses in both mucosal and systemic lymphoid tissue compartments, it is essential to employ an appropriate mucosal adjuvant through the appropriate route (Langermann et al. 1994; Vadolas et al. 1995). Several mucosal adjuvants have been developed (Hagiwara et al. 2003; Yoshino et al. 2004). Nasal route of delivery has been shown to preferentially induce antigen (Ag)-specific antibody (Ab) responses in mucosal lymphoid tissues (Fujihashi et al. 1996; Kurono et al. 1999). Mucosal administration of CpG-ODN stimulates toll-like receptor (TLR) 9 ligand induced protection (Krieg et al. 2004) and targets mucosal DCs to enhance both innate and acquired immune responses (Krug et al. 2001). Additionally, administration of CpG-ODN through the nasal route had been shown to prolong Ag-specific mucosal IgA Ab responses with a balanced Th1 and Th2 type cytokine response (Fukuiwa et al. 2008).

Since *T. gondii* transmission between intermediate hosts is dependent on oral ingestion of walled cysts (Jensen et al. 2013) it would be of pivotal importance if we are able to induce *Toxoplasma*-specific mucosal immune responses. We previously reported the efficacy of CpG-ODN as an adjuvant (El-Malky et al. 2005) when combined with *Toxoplasma* lysate antigen (TLA) against toxoplasmosis through intramuscular administration. We designed this study to determine if intranasal administration of *Toxoplasma* lysate antigen with CpG-ODN as an adjuvant could enhance mucosal immunity and protect genetically susceptible C57BL/6 mice against infection by the moderately virulent Beverley strain of *T. gondii*.

Materials and methods

Mice and parasites

Female, 6–8 weeks old, C57BL/6 mice were obtained from experimental animal unit of Umm AL-Qura University,

Makkah, Saudi Arabia. The experimental animals were kept and handled under the guidelines of the Animal Care Committee of Umm AL-Qura University. The moderately virulent cyst-forming Beverley strain of *T. gondii* was maintained and used for experimental infections as previously described (El-Malky et al. 2005).

Preparation of *Toxoplasma* lysate antigen

TLA was prepared according to the method described before (Lee et al. 1999) with slight modification. Briefly, tachyzoites of the virulent *T. gondii* RH strain were obtained from the peritoneal fluid of intraperitoneally infected mice. The material then passed twice through a 25-gauge needle. The parasites were washed, resuspended in phosphate-buffered saline (PBS), sonicated on ice, and filtered through a 0.22- μ m-pore-size filter. The protein concentration of the TLA was determined using the Bio-Rad protein assay and bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA, USA). The TLA was aliquoted and stored at -20°C until further use.

Oligodeoxynucleotides

Phosphorothioates-modified ODNs were obtained from HSS (Hokkaido System Science, Hokkaido, Japan). The ODNs used in these studies were the CpG 1826 containing two CpG motifs (underlined 5'-TCCATGACGTTCCCTGACGTT-3') and reversed non-CpG 1745 (5'-TCCAATGAGCTTCC TGAGTCT-3'), which are non-stimulatory and used as a control. CpG 1826 has been well characterized for its adjuvant activity with protein antigens (Chu et al. 1997).

Vaccination

Seventy female C57BL/6 mice were assigned into five groups; CpG/TLA intranasal (I.N) vaccinated group, non-CpG/TLA I.N vaccinated group, CpG/TLA intramuscular (I.M) vaccinated group, non-CpG/TLA I.M vaccinated group, and non-vaccinated group. For I.N vaccination, mice were anesthetized with 0.3 ml of ketamine (10 mg/ml) and xylazine (1.0 mg/ml), diluted in 0.9 % NaCl₂, and immunized by slowly pipetting onto the tip of the nose 15 μ l of phosphate-buffered saline (PBS) containing the required dose of TLA (20 μ g) plus 50 μ g of CpG or non-CpG into each nostril. The mice were kept on their backs until a complete recovery from anesthesia. Intramuscular vaccinated mice were immunized into the alternate tibialis anterior muscle with 50 μ l of phosphate-buffered saline (PBS) containing 20 μ g of TLA plus 50 μ g of CpG or non-CpG. non-immunized mice were used as negative controls. Mice were immunized three times at 2-week intervals.

Challenge infection

Brains from mice infected 17 to 21 weeks earlier were harvested and homogenized in 2 ml of PBS (pH 7.4) by multiple passages through a 21-gauge needle. The number of cysts was counted in the brain homogenate. We adjusted LD₅₀ of Beverley strain in our laboratory, and it was found to be 85 cysts. Mice were infected orally 2 weeks after the third booster immunization by gavage, with 150 µl of the brain homogenate containing 85 cysts.

Serum samples

Sera were collected from the mice by retro-orbital puncture 10 days after the third booster dose and 25 and 35 days after challenge infection. Sera were stored at -70 °C for determination of antibodies and cytokine levels as described below.

Specific fecal IgA

Small pieces of freshly voided feces were collected 10 days after the third booster immunization, weighed, and 1 ml of the extraction cocktail was added for every gram of stool. The extract cocktail used was PBS containing 1 % BSA, 5 % fetal bovine serum, 0.05 % sodium azide, and protease inhibitor cocktail (Sigma) (Tochikubo et al. 1998). Mixtures were then vortexed and centrifuged at 15,000 rpm at 4 °C. Supernatants were collected and stored at -80 °C until analyzed. To measure IgA in the stool specific to TLA antigen, 96-well microtiter plates were coated with 10 µg/ml of TLA, blocked with 1 % BSA in PBS, and incubated with stool extracts diluted at 1 to 100. Then peroxidase-conjugated goat anti-mouse IgA α was added. ABTS was used as a substrate. Optical densities were measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 405 nm. For descriptive purposes, anti-TLA titers were expressed as group means \pm SD of individual group OD values, which were themselves the average of duplicate assays.

Measurement of antibody responses

Total IgG, IgG1, and IgG2a specific to TLA were quantified by ELISA assay on individual mice serum samples. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with TLA in a concentration of 10 µg/ml. After washing, the plates were blocked with 1 % BSA in Tris-buffered saline (TBS) for 3 h at room temperature and then overlaid with 2,000 diluted mouse sera for total IgG or 1,000 diluted sera for IgG1 and IgG2a. Detection was carried out with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, or IgG2a (2,000 dilution) (Kirkegaard and Perry laboratories, Inc., Gaithersburg, MD, USA). ABTS (2,2-azino-di [3-ethyl-benzthiazoline

sulfonate]; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was used as a substrate. Optical densities were measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 405 nm. For descriptive purposes, anti-TLA titers were expressed as group means \pm SD of individual animal OD values, which were themselves the average of duplicate assays.

Measurement of cytokine levels

Two weeks after the third booster immunization, four mice from each group were sacrificed and their mesenteric lymph nodes (MLN) and spleens were removed aseptically and placed in 5 ml of RPMI 1640 medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 0.05 mM mercaptoethanol. Cell suspensions were centrifuged at 1500 \times g at 4 °C for 5 min. The supernatant was decanted, and the erythrocytes were lysed by resuspension of the pellet in 5 ml 0.17 M NH₄CL. Following four washes in RPMI 1640 medium, the cells were resuspended in 2 ml of RPMI 1640 medium supplemented as above and viable cells were counted by trypan blue exclusion. Cell suspensions were adjusted to 2 \times 10⁶ cells per milliliter, and aliquots of 100 µl each, containing 2 \times 10⁵ cells, were added to the wells of 96-well flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark) which contained either 100 µl of TLA per well at concentrations of 40 µg/ml, ConA 10 µg/ml (Sigma, St Louis, Mo, USA), or RPMI 1640 medium alone. Thus, final concentrations of TLA were 20 µg/ml and that of ConA was 5 µg/ml (Gazzinelli et al. 1991). Spleens were examined individually from each mouse in triplicate. The cultures were incubated for 48 h at 37 °C and 5 % CO₂. Supernatants were collected from cultures and stored at -70 °C till cytokine quantification. Cytokine (INF- γ and IL-4) levels were measured in the supernatants of cell cultures and at day 35 sera by capture ELISA using AN'ALZA kit (TECHNE, MN, USA), according to the manufacture instructions.

Enumeration of *T. gondii* brain cyst burden

At day 35 postinfection, survived mice were sacrificed and their brains were homogenized with a mortar and pestle in 3 ml of PBS. Total brain cyst burden was counted as before.

Statistical analysis

SPSS software was used for data analysis. Descriptive statistics including the mean \pm standard deviation (SD) were used. A non-parametric Mann-Whitney test was used to test for significant differences between groups. The data were considered significant if *P* values were less than 0.05.

Results

All of these experiments have been repeated twice with almost similar results.

Protection against LD₅₀ challenge

Protection against challenge infection was assessed by monitoring survival rate and counting cysts in the brains of mice.

Survival rate

Survival rate was monitored following challenge with 85 *Toxoplasma* cysts. All I.N and I.M CpG/TLA vaccinated mice survived infection and were healthy throughout the observation period (Fig. 1). non-CpG/TLA I.N immunized group survived early infection and 20 % died at day 28. Non-CpG/TLA I.M immunized group survived early infection and 30 % died at day 21. On the other hand, 40 % of non-immunized group died from acute illness 14 days after challenge and 30 % died at day 22.

Brain cyst burden

At day 35 following challenge, survived mice were sacrificed, and total brain cyst burden was counted. A significantly fewer brain cyst load ($P < 0.01$) was detected in CpG/TLA I.N immunized group compared to the other groups of mice (Fig. 2).

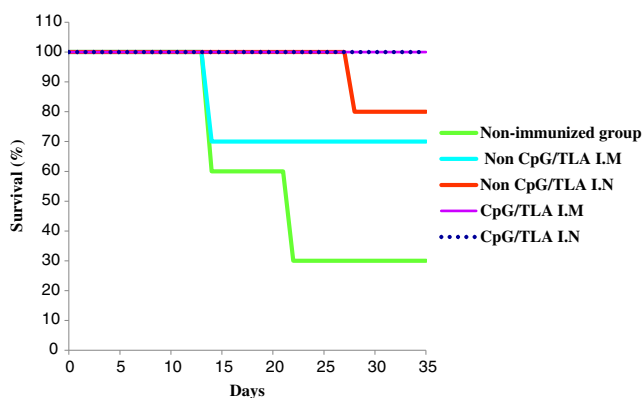


Fig. 1 The percent survival of mice infected with *T. gondii* cyst (RRA strain). Groups of mice were infected 2 weeks after the third booster vaccination and monitored thereafter. All I.N and I.M CpG/TLA vaccinated mice survived infection and were healthy throughout the observation period (Fig. 1). Non-CpG/TLA I.N immunized group survived early infection and 20 % died at day 28. Non-CpG/TLA I.M immunized group survived early infection and 30 % died at day 21. On the other hand, 40 % of non-immunized group died from acute illness 14 days after challenge and 30 % died at day 22

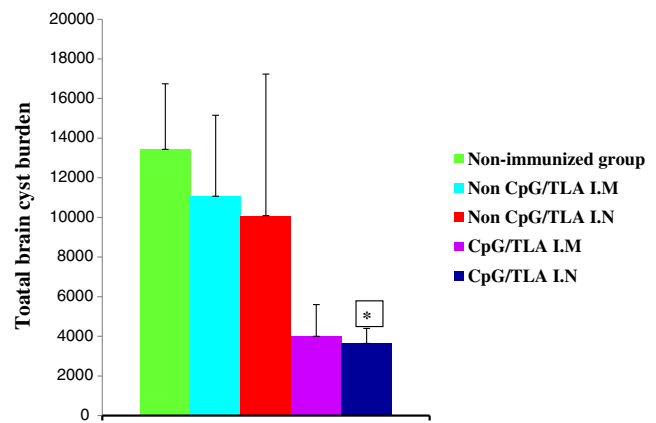


Fig. 2 Comparison of the number of brain cysts of *T. gondii* in five groups of mice. Mice were sacrificed 35 days after challenge infection, and total cysts in each brain were counted, results are expressed as mean \pm SD. CpG/TLA I.N vaccinated mice showed significant decrease in number of brain cysts ($P < 0.01$) when compared with other groups of mice

Fecal specific IgA

We estimated fecal IgA as an index for mucosal humoral immune responses before challenge infection. CpG/TLA I.N vaccinated mice showed a significant increase in fecal-specific IgA ($P < 0.05$) when compared to other of groups of mice (Fig. 3).

Antibody subclass responses to TLA

Sera collected 10 days after the third booster, 25 and 35 days postinfection were assessed for total IgG, IgG1, and IgG2a. The CpG/TLA I.M immunized group induced significant levels of total IgG and IgG2a response at all-time points examined ($P < 0.01$ and < 0.05 , respectively) compared with the other groups of mice examined (Figs. 4 and 5). During early

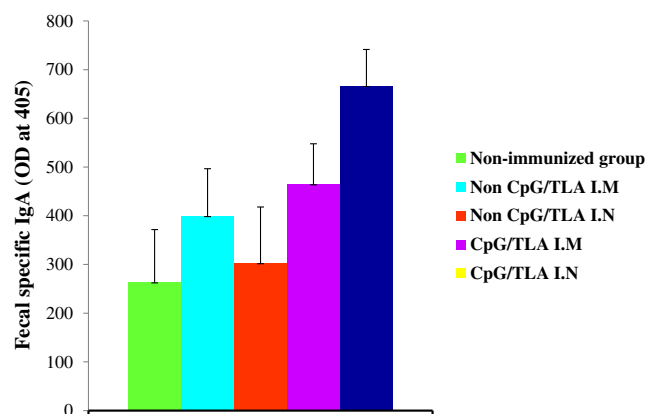


Fig. 3 Comparison of *T. gondii* fecal-specific IgA in five groups of mice. Results are expressed as mean \pm SD. CpG/TLA I.N vaccinated mice showed a significant increase in fecal-specific IgA ($P < 0.05$) when compared with other groups of mice

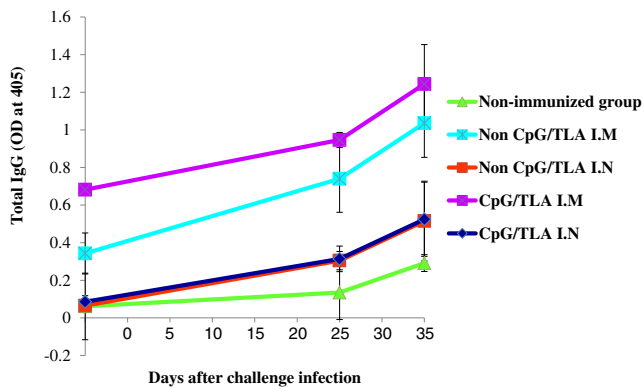


Fig. 4 Kinetics of total IgG of *T. gondii* in five groups of mice. Sera collected at the time points indicated, results are expressed as mean \pm SD. CpG/TLA I.M vaccinated mice showed significant increase in total IgG of *T. gondii* versus all groups at the time points examined ($P < 0.01$)

infection, none of the examined groups elicited a detectable IgG1 response; on the other hand, at 25 and 35 days after infection, non-vaccinated mice showed a significant increase in IgG1 of *T. gondii* versus other groups examined ($P < 0.01$) (Fig. 6).

Cellular immune response

Two weeks after the third booster dose, spleen and mesenteric lymph nodes (MLN) cell suspensions from four individual mice/groups were stimulated in vitro with TLA. Spleen cells and MLN from CpG/TLA I.N vaccinated mice produced significant levels ($P < 0.01$) of INF- γ when stimulated with TLA (Fig. 7). Serum INF- γ level was undetectable in any of the examined groups before infection and was significantly high ($P < 0.001$) in both CpG/TLA I.N- and I.M-treated groups at the end of the observation period (day 35) (Fig. 7). We investigated whether splenocytes or MLN secreted the Th2-associated cytokine IL-4 when stimulated with TLA.

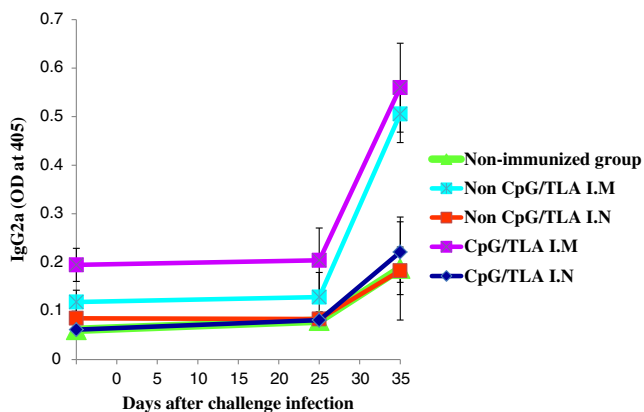


Fig. 5 Kinetics of IgG2a of *T. gondii* in five groups of mice. Sera collected at the time points indicated, results are expressed as mean \pm SD. CpG/TLA I.M vaccinated mice showed a significant increase in IgG2a of *T. gondii* versus all groups at the time points examined ($P < 0.05$ for each)

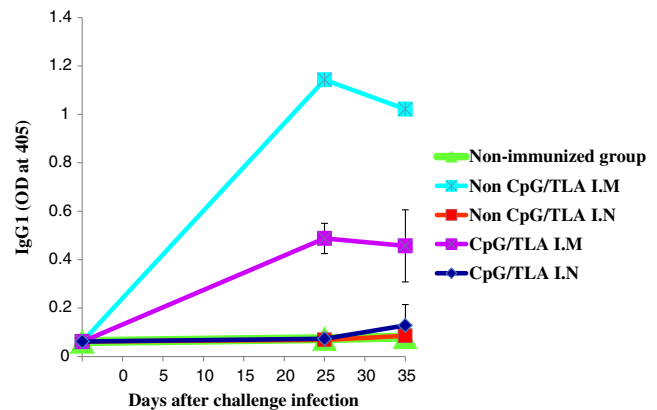


Fig. 6 Kinetics of IgG1 of *T. gondii* in five groups of mice. Sera collected at the time points indicated, results are expressed as mean \pm SD. There were no significant differences in IgG1 of *T. gondii* between the five groups of mice before challenge infection. At days 25 and 35 after infection, non-vaccinated mice showed a significant increase in IgG1 of *T. gondii* versus other groups examined ($P < 0.01$)

IL-4 was undetectable in supernatants or serum from any of the group's analyzed (Unpublished data).

Discussion

The possibility of successful vaccine development in toxoplasmosis has been strengthened by recent advances in our understanding of the nature of protective immunity to *T. gondii* and pathogenesis of the disease. In addition, accumulating evidence indicates that multi-antigen immunizations are needed for the development of an effective vaccine against complex pathogens such as parasites (Angus et al. 2000; Fachado et al. 2003). Previously, we confirmed that *Toxoplasma* lysate antigen is an antigen mixture with enough

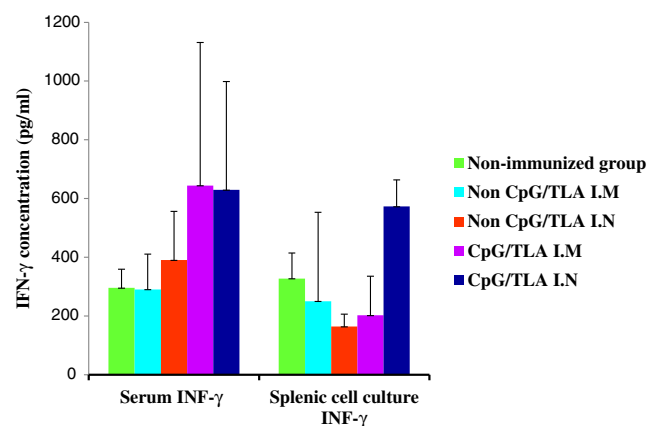


Fig. 7 Serum and splenic cell culture IFN- γ production from five groups of mice, results are expressed as mean \pm SD. Spleen cells and MLN from CpG/TLA I.N vaccinated mice produced significant levels ($P < 0.01$) of INF- γ when stimulated with TLA. Serum INF- γ level was undetectable in any of the examined groups before infection and was significantly high ($P < 0.001$) in both CpG/TLA I.N- and I.M-treated groups at the end of the observation period (day 35)

antigenicity for murine immune system (El-Malky et al. 2005). CpG when used as an adjuvant can stimulate multiple types of immune cells, leading to enhanced Th1 response characterized by the production of INF- γ , IL-6, IL-12, IL-18, and tumor necrosis factor alpha (Krieg 2002). The production of these cytokines represents an early event in the defense mechanisms against toxoplasmosis (Cai et al. 2000; Jebbari et al. 1998). Since *T. gondii* transmission between intermediate hosts is dependent on oral ingestion of walled cysts (Jensen et al. 2013) it would be of great importance if we could enhance *Toxoplasma*-specific mucosal immune responses. Accordingly, we constructed this experimental study to compare immune responses elicited in *Toxoplasma* susceptible mice following intranasal vaccination with CpG/TLA versus that elicited following intramuscular administration.

In the current study, all I.N and I.M CpG/TLA vaccinated mice survived acute infection and were healthy throughout the observation period compared with other groups of mice examined. Furthermore, significantly fewer cysts were recovered from brains of I.N CpG/TLA vaccinated mice compared to other groups, which indicate the superiority of I.N route over I.M route of administration. To reveal the possible underlying reasons for such superiority, we analyzed mucosal, humoral, and the cell-mediated immune responses in the different groups of mice.

The cellular arm of the Th1 response is essential for controlling intracellular pathogens (Corral and Petray 2000); therefore, we examined the cytokines produced after the third immunization and before challenge. MLN and splenocytes from mice I.N vaccinated with CpG/TLA adjuvant were able to proliferate and produced significantly higher amounts of INF- γ when stimulated in vitro with their specific antigen (TLA) compared to other groups of mice. However, by the end of the observation period, serum INF- γ was high and comparable in both CpG I.N- and I.M-treated mice. According to the results of this experiment, we concluded that I.N vaccination of CpG/TLA in comparison with I.M route was more effective to stimulate early Th1 cellular immune responses.

Although cellular immunity is considered the most important part of the immune response to *T. gondii* (Liu et al. 2009), antibodies do play a role in limiting its spread because specific antibodies inhibit the attachment of the parasite to the host cell receptors, and macrophages kill intracellular parasites coated with antibodies (Kang et al. 2000).

It is well accepted that pathogen-specific secretory IgA antibodies play a key role as the first defense line against infectious diseases (Asanuma et al. 2012). IgA is the most abundant antibody isotype in mucosal secretions and owes its success in frontline immunity to its ability to undergo transcytosis across epithelial cells (Cerutti et al. 2011). Mucosal IgA comprises antibodies

that recognize antigen with high- and low-affinity binding modes (Macpherson et al. 2008). Moreover, IgA neutralizes inflammatory microbial products inside epithelial cells (Fernandez et al. 2003). Interestingly, in accordance with these concepts about IgA, in the current experiment, *Toxoplasma*-specific fecal IgA was significantly higher in I.N CpG/TLA compared to other groups of mice

We measured the titers of total IgG, IgG1, and IgG2a antibodies raised against TLA. We detected significant levels of total IgG and IgG2a from the sera of mice immunized I.M with CpG/TLA at all-time points examined, this is consistent with the enhancement of IgG2a isotype switching previously reported for CpG ODN adjuvant administrated I.M (Corral and Petray 2000; Freidag et al. 2000; Kumar et al. 2004; Tewary et al. 2004). I.N vaccinated mice induced weak IgG and IgG2a antibody responses. There were no significant differences in IgG1 of *T. gondii* between the five groups of mice before challenge infection. At days 25 and 35 after infection, non-vaccinated mice showed a significant increase in IgG1 of *T. gondii* versus other groups examined ($P < 0.01$). We concluded that I.N administration was able to enhance mucosal IgA production but couldn't enhance humoral arm of Th1 response. On the other hand, I.M administration enhanced humoral Th1 response. Despite of higher levels of Th1 humoral response induced via I.M route, brain cyst burden was significantly fewer in I.N vaccinated mice which points to the importance of mucosal IgA in the protection induced in I.N vaccinated mice. We suggest that the pre-challenge high levels of intestinal IgA could neutralize or block the orally administrated *Toxoplasma* cysts.

Together with the cytokine data, these results clearly show that TLA with CpG adjuvant administrated I.N induced a Th1-oriented immune response in C57BL/6 mice before infection. After challenge, Th1 responses remained dominant in I.N- and I.M-treated groups till the end of observation period as manifested by high serum INF- γ compared to residual groups. Like other studies, our results confirm that CpG ODNs are excellent Th1 adjuvants. In a study conducted by Liu et al. (2009), they showed that the co-delivery of CpG ODN with DNA plasmid vaccine has yielded poor and, in some cases, impaired immune responses. Authors hypothesized that such inhibitory effect may be the result of inhibition of cellular uptake of plasmid by CpG-ODN.

Altogether, our data indicate that the protection observed here is well linked to the combination of a multi-*Toxoplasma* antigen (TLA) and an adequate adjuvant (CpG ODN) through an appropriate route of administration.

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