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Adjuvant Effect of Oligomannose-Coated Liposome-Based Platform for Vaccine against African Trypanosomosis

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ABSTRACT

Since an oligomannose-coated liposome (OML) vaccine strategy has been shown to induce a T helper-type 1 (Th1) immune response, it might be a suitable method to control the early stage of African trypanosomosis. In order to evaluate the adjuvant effect of OML, the present study was conducted to investigate the effect of bovine serum albumin (BSA)-encapsulating OML (BSA + OML) on host immunity and the *Trypanosoma brucei gambiense* infection in mice. Immunization with the BSA + OML induced strong *ex vivo* proliferation of lymphocytes, and the cultured cells notably produced interleukin (IL)-2, interferon (IFN)- γ , tumor necrosis factor (TNF) - α , IL-6, IL-10, IL-4, and IL-5. The sera from the mice immunized with BSA + OML displayed the similar cytokines profile, but, in addition, they contained IL-12p70. Following *T. b. gambiense* infection, the BSA + OML group exhibited a significantly reduced number of the parasites, slightly delayed survivals, and the reduction of IL-10 in the sera as compared to the other groups, possibly due to a skewed Th1 immune response. Therefore, the OML-based vaccine described here could provide a platform to apply for various protozoan diseases susceptible to Th1-immunity as a vaccine adjuvant.

Key words: Adjuvant; African trypanosome; Immunization; Oligomannose-coated liposome (OML)

INTRODUCTION

Oligomannose-coated liposome (OML) can be used as a potential delivery carrier by targeting host macrophages (Ikehara and Kojima, 2007). When administered to mice, the OML, which is made of a neoglycolipid composed of oligomannose and dipalmitoylphosphatidylethanolamine (DPPE) (Mizuochi, 1991), is rapidly and effectively incorporated by intraperitoneal macrophages probably via the mannose receptor (Shimizu *et al.*, 2007). Subsequently, the OML-incorporated macrophages move to and accumulate in lymphoid tissues (Ikehara *et al.*, 2006). Finally, administration of the OML encapsulating antigens is able to induce a cellular immune response characterized by delayed hypersensitivity to the antigens without any detectable toxicity (Mizuochi *et al.*, 1989a). Especially, immunization with the soluble leishmanial antigen encapsulated in the OML was shown to lead to clear protection against *Leishmania major* infection, concomitant with a dominant induction of the protozoa-specific T helper-type 1 (Th1) immune response (Shimizu *et al.*, 2007; Shimizu *et al.*, 2003). Taken together, our results indicate that the OML functions in (i) delivery of the encapsulated antigens to macrophages, (ii) homing of the incorporating macrophages to

lymphoid tissues, and (iii) subsequent induction of Th1 immune response specific for the antigens.

Since an OML vaccine has been shown to induce a Th1 immune response, it might offer a suitable strategy to control several protozoan diseases that are susceptible for Th1 immunity (Gazzinelli and Denkers, 2006). African trypanosomes, which are extracellular parasites, are well-known to evade the host's humoral (Th2) immune responses by constantly modifying their variant surface glycoproteins (VSG) (Barbet and McGuire, 1978; Donelson *et al.*, 1998). On the other hand, many investigators have described the involvement of the Th1 immune response in the resistant phenotype to African trypanosome infection (Hertz *et al.*, 1998; Magez *et al.*, 1993; Magez *et al.*, 1999; Uzonna *et al.*, 1999). Indeed, the production of interferon (IFN)- γ has been shown to be linked with resistance to *Trypanosoma brucei rhodesiense* infection (Hertz *et al.*, 1998; Schleifer *et al.*, 1993). Furthermore, the IFN- γ , in association with the interleukin (IL)-12-dependent synthesis of IgG2a and the secretion of tumor necrosis factor (TNF)- α , was reported to play an important role in the protection against *T. congolense* infection in mice (Uzonna *et al.*, 1998a, 1999). Recently, in our preliminary experiment, immunization with the soluble trypanosome antigen (the soluble lysates of *T. b. gambiense*) encapsulated in the OML was tested in mice, but the OML vaccine did not provide any protection against *T. b. gambiense* infection (Kuboki *et al.*, 2007). Interestingly, however, bovine serum albumin (BSA)-encapsulated OML (BSA + OML) suppressed parasitemia development during the course of infection. Therefore, in order to evaluate the adjuvant effect of OML, the present study was conducted to investigate the effect of the BSA + OML on the host immunity and prophylactic effect against *T. b. gambiense* infection in mice.

MATERIALS AND METHODS

Parasites and culture

The bloodstream form (BSF) of *T. b. gambiense* IL2343 was previously obtained from the International Livestock Research Institute, Nairobi, Kenya, and maintained at the National Research Center for Protozoan Diseases (Inoue *et al.*, 2000). The parasites were cultured *in vitro* using an HMI-9 growth medium (Hirumi and Hirumi, 1989, 1991) composed of Iscove's modified Dulbecco's medium (IMDM; Sigma, St. Louis, MO) supplemented with 20% fetal calf serum (FCS; Biological Industries, Ashrat, Israel), 100 U - 100 μ g/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Sigma), 0.1 mM bathocuproine (Sigma), 1 mM pyruvic acid (Sigma), 10 ml/l HT supplement (0.1 mM sodium hypoxanthine and 0.016 mM thymidine) (Invitrogen), 0.4 mg/ml BSA (Sigma), 1 μ g/ml bovine holo-transferrin (Sigma), 0.2 mM 2- β -mercaptoethanol (Wako, Osaka, Japan), 2 mM L-cysteine (Sigma), and 60 mM HEPES (Sigma) at pH 7.2.

Preparation of liposomes

Dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylcholine (DPPC), and cholesterol were purchased from Sigma. Mannotriose, referred as M3 [$\text{Man}\alpha\text{1-6}(\text{Man}\alpha\text{1-3})\text{Man}$], was purchased from Dextra Laboratories (Reading, UK). Neoglycolipids (M3-DPPE) were prepared by conjugating the M3 with DPPE as described previously (Mizuochi, 1991; Mizuochi *et al.*, 1989b). Liposomes were prepared as described previously (Fukasawa *et al.*, 1998; Shimizu *et al.*, 2003). Briefly, a chloroform-methanol (2:1, v/v) solution containing 1.5 μ mol of DPPC and cholesterol was placed in a conical flask and then dried by rotary evaporation. Subsequently, 2 ml of ethanol containing 0.15 μ mol of M3-DPPE was added into the flask and then evaporated to prepare a lipid film containing the neoglycolipid. Two hundred microliters of PBS containing 2 mg/ml BSA was added to the dried lipid film, and multilamellar vesicles were prepared by intense vortex dispersion. The multilamellar vesicles were then

extruded 10 times through a 1- μ m pore polycarbonate membrane (Nucleopore, Pleasanton, CA). The OML encapsulating BSA was separated from free BSA by 3 successive washes in PBS with centrifugation (20,000 x g, 30 min, at 4°C).

Immunization and infection

BALB/c mice (n = 10 per group) were immunized twice subcutaneously with 3 μ g BSA encapsulated within OML (BSA + OML group), 3 μ g BSA in 100 μ l PBS (BSA group), or PBS (PBS group). A booster immunization was administered after a 7-day interval. Seven mice were injected intraperitoneally with 1,000 cells of *T. b. gambiense* BSF on day 7 after the second immunization. Parasitemia was monitored daily by tail vein puncture. All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

Splenocyte preparation and sera collection

Seven days after the second immunization, 3 mice were sacrificed from each group, and a single cell suspension was prepared from the spleen of each mouse as described previously (Hertz *et al.*, 1998). Blood was also collected by heart puncture, and the serum of each mouse was prepared and stored at -80°C until use. Furthermore, serum samples were also obtained from the tail vein of mice on day 5 post-infection (DPI).

Lymphoproliferation assay

The lymphocyte proliferative response of the collected splenocytes (2.5 x 10⁵ cells/well) was performed in triplicates in 96-well cell culture plates (Nunc A/S, Denmark). The splenocytes were cultured in an RPMI1640 (Sigma) medium supplemented with 10% FCS with or without 5 μ g/ml concanavalin A (Con A; Sigma) at 37°C for 72 hrs in a humidified atmosphere containing 5% CO₂ as described previously (Okamura *et al.*, 2004). Lymphoproliferation was determined using a non-radioactive Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

Determination of cytokine concentrations in the supernatant of splenocyte culture and in sera

For the evaluation of cytokine secretion from prepared splenocytes, the cells were first adjusted to 2.5 x 10⁶ cells/well in a final volume of 1 ml in 24-well cell culture plates (Nunc A/S, Denmark). After the cells were stimulated *ex vivo* with or without 5 μ g/ml of Con A at 37°C for 72 hrs in a humidified atmosphere containing 5% CO₂, the culture supernatants were collected and frozen at -80°C until use. Cytokine concentrations (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF- α and IFN- γ) were quantified in the cell culture supernatants and in collected sera using a cytometric bead array (CBA; corresponding mouse flex sets and mouse/rat soluble protein master buffer kit, Becton Dickinson, San Diego, CA), following the manufacturer's suggested protocols.

Statistical analyses

P-values were calculated using the Student's *t*-test for statistical analyses of parasitemia, lymphoproliferation, and cytokine concentrations. The survival rates of all groups were plotted according to a Kaplan-Meier method and compared statistically by a log-rank test (Mantel-Cox).

RESULTS

Lymphoproliferation of splenocytes via OML-based immunization

The proliferative ability of splenocytes obtained on day 7 after the second immunization was examined in the absence or presence of Con A. The culture was conducted under an appropriate condition of the cultured medium containing 10% FBS. Accordingly, in the absence of Con A, the splenocytes

proliferated more strongly in the BSA + OML group than in the PBS group (*, $P < 0.05$, Fig. 1A). Furthermore, Con A stimulation also appeared to enhance the proliferative ability at higher levels in the BSA + OML group than in the other BSA and PBS groups, although the differences were not significant (Fig. 1B). The results indicate that OML-based immunization induces *ex vivo* lymphocyte proliferation.

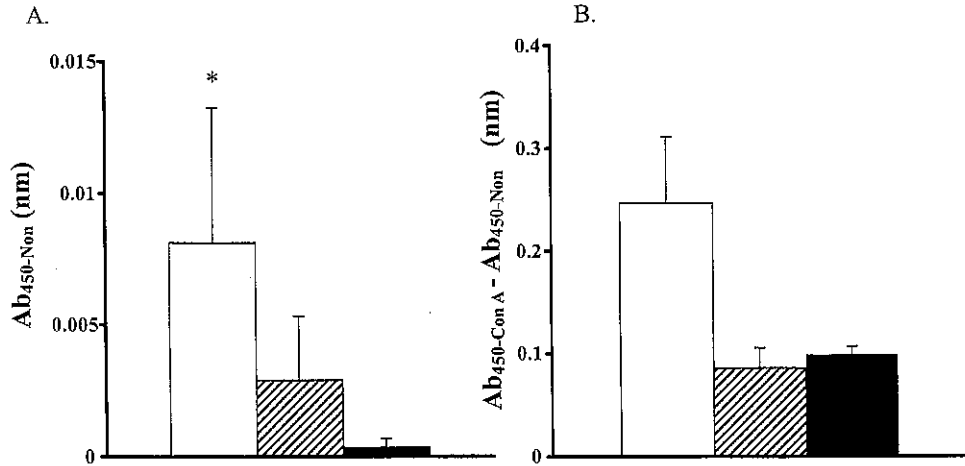


Fig. 1. Proliferation effect of splenocytes via immunization of BSA in OML. Splenocytes were collected on day 7 after the second immunization. The ability of cells to proliferate in the absence (A) or presence of 5 $\mu\text{g/ml}$ Con A (B) was evaluated using the CCK-8, and the absorbance was measured at 450 nm ($\text{Ab}_{450\text{-Non}}$ or $\text{Ab}_{450\text{-Con A}}$, respectively). The degree of lymphoproliferation under a Con A-stimulation was determined by subtracting the $\text{Ab}_{450\text{-Con A}}$ from $\text{Ab}_{450\text{-Non}}$. The vacant, striped, or filled column indicates the values from the BSA + OML-, BSA-, or PBS-immunized group. $P < 0.05$ was considered statistically significant. Data are presented as the mean \pm standard error (SE). * indicates a significant difference from the PBS group. The data are representative of three separate experiments.

Cytokine production via OML-based immunization

The levels of cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF- α and IFN- γ) produced by the cultured splenocytes were examined in the supernatant following stimulation with or without Con A (Table 1, Supernatant in splenocytes). Accordingly, under the non-stimulated condition, the cells from the BSA + OML group produced a significantly higher level of IL-2 (* and #, $P < 0.05$) and apparently higher induction of IFN- γ , TNF- α , IL-6, IL-10, IL-4, and IL-5 than the cells from the BSA and PBS groups, which had undetectable or rarely detectable levels. In response to Con A, the cells from the BSA + OML group also displayed more significant induction of IL-5 (* and #, $P < 0.05$) and apparently higher induction of IFN- γ , IL-6, and IL-10 than those from the BSA and PBS groups. In the BSA and PBS groups, Con A stimulation similarly showed the induction of IFN- γ , TNF- α , IL-6, IL-10, and IL-5. The BSA group exhibited the highest level of IL-4 among the three groups. The IL-12p70 was below the detectable level in all groups with and without stimulation.

Table 1. Cytokine responses in the splenocyte supernatants, and the sera^a.

Cytokines	Groups	Supernatant of splenocyte cultures ^b		Sera ^c	
		Unstimulated	Stimulated with Con A (5 µg/ml)	Pre-infection	Post-infection
IFN-γ	BSA+OML	2,429 ± 1,463	1,379 ± 735	67 ± 9 ** [†]	1,093 ± 142 [‡]
	BSA	< 5	427 ± 128	23 ± 1	625 ± 51 ** [‡]
	PBS	< 5	467 ± 73	12 ± 4	1,074 ± 57
IL-12p70	BSA+OML	< 9	< 9	333 ± 53 * [‡]	< 9
	BSA	< 9	< 9	52 ± 10 * [‡]	149 ± 71
	PBS	< 9	< 9	< 9	< 9
IL-2	BSA+OML	246 ± 50 * ^d	5 ± 1	56 ± 4 ** [‡]	< 2
	BSA	13 ± 4	11 ± 6	20 ± 2 * [‡]	39 ± 14
	PBS	11 ± 3	6 ± 1	< 2	< 2
TNF	BSA+OML	469 ± 147	1,066 ± 86	2,118 ± 298 * [‡]	< 17
	BSA	< 17	802 ± 90	369 ± 75 * [‡]	1,282 ± 679
	PBS	< 17	965 ± 116	< 17	< 17
IL-6	BSA+OML	880 ± 445	657 ± 309	125 ± 14 ** [‡]	419 ± 114
	BSA	12 ± 9	193 ± 47	33 ± 4	295 ± 87
	PBS	12 ± 6	281 ± 18	20 ± 7	419 ± 114
IL-10	BSA+OML	1,666 ± 1,166	2,148 ± 474	548 ± 51 ** [‡]	161 ± 57 *
	BSA	< 16	1,267 ± 551	90 ± 8 * [‡]	597 ± 155
	PBS	< 16	1,457 ± 528	< 16	445 ± 60
IL-4	BSA+OML	26 ± 14	24 ± 6	92 ± 10 * [‡]	< 1
	BSA	< 1	103 ± 81	35 ± 5 * [‡]	68 ± 22
	PBS	< 1	57 ± 33	< 1	< 1
IL-5	BSA+OML	686 ± 400	262 ± 59 * [‡]	176 ± 18 * [‡]	< 5
	BSA	< 5	82 ± 20	57 ± 5 * [‡]	114 ± 47
	PBS	< 5	73 ± 16	< 5	< 5

^a Values are expressed as pg/ml.

^b Supernatant were collected from the splenocytes that were derived from the mice 1 wk after the second immunization.

^c Sera were collected 1 wk after the second immunization (Pre -infection) and 5 days after the infection (Post-infection).

^d Statistical significance was observed as compared to PBS-immunized group (*, $P < 0.05$, **, $P < 0.01$) or BSA+OML immunized group (†, $P < 0.05$, ‡, $P < 0.01$).

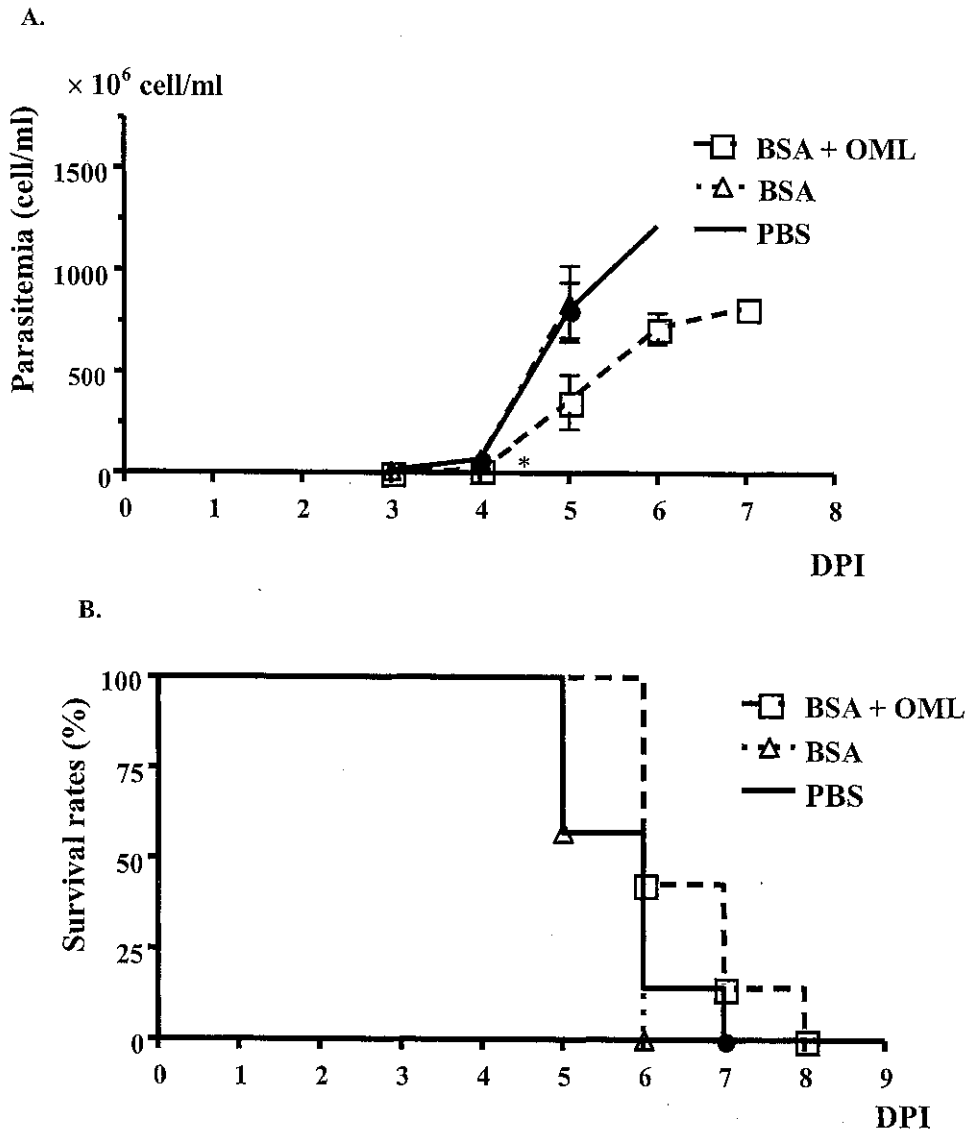


Fig. 2. Anti-trypanosome effect of BSA in OML. BALB/c mice were immunized subcutaneously twice with 3 μ g of BSA + OML, BSA, and PBS at 7-day intervals. Seven days after the second immunization, each mouse was injected intraperitoneally with 1,000 cells of *T. b. gambiense* IL2343. The parasitemia (A) and survival rates (B) were monitored as described in Materials and Methods. Data are presented as the mean \pm SE. Statistical analyses were assessed by the Student's *t*-test (parasitemia) or by a log-rank test (Mantel-Cox) (survival rates). $P < 0.05$ was considered statistically significant. * indicates significant differences between the BSA in OML and PBS groups ($P < 0.05$). The data are representative of three separate experiments.

The levels of cytokines were also examined in the sera obtained on day 7 after the second immunization (Table 1, Sera, Pre-infection). Interestingly, the BSA + OML group showed significantly higher levels of IFN- γ , IL-2, IL-6, and IL-10 (**, $P < 0.01$) and more significant increases of IL-12p70, TNF- α , IL-4, and IL-5 (*, $P < 0.05$) than the PBS group. In comparison, the BSA group showed more significantly selective inductions of IL-12p70, IL-2, TNF- α , IL-10, IL-4, and IL-5 than the PBS group (*, $P < 0.05$), but the levels

were significantly lower than those in the BSA + OML group (^{##}, $P < 0.01$).

Anti-trypanosome effect of OML-based immunization

The ability of BSA + OML to protect against acute *T. b. gambiense* infection was tested in mice. Immunization with BSA + OML resulted in a significantly greater reduction of parasitemia on 4 DPI (*, $P < 0.05$) than that observed in the PBS group (Fig. 2A). Although significance of the survival was not observed among the three groups, the BSA + OML group was shown to delay the average mortality by about 6.6 days, while the BSA and PBS groups had delays of about 5.6 and 5.7 days, respectively (Fig. 2B). These results indicate that immunization with BSA + OML showed a slight, but significant anti-trypanosome effect.

Cytokine levels after *T. b. gambiense* infection

The cytokine levels were examined in the sera collected on 5 DPI (Table 1, Sera, Post-infection). The BSA + OML group displayed a more significant decrease of IL-10 than the PBS group (*, $P < 0.05$), although other cytokine levels were similar between the two groups. The BSA group showed a significant decrease of IFN- γ (*, $P < 0.05$) and an increase of IL-4 (*, $P < 0.05$) as well as apparent increases of IL-12p70, IL-2, TNF- α , and IL-5, which is in contrast to the findings in the PBS group. The BSA + OML group showed more significant IFN- γ secretion ([#], $P < 0.05$) but relatively less IL-10 suppression than the BSA group.

DISCUSSION

In the present study, we examined the adjuvant effect of OML on the host Th1 cytokine induction and protection of immunized mice against the lethal infection of *T. b. gambiense*. BSA + OML induced strong *ex vivo* proliferation of lymphocytes. An incidental response to BSA in an FBS-containing culture medium was considered to cause the proliferation. The lymphocytes from the BSA + OML group also notably produced IL-2, IFN- γ , TNF- α , IL-6, IL-10, IL-4, and IL-5, indicating that OML has adjuvant ability to activate splenocytes capable of strong induction of the above cytokines in immunized mice. In fact, the sera from mice immunized with BSA + OML also displayed a similar profile of these cytokines, together with IL-12p70, which is an important Th1 cytokine (Szabo *et al.*, 1995). In comparison, BSA immunization induced only selective increases of IL-12p70, IL-2, TNF- α , IL-10, IL-4, and IL-5, but the levels were significantly lower than those of the BSA + OML group.

Following *T. b. gambiense* infection, the BSA in the OML group exhibited a significantly reduced number of parasites and slightly delayed survivals when compared to the other control groups. At 4 DPI, the BSA + OML group showed similar cytokine responses to the PBS group but a significantly lower induction of IL-10. The results suggest that the reduction of IL-10, as well as the general response against the infection, can give a resistant phenotype to the acute protozoan infection in immunized mice. Even though the role of IL-10 against trypanosomiasis remains controversial (Namangala *et al.*, 2001; Shi *et al.*, 2003; Shi *et al.*, 2006; Uzonna *et al.*, 1998a, 1998b; Uzonna *et al.*, 1998c), as shown in a previous study (Namangala *et al.*, 2007), the study here indicates that suitable reduction of IL-10 leads to resistance against *T. congolense* infection by skewing to a Th1 immune response. In sharp contrast, the BSA group showed a significant reduction of IFN- γ and prominent induction of IL-4 but was ineffective against the infection, possibly because of a skewed Th2 immune response.

The present study showed that OML has adjuvant efficacy that is strong enough to induce multiple cytokine inductions in immunized mice. Although the efficacy might be due to the heterogeneous response to an antigen of *T. b. gambiense*, OML showed an adjuvant effect capable of giving a resistant phenotype to immunized mice via a Th1 immune response. Therefore, the OML-based vaccine described here could

provide the platform to apply for Th1-immune susceptible protozoan diseases as a vaccine adjuvant.

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