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Research Article

Enhanced Humoral Response Induced against *Plasmodium falciparum* Asexual Blood Stage Immunogens in Mice after Complementary DNA Primed-Recombinant Hybrid Qβ Phage Boost

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Abstract

Dendritic cells are vital in the initiation and regulation of immune responses against invading pathogens. Here we asked whether the immune response against asexual blood stage malaria can be improved by combining DNA vaccine targeting UB05-MSP3 to the dendritic cells *in situ* with a recombinant QβUB05MSP3 hybrid phage boost.

We generated a DNA vaccine candidate encoding for fusion of *Plasmodium falciparum* UB05MSP3 antigens and single-chain antibody specific to antigen uptake receptor DEC-205 expressed on both mouse and human DCs (scDEC205). DNA vaccine (scDEC205-UB05MSP3) was administrated in mice by conventional intramuscular injection. Animals were boosted intranasally either with recombinant QβUB05, QβMSP3 or QβUB05MSP3 phage. The parasite invasion inhibition assay was done using mice antisera against 3D7 *Plasmodium falciparum* strain. The reactivity of specific IgA and IgG subclasses were determined by ELISA. The complementary prime-boost immunization with DNA and QβUB05MSP3 induced more pilly potent antibody response that resulted to two time more effective parasite invasion inhibition than the monoclonal antibodies (positive control) and antisera of others mice groups ($P<0.0001$). The mice boosted with QβUB05MSP3 phage had significantly higher IgA and IgG antibody responses than those boosted with DNA, QβUB05 or QβMSP3 ($P<0.01$). The significantly higher reactivities of IgG2b and IgG3 against MSP3 and UB05 antigens separately were shown in QβUB05MSP3 and QβUB05 boosted mice compared to the others groups ($p<0.05$). These findings showed that complementary QβUB05MSP3 hybrid phage boost vaccination enhances plasmid DNA immunogenicity of asexual blood stage antigen, resulting to an effective parasite invasion inhibition through a significant reactivity of IgG2b and IgG3 antibody against surface antigens.

Keywords: Complementary; Prime-boost strategy; Qβ Phages; Vaccination; Malaria.

Introduction

The fundamental initial step in vaccination is that the vaccine molecule must be taken up, processed, and presented by dendritic cells (DCs) which are vital for initiating and regulating many aspects of immune response [1-4]. DEC-205 is an endocytic receptor expressed at the highest level by dendritic cells within the T-cell areas of lymphoid tissues which are necessary for

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generating immunity and tolerance [5-7]. The Generation of anti-DEC205/CD205 monoclonal antibodies that recognize conserved epitopes in different mammals, and its incorporation in vaccine molecule has been reported to enhance the antigen presentation on major histocompatibility complex (MHC) class I and class II as well as strong protective immunity [4, 5, 8-10]. A particular attention is being focused on the development of DNA vaccines targeting DCs as a potential immunogenic strategy to elicit both humoral and cellular immune reactions even at a considerably reduced dose [4, 11, 12]. Since asexual blood-stage parasites of Plasmodium are the main cause of malaria pathogenesis and mortality in infected individuals, immune responses targeting blood-stage antigens was reported with a crucial importance for malaria control [13]. Merozoite surface Protein 3 (MSP3) and UB05 protein are two merozoite blood stage antigens that are being considered as potential malaria vaccine candidates [14-16]. MSP3 was selected on basis of antibody-dependent cellular inhibition (ADCI) of parasite growth in vitro [14, 15] and has been evaluated in several clinical studies [17-19]. UB05 was first isolated and characterized in Cameroon, and showed a significant reaction with sera of not only semi-immune adults, but also of children having low or non-detectable parasitaemia. [16]. Although several malaria blood-stage vaccine candidates are at different stages of development demonstrating their capacity to induce immune responses, they failed to achieve efficacy outcomes even [20, 21]. In context of vaccine development, the use of heterogeneous prime-boost strategies with DNA and recombinant protein is actually a beneficial approach to induce a potential protective immune response against infectious diseases. However, the virus like particles-based vaccine has taken the first place in term of subunit immunogen compared to the single recombinant proteins. In fact, genetically linking of promising immunogens to the surface of Qβ phages allowed them to be displayed at high density [22-24]. This high-density display, as previously reported in our group enhanced antigenicity and immunogenicity of the desired immunogen [24]. The present study aimed to assess the humoral immune response of DNA encoding scDEC-UB05MSP3 in a complementary prime-boost immunization with Qβ phages (QβMSP3, QβUB05 or QβUB05MSP3) in mice.

Materials and Method

Mice

Six to eight weeks old BALB/c mice (with 20-30 g) were purchased from Laboratory of Animal Science Department of "Université des montagnes" of Bangangte (Cameroon). They were maintained under standard conditions with 12/12hrs of the dark and light cycle. Mice were fed with standard commercial die (50% corn flour, 20% wheat bran, 23% fish flour and 70% bone flour) and water was provided *ad libitum*. The mice were maintained under pathogen-free animal facility of the same laboratory throughout the study period. Mice were acclimatized for two weeks before immunization with the vaccine candidates. The experimental protocol was done according to the guidelines of the Organisation for Economic Co-operation and Development (OEDC, 2011).

Cell lines, media, and antibodies.

293A and CHOhuDEC205 cells lines were maintained in DMEM supplemented medium (10% foetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine). 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) liposome was used for stable 293A cell transfection. Antibodies used in the study included mouse monoclonal antibodies X-Q38G73-N (anti-UB05 N-terminal IgG, Abmart), X-Q38G73-C (anti-UB05 C-terminal IgG, Abmart), X-Q0KGH2-M (anti-MSP3 M-terminal IgG, Abmart) and X-Q0KGH2-C (anti-MSP3 C-terminal IgG, Abmart), horseradish peroxidase (HRP)-conjugated anti-mouse IgA, IgG, IgG1, IgG2a, IgG2b, and IgG3 antibodies (Southern Biotech), Biotin-conjugated Mouse anti-human IgG (Southern Biotech), APC-conjugated streptavidin (BD Biosciences) and Goat anti-mouse IgG1-APC (BD Biosciences).

Construction and characterization of DNA vaccines

The full-length region coding for fusion of *Plasmodium falciparum* UB05-MSP3 protein and a single-chain antibody specific to DEC205 (scDEC205-UB05MSP3) was designed and inserted in the pCMV-IRES-EGFP (kanamycin) vector. The pCMV-SFL HD83UB05MSP3-IRES-EGFP plasmid was then cloned within the NEB 5-alpha competent E. coli (Biolabs Inc. New England) in LB medium Giga preparation (invitrogen, USA). The cloned product was extracted using the DNA Qiagen kit as recommended by the manufacturer. The ScDEC205-UB05MSP3 recombinant protein was expressed from stably transfected 293A cells with encoding DNA and DOTAP liposome in supplemented DMEM medium. The recombinant protein produced in the supernatant was collected and purified by anti-FLAGRM1 Purification technique according to SIGMA-ALDRICH (USA) recommendations.

Biological activity assessment of scDEC205-UB05MSP3 protein for its binding CHO cells-expressed human DEC-205

This experiment consisted to investigate the integrity of synthetized SCDEC205-UB05MSP3 protein through assessing its capacity to be specifically directed to DCs via DEC-205 receptor. The Chinese hamster ovary (CHO) cell lines expressing human DEC205 (CHO/hDEC205) were used to performed this experiment as previously described [4]. CHO/hDEC205 cells stored in liquid nitrogen were

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thawed, washed and transferred into 20 ml pre-warmed supplemented DMEM medium in T-75 cm2 tissue culture flasks (Thermo Scientific, Denmark). The cells were cultured for seven days at 37°C in 5% CO_2 incubator with daily renewing of Complete DMEM medium for revitalization. After seven days of culture, the cells were transferred into 15 ml falcon tube and washed with FACS buffer (2% FBS in PBS v/v). The cells were incubated for 20 min at 4°C with graded dilutions of scDEC205-UB05MSP3 protein solution (1; 1/3; 1/30; and 0) in FACS. The cells were washed with FACS buffer and incubated (for 20 min at 4°C) with 100 µl/well of diluted specific mouse anti-MSP3 and UB05 IgG in FACS buffer (1/200). The unbound human antibodies were removed by washing the plate, and the cells were suspended with 100 µl/well of diluted (1/1000) Goat Biotinilated-anti-mouse IgG (Southern Biotech, USA) in FACS buffer and incubated as previously mentioned. The cells were washed and incubated (20 min at 4°C in dark) 100µl of diluted (1/1000) anti-biotin APC (Miltenyi Biotec, USA). The cells were then washed and transferred into labeled FACS tubes in 450µl of FACS buffer. The acquiring of cells was performed using BD FACSCantoTM II cytometer. The acquired data were managed using FlowJo software (version 9.8.5).

Characterization of scDEC205-UB05MSP3 protein by western blot analysis

The western blot analysis was carried out as we have previously described [25]. About 92.5 µl of culture supernatant, lysate or purified DEC205-UB05MSP3 protein was mixed with 2.5 µl of β-Mercaptoethanol and 5 µl SDS (20%) then incubated for 1 hr at 37°C. Then 100 µl of 2x Laemmli SDS buffer was added and further incubated for 10 mins at 100°C. The samples were loaded in a 10% polyacrylamide gel and allowed to run for 1hr intervals at 30, 50 and 70 volts. The separated proteins were then transferred unto a nitrocellulose membrane, blocked with 1x roti block and probed with Plasma containing high titres of MSP3 and UB05 specific polyclonal antibodies. Specific recognition of the immunogens was revealed with Horse radish peroxidase-conjugated Mouse anti-human IgG (Southern Biotech, Birmingham, USA) diluted 1:4000 in 1x roti block. Bound conjugate was detected using 1-step ultra TMB blotting solution (Thermo scientific, USA) and the HRP reaction stopped by washing with molecular grade pure water.

Reactivity of purified scDEC205UB05MPS3 protein with monoclonal antibodies targeting the N-and C-terminal regions of UB05 and MSP3 antigens

The purified scDEC205-UB05MPS3 protein was diluted in PBS so that 100 µl containing 50ng of protein were added/well to high binding 96-well flat bottom (Thermo Fisher scientific) ELISA plates and incubated overnight at 4°C. The following day, plates were washed 3x with PBST (PBS with 0.05% Tween-20) and blocked with 2% BSA for one hour at 37°C. The coated plates were then probed with 100µl/well of graded concentrations (1000, 100, 10, 1 and 0.1 ng/ml diluted in 2% BSA) of monoclonal antibodies X-Q38G73-N (anti-UB05 N-terminal), X-Q38G73-C (anti-UB05 C-terminal), X-Q0KGH2-M (anti-MSP3 M-terminal) and X-Q0KGH2-C (anti-MSP3 C-terminal), and incubated for two hours at 37°C. Unbound antibodies were removed by washing 5x (198 µl/well) with PBST. Reactivity of the monoclonal antibodies with the scDEC205-UB05MPS3 protein was probed with Horse radish peroxidase-conjugated Goat anti-mouse IgG (Southern Biotech, Birmingham, USA) diluted at 1:4000 in 2% BSA. Bound conjugate was detected using ABTS substrate and the HRP reaction was stopped by adding 100 µl a stop solution according to the manufacturer’s protocol (Southern Biotech, Birmingham, USA). The colorimetric signal was measured at 405 nm using a multiscan FC microplate reader (Thermo Fisher 6cLentLic, USA).

Engineering of recombinant phages QβMSP3, QβUB05 and QβUB05MSP3

UB05, MSP3 and UB05-MSP3 fusion proteins were engineered in frame with the minor coat protein A1 of RNA-coliphage Qβ as described in our previously work [25].

Mice immunization

Mice immunization was performed as previously described with some modifications at the level of the way and the period of immunization [4, 8]. After two weeks of acclimatization, BALB/c mice were randomly divided into seven groups of five animals/ group (Table 1). Five groups of mice received an intramuscular administration of the DNA encoding for scDEC205-UB05MSP3 protein (50 µg/mice) in both anterior foot pads in a total volume of 100 µl of endotoxin-free PBS (CORNING, Manassas, USA). One mice group received 100 µl of endotoxin-free PBS as negative control. Beside the negative control group, there was one group of five mice called the pré-immunized mice (Group 7) that did not receive anything. Two weeks later, four mice groups previously vaccinated with DNA were boosted by either intramuscular route with scDEC205-UB05MSP3 DNA (50 µg/100µl PBS), or intranasal route with recombinant phages QβUB05, QβMSP3 or QβUB05MSP3 in 35 µl PBS (2•108 particles/mice). The control mice were treated with the same component as previously done during the first immunization. At any vaccine candidate administration, the cleaning of the injection site was done by rubbing with a sterile piece of gauze soaked in 70% ethanol. All injections were done using a sterile syringe fitted with 25gauge needle. Two weeks later mice blood samples were collected in labelled anticoagulant-free tubes by retro-orbital sinus bleeding using capillary tubes, and serum samples were extracted and used for antibody measurement.

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Table 1: Immunization protocol of BALB/c mice with the malaria vaccine candidates

Groups	First Immunization: Week-0	Boost Immunization: Week-2
Group 1	scDEC205-UB05MSP3 DNA	-
Group 2	scDEC205-UB05MSP3 DNA	scDEC-205-UB05MSP3 DNA
Group 3	scDEC205-UB05MSP3 DNA	QβUB05
Group 4	scDEC205-UB05MSP3 DNA	QβMSP3
Group 5	scDEC205-UB05MSP3 DNA	QβUB05MSP3
Group 6	PBS	PBS
Group 7	-	-

In vitro Parasite invasion inhibition assay

This experiment was carried out to assess the re-invasion/growth inhibition activity of the mouse anti- UB05 and MSP3 antisera using a modified protocol [16, 26]. Briefly, human group O^+ erythrocytes infected with *P. falciparum* 3D7 strains were maintained in vitro at pH 7.4 in plastic petri dishes at 3% hematocrit in Malaria culture medium (MCM) in 5% CO_2 and 95% N_2 humidified incubator at 37°C. Cultures were synchronized three times per week by re-suspending culture pellets in MCM with human group O+ erythrocytes. In effect, the parasite load was determined using microscopy after thick and thin blood smear staining with 10% diluted Giemsa. *P. falciparum*-infected red blood samples were centrifuged at 1200 rpm for 10 min. The supernatant was removed and the pellet containing the RBC was washed twice at 1200 rpm for 10 min with an equal volume of MCM. The pellet of red blood cell was resuspended in MCM at 1% haematocrit and then cultured for 24 h at 37°C in humidified incubator. Parasite cultures were synchronized the day before starting the assay. The cells were harvested at late-stage schizonts and washed three times with MCM. Infected red blood cells suspension with initial parasitaemia of 0.4% (with 1% hematocrit) was then distributed in 96 wells (100µl/well) round bottom culture plate (Corning, USA). The duplicate of mice sera, plasma samples from malaria exposed and non-exposed human as well as Purified Mouse anti-MSP4 IgG antibody sample (positive control) were added in appropriate wells of infected red blood cells suspension. The plate was incubated at 37°C in 5% CO_2 humidified incubator. After 48 hrs, 5µl of culture medium was gently mixed into all wells and incubation continued. After 80 hrs, parasitaemia of each culture well was determined on the 10% Giemsa-stained thin films with microscope. The slides were read by two technicians and the mean parasitaemia was used to determine the percentage of parasite invasion inhibition. The Percentage of parasitic inhibition was determined as follow: [1– (proportion of target cells invaded in presence of immune sera/proportion of target cells invaded in MCM)] × 100.

Specific antibodies measuring in mice serum

The sera levels of antibodies specific to the malaria immunogens UB05, MSP3 and UB05-MSP3 were determined by indirect ELISA assay. Briefly high binding 96-well Costar assay plates (CORNING, USA) were coated with 10^7 particles/well of recombinant QβUB05, QβMSP3 or QβUB05MSP3 and incubated at 4°C overnight. The following day, the plates were washed 3x with PBST (198µl/well; PBS with 0.05% v/v Tween 20), and blocked with 200µl/well of 2% bovine serum albumin (BSA) for 1 hour at 37°C. The mice sera were diluted serially in PBS (1/100 to 1/204800 dilution) then 100 µl/well added in triplicate and incubated for 2 hours at 37°C. The plates were washed 5x (198 µl/well) with PBST after which the bound antibodies were probed with the HRP-conjugated anti-mouse IgA, IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech, Birmingham, USA) diluted 1:4000 in PBS. Bound conjugate was detected using ABTS substrate and stop solution according to the manufacturer's protocol (southern biotech, Birmingham USA). The colorimetric signal was measured at 405 nm using a multiscan FC microplate reader (Thermo Fisher Scientific, USA). The final values were calculated by subtracting the OD of pré-immunized mice sample considered as blank.

Statistical analysis

Data analysis was performed with Graphpad Prism Software version 6.1. Data were expressed as mean ± standard deviation (SD). Comparisons of means among the animal groups were performed using ANOVA test with turkey multiple comparisons post-test. Statistical difference was confirmed at $P < 0.05$.

Results

scDEC205-UB05-MSP3 DNA Construct and expression of the recombinant protein

The DNA coding for the UB05-MSP3 fusion protein was clone in frame with a single chain antibody gene targeting DEC205 (scDEC205-UB05MSP3) on dendritic cells. The scDEC205-UB05MSP3 expression cassette was then inserted upstream of the IRES sequence of EGFP reported plasmid (figure 1A). To express scDEC205-UB05MSP3, 293A cells were stably transfected with the encoding DNA. The fluorescence microscopy analysis showed an effective production of the recombinant protein by the transfected cells (characterized by persistent expression of enhanced green fluorescent protein (EGFP)), compared to the non-transfected control cells (figure 1A). The supernatants of the stable transfectant were tested for the scDEC205-UB05MSP3 binding to Chinese hamster ovary (CHO) cell lines stably expressing the human DEC205 receptor upon their surface (CHOhuDEC205). Here a selective binding of CHOhuDEC205 was observed only with scDEC205-

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UB05MSP3, but not with PBS (figure 1B). As shown in figure 1C, the C and N- terminal part of MSP3 and UB05 were specifically well recognized by their appropriate antibodies on the scDEC205-UB05MSP3. However, the anti-UB05 N-terminus and anti-MSP3 M-terminus antibodies showed superior reactivity with UB05 and MSP3 antigen respectively, compared to anti-UB05 C-terminus and anti-MSP3 C-terminus antibodies.

(A) scDEC205-UB05MSP3 expression after 293A cells stably transfected with scDEC205-UB05MSP3 DNA. (B) Binding assay to CHO-hDEC205 cells, using graded volumes (3–100 µl) of scDEC205-UB05MSP3 transfected stable cell line supernatant. (C) Specific recognition of UB05MSP3 protein transfectants supernatant by purified mouse antibodies specific to N, M and C-terminal.

Assessment of parasite invasion inhibition with sera from immunized mice

The invasion inhibition activity of antisera specific to UB05/MSP3 antigens from immunized mice was assessed to determine the biological activity of antibodies induced in immunized mice. Figure 2 shows that the antisera of mice primed and boosted with only scDEC205-UB05MSP3 DNA significantly induced a higher parasite invasion inhibition compared to the control group of mice (P= 0.0126), thought it was lower than the positive control (anti-MSP4), but not statistically significant (P= 0.0898). The antisera of mice primed with scDEC205-UB05MSP3 DNA and boost with either QβUB05 or QβMSP3 induced the percentage of invasion inhibitions comparable to the positive control (anti-MSP3). A significantly higher parasite invasion inhibition (39.1 ± 2.9%) was shown with antisera of mice vaccinated with scDEC205-UB05MSP3 DNA + QβUB05MSP3 compared to all the groups (P<0.0001).

Complementary scDEC205-UB05MSP3 DNA prime and QβUB05MSP3 boost enhanced the immunogenicity of malaria blood stage vaccine

To assess the immunogenicity of scDEC205-UB05MSP3 DNA, the determination of endpoint titres of IgA and IgG antibodies specific to UB05MSP3 protein was carried out in mice sera vaccinated with scDEC205-UB05MSP3 DNA once, a DNA/DNA prime-boost, a scDEC205-UB05MSP3 DNA and recombinant phage QβUB05MSP3 prime-boost, or only received PBS. It was shown that scDEC205-UB05MSP3 DNA elicited a higher antibody response in mice just after the first vaccine candidate administration compared to PBS vaccinated mice (P < 0.05) (figure 3). The significantly higher titres of specific IgA, IgG1, IgG2a and IgG2b antibodies were shown in mice that received scDEC205-UB05MSP3 DNA prime and Qβ-UB05MSP3 boost as compared to the mice that were prime-boosted with scDEC205UB05MSP3 DNA (P< 0.01). Compared to the other antibodies, the titre of specific IgG3 isotype was revealed gradually important in mice receiving one dose of scDEC205-UB05MSP3 DNA (4800 ± 2263), two dose of scDEC205-UB05MSP3 DNA (13600 ± 8314), and then in mice immunized with scDEC205-UB05MSP3 DNA + QβUB05MSP3 (2565000 ± 70.71) (figure 3E).

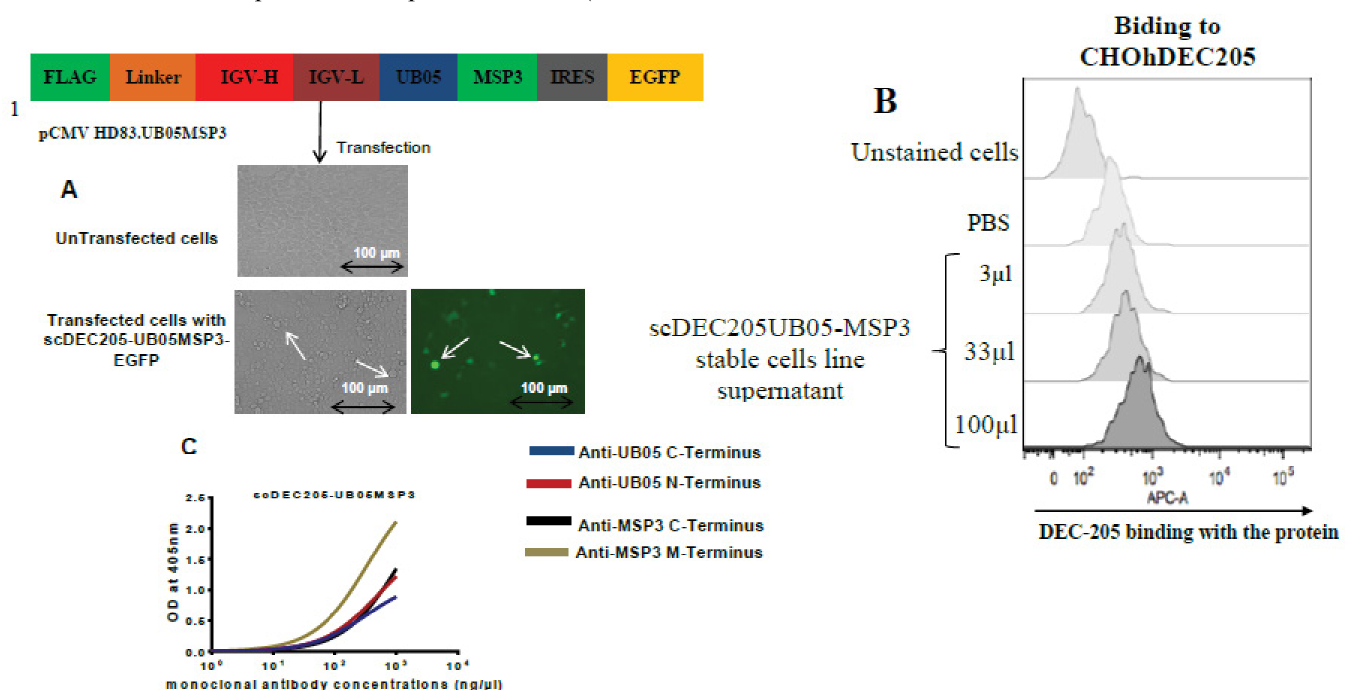

Figure 1: Genetic engineering of UB05MSP3 with the single chain of the anti-DEC205 antibody.

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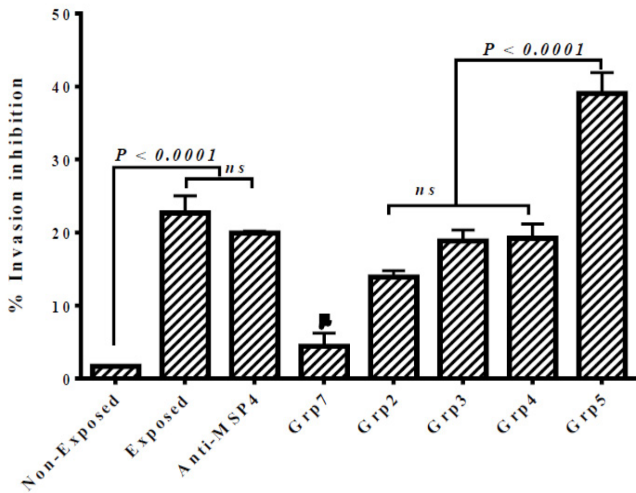

Figure 2: Invasion inhibition studies with antisera of immunized mice.

Reactivity of specific IgA responses against MSP3/UB05 protein in mice after complementary prime boost

Overall, IgA reactivity against MSP3/UB05 antigens was lower in mice immunized with only scDEC205UB05MSP3 DNA compared to those receiving a complementary prime-boost immunized with scDEC205UB05MSP3 DNA and recombinant phages ($p<0.05$) (figure 4). The anti-MSP3 IgA level was significantly higher in QβUB05MSP3 boosted mice (0.232 ± 0.006) compared to those boosted with QβMSP3 (0.114 ± 0.026) or QβUB05 (0.096 ± 0.021) phages ($p<0.0001$) (figure 4A). The same results were observed when determining the IgA reactivity against UB05 or UB05MSP3 ($p<0.0001$) in complementary prime-boost immunized mice (figure 4B&C).

Figure 3: Titre of IgA and IgG subclasses specific to QβUB05MSP3 in immunized mice.

The titres of antibodies specific to QβUB05MSP3 were determined by ELISA in mice sera (n = 5) after scDEC205UB05MSP3 DNA prime, or scDEC205UB05MSP3 DNA prime and recombinant phage QβUB05MSP3 boost. Results were expressed as mean of endpoint titre + standard deviation (SD). The comparison of antibody titres was done according to the protocol of immunization. (A) Specific IgA response to UB05MSP3. In (B), (C), (D) and (E) same as in (A) but the response against UB05MSP3 is assessed with IgG1, IgG2a, IgG2b and IgG3 respectively. *P< 0.05; **P< 0.01;***P <0.001; ****P <0.0001.

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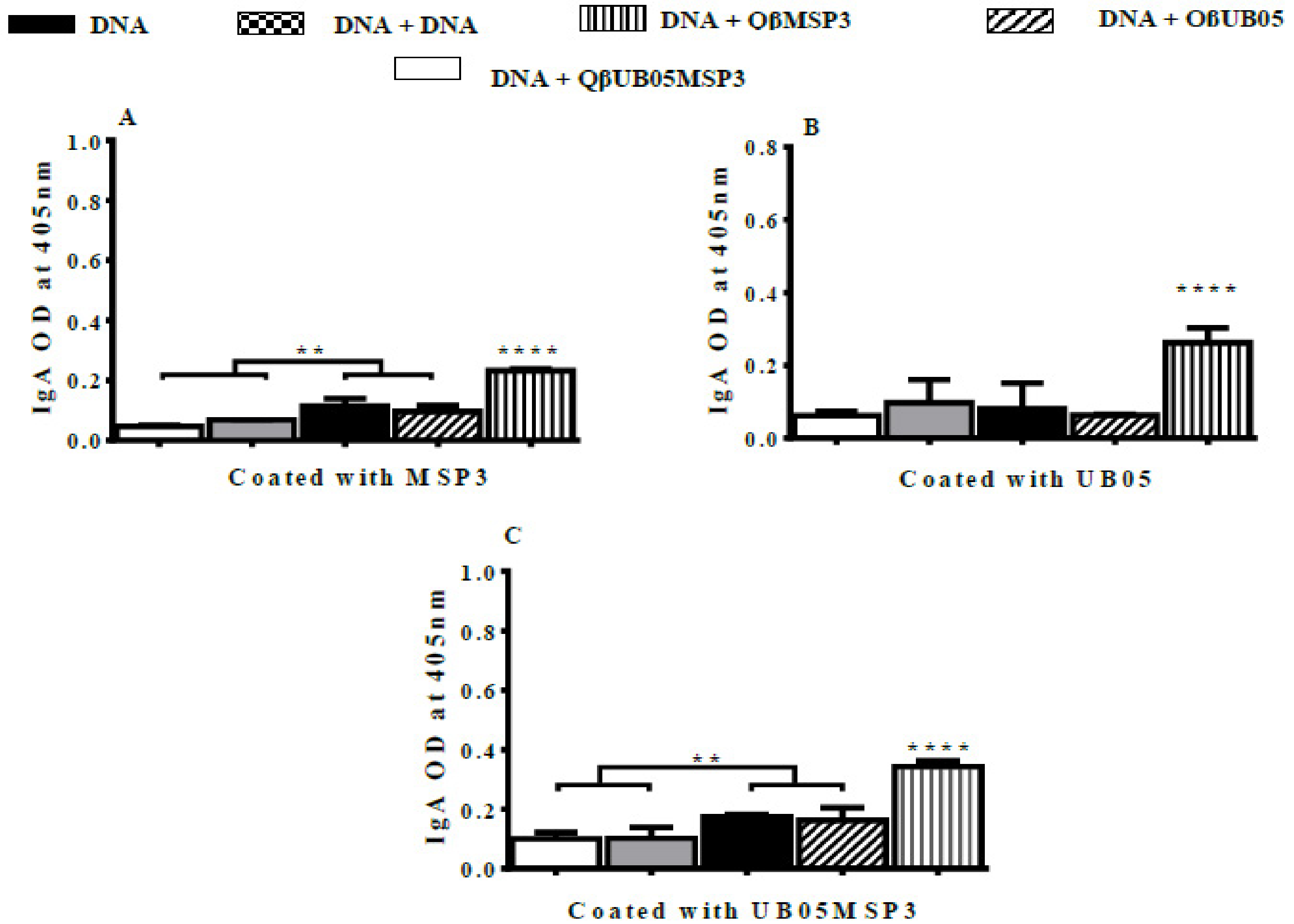
Figure 4: IgA isotypes profile against UB05 and MSP3 antigens in prime boosted mice.



The comparison of mean OD ± SD of antibodies was performed according to protocol of immunization. A: specific IgA reactivity to UB05 antigen B: specific IgA reactivity to MSP3 antigen; C: specific IgA reactivity to UB05-MSP3 antigen, Results are expressed as mean OD405nm + S.D. **: p< 0.01;****: p<0.0001.

Reactivity of specific IgG1 and IgG3 responses against MSP3/UB05 protein in mice after complementary prime boost

The assessment of IgG1 and IgG3 response against MSP3/UB05 antigens showed that the sera levels anti-QβUB05 IgG1 of mice treated with DNA/DNA prime-boost or with scDEC205UB05MSP3 DNA prime and QβUB05MSP3 boost were significantly higher than those receiving or scDEC205UB05MSP3 DNA and QβMSP3/ or QβUB05 (P <0.0001) (figure 5B). However, the scDEC205UB05MSP3 DNA prime and QβUB05MSP3 boost immunization have elicited a significantly higher level of IgG3 subclass specific to QβUB05 as compared to the other immunization protocol in mice (P<0.001) (figure 6B). The reactivity against MSP3 antigen revealed that the sera levels of IgG1 subclass specific to MSP3 were significantly higher (P <0.0001) in mice immunized with scDEC205UB05MSP3 DNA than those receiving the DNA prime and recombinant phages boost (figure 5A). However, the level of IgG3 subclass was shown significantly higher only in mice that were immunized with scDEC205UB05MSP3 DNA prime + QβUB05MSP3 boost (figure 6A). The sera levels of IgG1 and IgG3 specific to UB05MSP3 were significantly higher (P <0.05) in mice that received scDEC205-UB05MSP3 DNA prime and QβUB05MSP3 boost compared to all the other vaccinated mice (figure 5C& 6C).

Reactivity of specific IgG2a and IgG2b responses against MSP3/UB05 protein in mice after complementary prime boost

The reactivity of IgG2a and IgG2b antibodies with malaria MSP3/UB05 recombinant antigen was also assessed in mice sera according to the vaccine treatment. Overall, the reactivity of MSP3/UB05 antigens with mice sera was predominantly associated with IgG2a and IgG2b responses in mice boosted with QβUB05 or QβUB05MSP3 (complementary figure). The reactivity of IgG2a and IgG2b with UB05 was significantly

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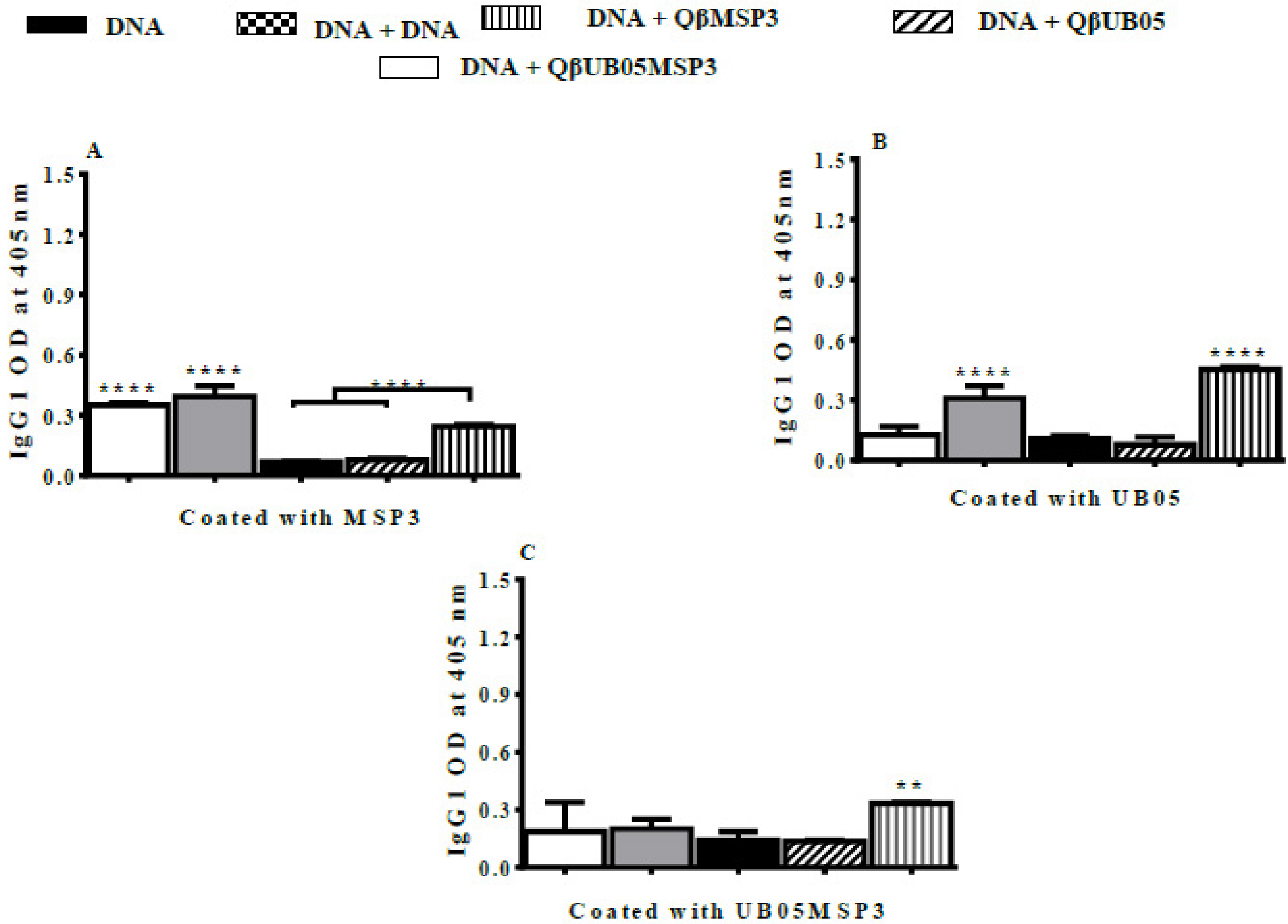
Figure 5: IgG1 isotypes profile against UB05 and MSP3 antigens in prime boosted mice.



The comparison of mean OD ± SD of antibodies was performed according to protocol of immunization. A: specific IgG1 reactivity to UB05 antigen B: specific IgG1 reactivity to MSP3 antigen; C: specific IgG1 reactivity to UB05-MSP3 antigen, Results are expressed as mean OD405nm + S.D. **: p<0.01;****: p<0.0001.

higher in mice vaccinated with scDEC205UB05MSP3 DNA prime and QβUB05 boost (1.140 ± 0.374; 1.526 ± 0.378 respectively) followed by the scDEC205UB05MSP3 DNA prime-QβUB05MSP3 boost group (0.722 ± 0.048; 1.394 ± 0.214 respectively) and then scDEC205UB05MSP3 DNA prime- QβMSP3 boost mice (0.430 ± 0.141; 0.854 ± 0.071 respectively) as compared to the mice vaccinated with only that DNA (P<0.05) (figure 7B & 8B). Both for MSP3 (figure 7A & 8A) and UB05MSP3 (figure 7C & 8C), the levels of IgG2a and IgG2b were significantly higher in QβUB05 or QβUB05MSP3 boost immunized mice after DNA prime compared to the other vaccinated mice (P<0.01).

Balance of Th1/Th2 mediated immune response

The ratio of T helper mediated IgG subclass responses in immunized mice were determined with respect to the MSP3, UB05 and UB05MSP3 antigens, in order to assess the balance of Th1/Th2 mediated IgG subclass responses regulation. The Th1/Th2 index was determined by the formula: ((IgG2a+IgG3)/2)/IgG1, assuming that the Th1/Th2 Index < 1 corresponds to a Th2-mediated immunity and the Th1/Th2 Index > 1 corresponds to a Th1-mediated immunity as previously described [27]. As shown in table 2, the antibody response against MSP3 was mediated by Th1 polarization in all immunized mice (Th1/Th2 index >1). The response of antibodies specific to UB05 in mice that were immunized with scDEC205UB05MSP3 DNA or scDEC205UB05MSP3 DNA/QβMSP3 or QβUB05MSP3 prime-boost showed the predominance of Th2-polarized immune response (Th1/Th2 index<1). In contrast, in mice immunized with scDEC205UB05MSP3 DNA/scDEC205UB05MSP3 DNA and scDEC205UB05MSP3 DNA/QβUB05 prime-boost the anti-UB05 antibodies responses were mediated by a balance of Th1/Th2 response and a polarization of Th1 response respectively. Concerning the antibody response directed to UB05MSP3 antigen, ia full skewing towards a Th1 polarization was observed in sera of all immunized mice (Th1/Th2 index >2). Considering the approach of DNA/recombinant phage prime-boost that has induced the antibody

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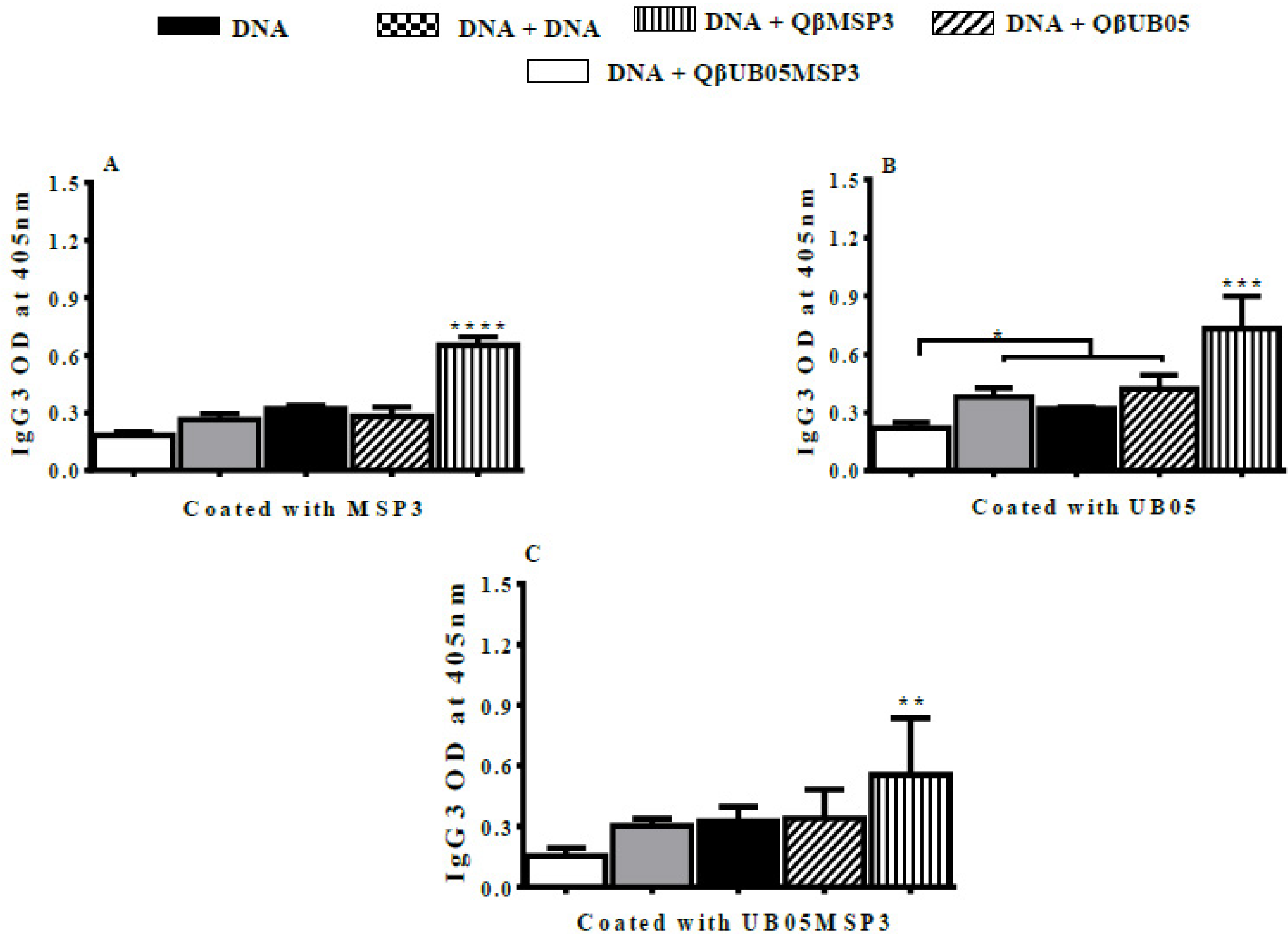
Figure 6: IgG3 isotypes profile against UB05 and MSP3 antigens in prime boosted mice.



The comparison of mean OD ± SD of antibodies was performed according to protocol of immunization. A: specific IgG3 reactivity to UB05 antigen B: specific IgG3 reactivity to MSP3 antigen; C: specific IgG3 reactivity to UB05-MSP3 antigen, Results are expressed as mean OD405nm + S.D. **: $p < 0.01$;***: $p<0.001$;****: $p<0.0001$.

production specific to antigen used as a boost, it was shown that the antibody responses fully oriented toward a Th1 polarization.

Mean endpoint titres for each of the three isotypes in each mice group were used to determine the Th1/Th2 index by the formula: ((IgG2a+IgG3)/2)/IgG1. The Index < 1 = Th2-mediated immunity. Index > 1 = Th1-mediated immunity.

Discussions

We aimed in this study to develop the fusion UB05MSP3 DNA vaccine candidate targeting dendritic cells and assess its immunogenicity in mice using different immunization strategies, including DNA prime, DNA/DNA prime-boost, and DNA prime and recombinant hybrid Qβ phage (QβUB05, QβMSP3 or QβUB05MSP3) boost. Our findings showed that recombinant scDEC205-UB05MSP3 protein was well expressed on 293A cell line after stable transfection with the encoding DNA. The assessment of the ability of the vaccine candidate to specifically bind to the Chinese hamster ovary (CHO) cell lines stably expressing the human DEC205 receptor upon their surface (CHOhuDEC205), suggest the capacity of this vaccine candidate to directly target the human dendritic cell throughout its DEC205 receptor. The HD83 encoding gene used in this study was previously proven to specifically target both mouse and human DEC205 receptors expressed on dendritic cell [28]. This means that the findings obtained in the assessment of the vaccine candidate fused with scDEC205 in animal model could inform about the vaccine activity in human beings. All these findings demonstrated that the immunization with the DNA vaccine candidate encoding for fused scDEC-UB05MSP3 protein will well express the appropriate recombinant protein that will specifically target DCs in situ via its DEC205 receptor. The findings of the studies carried out in our consortium previously demonstrated that the immunogens were specifically delivered to DCs when they were coupled to scDEC205, and this enhanced

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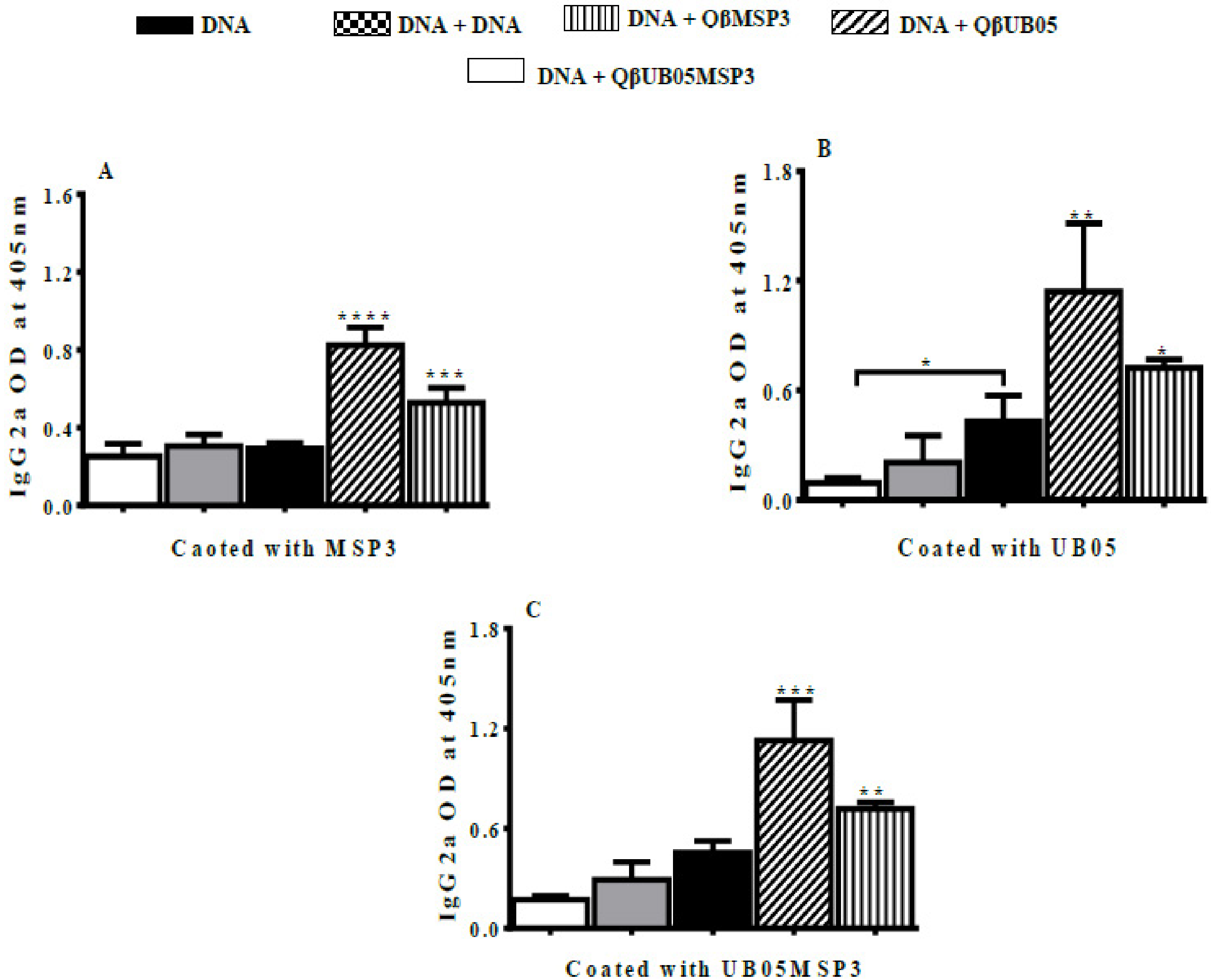
Figure 7: IgG2a isotypes profile against UB05 and MSP3 antigens in prime boosted mice.



The comparison of mean OD ± SD of antibodies was performed according to protocol of immunization. A: specific IgG2a reactivity to UB05 antigen B: specific IgG2a reactivity to MSP3 antigen; C: specific IgG2a reactivity to UB05-MSP3 antigen, Results are expressed as mean OD405nm + S.D. *: $p< 0.05$ **: $p< 0.01$;***: $p<0.001$;****: $p <0.0001$.

the immune response against the antigen and the challenge of the disease [4, 5, 8-10]. Further, it was shown that DNA vaccine candidate targeting DCs throughout DEC 205, significantly enhances the immunogenicity of the vaccine even at the largely reduced dose [4, 5, 8-10]. Yet, many research groups have reported an enhanced immune response by using heterologous prime-boost strategies which involved in sequential immunization of the same antigen with two or more different antigen delivery systems [29-31], suggesting that this strategy could be useful to strengthen cellular and humoral immune responses against malaria. We assessed the immunogenicity of our vaccine candidates in BALBc mice using DNA/DNA and DNA/recombinant phages prime-boost strategy. It was shown that the immunization of mice with scDEC205-UB05MSP3 DNA have induced a significant development of antibodies response (IgA and IgG subclasses) specific UB05MSP3 after the first and second vaccine administrations as compared to negative control group. These results are similar to those that Nchinda et al. found in 2008, demonstrating that a single intramuscular injection of DNA vaccine encoding HIV gag p41-scFv DEC205 fusion protein resulted in significant antibody responses [4]. In fact, during the intramuscular immunization of the mice, the transfected muscle cells with the DNA vaccine express recombinant proteins fused to a single chain antibody targeting DEC-205 receptors. These recombinant proteins are taken up by dendritic cells via DEC-205 receptor largely providing antigen presentation to MHC class I and II, and then induce a significant protective immune response [4, 32-35].

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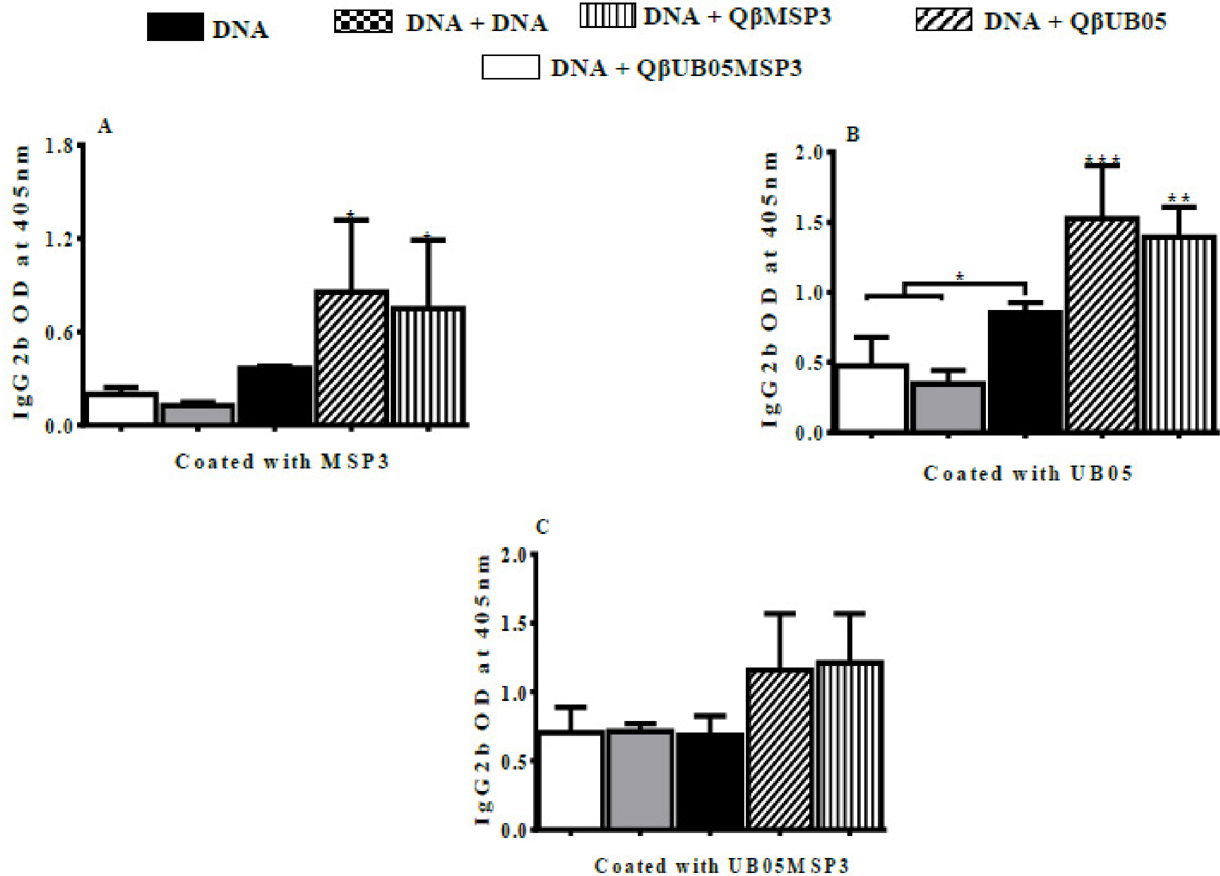
Figure 8: IgG2b isotypes profile against UB05 and MSP3 antigens in prime boosted mice.



The comparison of mean OD ± SD of antibodies was performed according to protocol of immunization. A: specific IgG2b reactivity to UB05 antigen B: specific IgG2b reactivity to MSP3 antigen; C: specific IgG2b reactivity to UB05-MSP3 antigen, Results are expressed as mean OD405nm + S.D. **: p< 0.01;***: p<0.001.

Table 2: Th1/Th2 mediated IgG subclasses antibodies ratio for UB05, MSP3 and UB05MSP3 antigens

	1X UB05MSP3 DNA	2X UB05MSP3 DNA	DNA + QβMSP3	DNA + QβUB05	DNA + QβUB05MSP3
MSP3					
IgG1 (Th2)	300	1066	500	300	1650
IgG2a (Th1)	600	1250	4000	8000	6400
IgG3 (Th1)	1000	1800	140445	57600	76800
Th1/Th2	**2,67**	**1,43**	**144,45**	**109,33**	**25,21**
UB05					
IgG1 (Th2)	600	450	2400	500	8000
IgG2a (Th1)	250	675	1200	7200	4800
IgG3 (Th1)	500	302	2400	4000	4800
Th1/Th2	**0,63**	**1,09**	**0,75**	**11,2**	**0,6**
UB05MSP3					
IgG1 (Th2)	1000	2200	1100	1200	8000
IgG2a (Th1)	2000	1200	9600	19200	9600
IgG3 (Th1)	4800	136000	4800	19200	25650
Th1/Th2	**3,4**	**31,18**	**6,55**	**16**	**2,20**

Mean endpoint titres for each of the three isotypes in each mice group were used to determine the Th1/Th2 index by the formula: ((IgG2a+IgG3)/2)/IgG1. The Index < 1 = Th2-mediated immunity. Index > 1 = Th1-mediated immunity.

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The anti-parasite activity of mice sera was assessed in-vitro using reinvasion/growth inhibition assay. It was shown that, the antisera of mice vaccinated with only scDEC-205UB05MSP3 DNA significantly induced a higher inhibition of parasite invasion compared to the control group of mice (P= 0.0126). Interestingly, a significantly higher inhibition of parasite invasion (39.1 ± 2.9%) was shown with antibodies of mice vaccinated with scDEC-205UB05MSP3 DNA + QβUB05MSP3 compared to all the groups (P<0.0001). These results correlate with the potential antibody responses observed in mice immunized with DNA/recombinant QβUB05MSP3 prime-boost regimen, supporting the idea that this strategy of immunization is more suitable. Previous studies reported that antibodies specific to P. falciparum UB05 antigen are involved in limiting parasite invasion of erythrocytes in people living in endemic area [16, 36]. However, the anti-sera of mice that received scDEC-205UB05MSP3 DNA + QβUB05MSP3 in this study showed a two-fold inhibition of invasion (40%) compared to the invasion inhibition reported by Titanji and collaborators (2009) (20%) at the same sample dilution, suggesting an antibody response enhancement with the immunization strategy used in this work. Studies reported that antibodies against MSP3 do not significantly inhibit parasite growth on their own; rather, they induce parasite killing via antibody-dependent cellular inhibition (ADCI) mechanisms [37-39]. The fact that the mice sera only induced around 40% of parasite inhibition in this study, suggest that these antibodies might use other mechanisms such as antibody-dependent cellular inhibition (ADCI) that would contribute to the parasite eradication. Further study needs to be done to sustain this way of antibody activity from sera of mice immunized with UB05MSP3-based vaccine candidate. The characterisation of humoral immune response showed the significantly higher titres of specific IgA, IgG1, IgG2a, IgG2b and IgG3 antibodies specific to UB05MSP3 after a complementary scDEC205-UB05MSP3 DNA prime and QβUB05MSP3 boost immunization compared to the mice prime-boosted with scDEC205UB05MSP3 DNA alone. These results corroborate with those of Mehrizi et al. when they assessed the immune responses elicited by P. vivax and P. falciparum MSP119 using prime-boost immunization strategies. They demonstrated that the immunization with plasmodium MSP-119 antigens at a single site generated a substantial specific antibody response in DNA/protein prime-boost immunization strategy as compared to the other strategies [40]. In addition, the nasal mucosa could be an attractive route for the administration of malaria recombinant antigens, due to its easy accessibility, lower enzyme distribution compared to other sites and immune cells dense population, often considered as the nasal associated lymphoid tissue [41-44]. We have also assessed the profile of IgA and IgG subclasses specific to recombinant QβMSP3, QβUB05 and QβUB05MSP3 phages in mice with respect to the DNA/DNA and DNA/recombinant phage prime-boost strategies. It was shown that the DNA/recombinant phage prime-boost elicited the significantly higher sera levels of IgA specific to MSP3 and UB05MSP3 as compared to DNA/DNA prime-boost (P<0.01). Yet, the mice immunized with scDEC205UB05MSP3 DNA and QβUB05MSP3 showed significantly higher level of anti-MSP3 and anti-UB05MSP3 IgA antibodies than the mice treated with DNA and recombinant QβMSP3 or QβUB05 phages (P <0.0001). Contrary to these results, the IgA specific to UB05 was highly expressed in mice immunized with scDEC205UB05MSP3 DNA/QβUB05MSP3 prime-boost (P<0.0001) compared to the other mice groups within which no significant difference was observed.

Taken together, these findings suggest that in addition to the efficient effect of DNA/recombinant phage prime-boost strategy, the fused QβUB05MSP3 immunogen is more suitable for intranasal boost immunization compared to recombinant phages QβMSP3 or QβUB05. It is also well known that the size of the vaccine molecule is one of the most important factors in inducing a significant immune response. Thus, the bigger the malaria antigen, the more it could easily be detected by immune cells, and then elicits a high immunogenicity. Mice, like humans, produce in general four different classes of IgGs, namely IgG1, IgG2a, IgG2b and IgG3, which are associated with malaria parasite clearance using several mechanisms [45]. The assessment of the profile of the patterns of IgG isotype specific to the recombinant phages in this study showed that the sera levels of IgG1 subclass specific to MSP3 was significantly higher in mice that were immunized only with scDEC205-UB05MSP3 DNA than those receiving the DNA/recombinant phages prime-boost immunization. On the contrary, the sera levels of anti-QβUB05 IgG1 subclass were significantly higher in mice immunized with a DNA/DNA or scDEC205-UB05MSP3 DNA/QβUB05MSP3 prime-boost than the mice receiving the other strategies of vaccine treatment. When looking at the level of this IgG1 subclass specific to fused QβUB05MSP3, it was revealed that anti-QβUB05MSP3 IgG1 antibody was highly produced in mice sera immunized with scDEC205-UB05MSP3 DNA/QβUB05MSP3 prime-boost regime with a significant statistical difference compared to the other mice groups. It has been shown that IgG1 can confer protection against lethal challenge infection with *P. yoelii YM* in mice immunized with MSP119 [46]. This IgG subclass was proven to be highly produced during the chronic stage of *P. chabaudi chabaudi AS* infection [47]. Regarding the profile of IgG3 subclass in vaccinated mice, it was shown that IgG3 subclass specific to QβMSP3, QβUB05 and to QβUB05MSP3 was significantly higher in mice immunized with a scDEC205-UB05MSP3 DNA/QβUB05MSP3 prime-boost regime as compared to the other mice groups. Other

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research groups have shown that IgG3 subclass monoclonal antibody (mAb) specific to 19-kilodalton carboxyl-terminal fragment of *Plasmodium yoelii* MSP1 confer protective immunity in normal naïve mice and in mice deficient in Fc receptor (FC-γRI), suggesting that antibody-dependent cell-mediated cytotoxicity and Fc-mediated phagocytosis are not necessary for parasite clearance by this antibody [48, 49]. The vaccine treatment using scDEC205-UB05MSP3 DNA/QβUB05 or scDEC205-UB05MSP3 DNA/QβUB05MSP3 prime-boost protocols particularly elicited the high levels of IgG2a and IgG2b subclasses specific to recombinant phages (QβMSP3, QβUB05 and QβUB05MSP3) with significant statistical difference compared to the other immunized mice groups. These cytophilic IgG2a and IgG2b antibodies were associated with protection against Plasmodium infection in mice [40, 45, 50]. The cytophilic IgG2a isotype was reported to predominate during the primary ascending parasitaemia in mice infected with *P. c chabaudi AS* [47], while IgG2b was reported to confer protection against lethal challenge infection with *P. yoelii YM* in immunized mice [51]. These results are in agreement with a previous study reporting that GMZ2 vaccine formulation (a hybrid MSP3/GLURP recombinant protein) induced higher IgG subclasses (IgG1, IgG2a, IgG2b & IgG3) antibody responses than individual MSP3 and Glutamate Rich Protein (GLURP) recombinant proteins [38, 50, 52, 53]. Furthermore, this research group had demonstrated that the immunogenicity of GMZ2 virosomal formulation was compared to vaccine candidate preparations of GMZ2 in Montanide ISA 720 and Alum in mouse, suggesting that the display of GMZ2 on the surface of the virosomes increases the accessibility of B-cell epitopes of the antigen. Hence, in addition to the fact that the fused UB05-MSP3 is suitable to increase the immunogenicity of individual corresponding antigens, the surface phage display of UB05-MSP3 recombinant protein could be an important factor enhancing antibody immune response with an excellent safety profile [54, 55]. The fact that scDEC205-UB05MSP3 DNA prime and QβUB05MSP3 boost highly elicited the production of all the patterns of IgG isotype suggests that the antibody responses directed against corresponding merozoite proteins may function either by blocking RBC invasion or by making the merozoites susceptible to phagocytosis via Ab-dependent cellular inhibition (ADCI) [38].

The IgG subclass expression is influenced by multiple factors, including the prevailing cytokine environment. To assess whether the immunization strategies elicited a specific IgG subclass profile or induced an increment in all subclasses, a Th1:Th2 index was calculated for each immunization groups, assuming that IgG2a and IgG3 subclasses are mediated by Th1 response while compared IgG1 subclass is under the regulation of Th2 response [27, 47, 56]. It was shown that, the response of antibodies specific to UB05 was Th2 polarized in sera of mice that were immunized with either scDEC205-UB05MSP3 DNA prime or scDEC205-UB05MSP3 DNA/QβMSP3 or QβUB05MSP3 phages prime-boost regimen (Th1/Th2 index<1). In contrast, in mice immunized with scDEC205UB05MSP3 DNA/scDEC205UB05MSP3 DNA and scDEC205-UB05MSP3 DNA/QβUB05 prime-boost the anti-UB05 antibody responses were mediated by a balance of Th1/Th2 response and a polarization of Th1 response respectively. However, the antibody responses specific to either MSP3 or fused UB05MSP3 showed a full skewing towards a Th1 polarization in sera of all immunized mice (Th1/Th2 index >2). Taken together, these findings suggest that Th1 immune response predominantly would regulate the antibodies response against UB05/MSP3 antigens, mainly when the animals are treated with complementary scDEC205-UB05MSP3 DNA primed-recombinant Qβ phage boost. Mehrizi and other workers (2011) also showed that the DNA/protein prime-boost immunization strategies generated a substantial plasmodial species-specific antibody response and an IFN-γ cytokine production [40]. In two different studies, Su and Stevenson demonstrated the central role of IL-12 and IFN-γ produced by Th1 cell during both acute and chronic phases of blood-stage malaria in mice. They showed that, IL-12 p40 gene knockout (KO) mice and IFN-γ-deficient mice produced significantly lower levels of Th1-dependent IgG2a and IgG3 but a higher level of Th2-dependent IgG1 compared to the wild type mice during primary and challenge infections [47, 56]. Other studies in P. yoelii-infected BALB/c and *P. berghei* XAT-infected CBA mice also demonstrated that Th1-associated IgG2a and IgG3 are the major protective Ab subclasses in Ab-mediated immunity against blood-stage malaria [57, 58]. Our study had some limitations, one of which being that the in situ targeting of scDEC-205UB05MSP3 recombinant protein to the dendritic cells has not been demonstrated in vivo after mice vaccination. Moreover, an infection challenge assay with Plasmodium parasite needs to be carried out in murine model to demonstrate the protective effect of our vaccine candidate. This study demonstrated in vitro the likely way of the vaccine candidate uptake by dendritic cells during immune response induction as well as the mechanism of parasite eradication.

Conclusions

We have shown that the developed DNA vaccine candidate encodes for the recombinant scDEC205-UB05MSP3 protein that can be specifically delivered to DEC205 receptors expressed on the surface of dendritic cells; suggesting an in situ targeting of our immunogens to dendritic cells and their processing and presentation to adaptive immune cells. The Combining scDEC205-UB05MSP3 DNA prime and QβUB05MSP3 boost has induced the high humoral immune responses with a significant parasite inhibition in vitro,

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compared to the other vaccination strategies. Thus, this approach of immunization was qualified as the best vaccine delivery strategy that enhances the immunogenicity of *Plasmodium falciparum*-derived UB05 and MSP3 antigens in vivo, and then contributes to immunity against malaria.

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Competing interests

The authors have declared that no competing interests exist.

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