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Blood Stage Merozoite Surface Protein Conjugated to Nanoparticles Induce Potent Parasite Inhibitory Antibodies

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Abstract

In this proof-of-concept study we report the use of <15nm, water soluble, inorganic nanoparticles as a vaccine delivery system for a blood stage malaria vaccine. The recombinant malarial antigen, Merozoite Surface Protein 1 (rMSP1) of *P. falciparum* served as the model vaccine. The rMSP1 was covalently conjugated to polymer-coated quantum dot CdSe/ZnS nanoparticles (QDs) via surface carboxyl groups, forming rMSP1-QDs. Anti-MSP1 antibody responses induced by rMSP1-QDs were found to have 2 to 3 log higher titers than those obtained with rMSP1 administered with the conventional adjuvants, Montanide ISA51 and CFA. Moreover, the immune responsiveness and the induction of parasite inhibitory antibodies were significantly superior in mice injected with rMSP1-QDs. The rMSP1-QDs delivered via intra-peritoneal (i.p.), intra-muscular (i.m.), and subcutaneous (s.c.) routes were equally efficacious. The high level of immunogenicity exhibited by the rMSP1-QDs was achieved without further addition of other adjuvant components. Bone marrow derived dendritic cells were shown to efficiently take up the nanoparticles leading to their activation and the expression/secretion of key cytokines, suggesting that this may be a mode of action for the enhanced immunogenicity. This study provides promising results for the use of water soluble, inorganic nanoparticles (<15nm) as potent vehicles/platforms to enhance the immunogenicity of polypeptide antigens in adjuvant-free immunizations.

Keywords

Adjuvant; Malaria Vaccine; Nanoparticles; Dendritic Cells; Inhibitory Antibodies

Introduction

A major obstacle in the development of subunit recombinant and peptide vaccines is the availability of adjuvants that can induce robust immune responses. The development of human blood stage malaria vaccines is a good example. One such vaccine is the *Plasmodium falciparum* Merozoite Surface Protein 1–42 (MSP1-42) [1–5]. MSP1-42 is a surface protein found on the invading merozoites of the erythrocytic stage [6, 7]. Vaccinations with MSP1-42 in animal models have demonstrated strong protection with the use of strong oil-

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water emulsion adjuvants such as Freund's Complete Adjuvant [1, 3–5, 8]. Parasite inhibitory antibodies specific for MSP1-42 are protective and correlate with clinical immunity [3, 4, 9–13]. Despite clear demonstration of protective immunity in animal models, a clinical trial using MSP1-42 showed no significant efficacy [14]. The inability of the MSP1-42 vaccine formulations to induce protection in clinical trials could be attributed to very low levels (titers) of parasite inhibitory antibodies [14, 15]. Two Phase 1 trials of MSP1-42 using Alum and Alum+CPG adjuvants also induced low levels of inhibitory antibodies [16, 17]. The failure to elicit protective immunity and/or high levels of parasite inhibitory antibodies in these clinical trials may be attributed partially to the choice of adjuvants (ASO2A, CPG, and Alum) [14, 16–18]. Currently, there are limited numbers of adjuvant formulations suitable for clinical testing; not only for malaria vaccines but also for vaccines against other infectious diseases. Alternative strategies need to be explored and developed to enhance vaccine immunogenicity. One such strategy is the use of particle-mediated delivery systems such as micro- or nanoparticles [19–23]. The types of particles currently being evaluated are lipid polymers (eg. PLGA, PGA, PLA) particles [24–27]; Virus-Like Particles (VLPs) [28, 29]; Immune Stimulating Complexes (ISCOMS) [30, 31]; chitosans [32–34]; and inorganic particles [35]. More recently, Self-Assembling, Polypeptide-based Nanoparticles (SAPN) have also been tested as a delivery platform for a peptide sporozoite malaria vaccine [36].

In this study, we focused on the use of the semiconductor nanoparticles, Quantum Dots (QDs) as an alternative vaccine delivery platform. QDs are small (<15nm) inorganic nanoparticles with a crystal shell of alternating cationic and anionic layers, which in this case is CdSe/ZnS [37–39]. QDs are non-immunogenic, stable, and when coated with an organic layer allow for an array of proteins, DNA, and other biomolecules to be conjugated to their surfaces [37–39]. Because of their small size and surface modification, QDs are highly soluble and behave as a true solution [40]. These characteristics may allow the particles to be rapidly dispersed in vivo, thereby readily reaching immunological sites and organs. Despite these advantages, the effectiveness of nanoparticles below 15 nm as vaccine delivery vehicles has not been thoroughly investigated. We used the recombinant malaria vaccine antigen, MSP1-42 (referred hereon as rMSP1) as a model immunogen to evaluate nanoparticles below 15 nm as a vaccine delivery platform in adjuvant-free immunizations. Results showed that rMSP1 conjugated to QDs (rMSP1-QD) was far superior to rMSP1 administered with CFA or with a clinically acceptable adjuvant, Montanide ISA51 in enhancing immunogenicity and efficacy. Our data provides promising proof-of-concept for the development of solid inorganic nanoparticles (<15 nm) as adjuvant-free vaccine delivery platforms.

Material and Methods

Mouse Strain

Outbred Swiss Webster (SW) mice and C57Bl/6 mice (female, 6–8 weeks old) were obtained from Charles River Laboratory (Wilmington, MA). The use of mice was approved by the University of Hawaii's Institutional Animal Care and Use Committee.

Recombinant MSP1-42 (rMSP1)

A truncated version of MSP1-42 was expressed in *Drosophila* cells [41] and purified by affinity chromatography [42]. Figure 1A shows SDS-PAGE profile of the purified protein. This recombinant MSP1-42 (rMSP1) has been shown to induce parasite growth inhibitory antibodies (Pusic et al manuscript in preparation).

Conjugation of rMSP1-42 to Quantum Dot Nanoparticles

The rMSP1-QD conjugates were prepared using N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) covalent coupling chemistry. In this method, QDs (4 μ M) with surface carboxyl groups were activated by incubating with sulfo-NHS (molar ratio 2000:1) and EDC (molar ratio 2000:1) for 5 minutes in borate buffer, pH 7.4, after which 2 mg of rMSP1 was added, vortexed thoroughly, and reacted for 2 hours at room temperature. At the end of 2 hours, the reaction was quenched by adding 5 μ l of Ocean's quenching buffer, a proprietary formulation in aqueous borate buffered solution at pH 9.5 ± 0.1 , and mixed for 10 minutes. The rMSP1-QD conjugates were stored at 4°C for about 12 hours and purified by ultracentrifugation at 60,000 rpm and 20°C for 30 minutes in order to separate and remove the unconjugated QDs from the supernatant.

The rMSP1-QD and unconjugated QDs were evaluated by agarose (1.5%) gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer, pH 8.5. For each well, 20 μ l of the QD samples at 100 nM were mixed with 5 μ l of 5XTAE loading buffer [5XTAE, 25% (v/v) glycerol and 0.25% (w/v) Orange-G at pH 8.5]. The gel was resolved at 100 V for 30 minutes (PowerPak Basic, Bio-Rad, USA) and then imaged with two exposures using a gel imaging system (Alpha Imager HP 2006, Alpha Innotech, USA).

Immunization of Mice with rMSP1-QD and rMSP1 with Conventional Adjuvants

SW mice (6 per group) were immunized with rMSP1-QDs using the intra-peritoneal (i.p.), intra-muscular (i.m.), and subcutaneous (s.c.) routes. Injection volume for the i.p. and s.c. routes was 100 μ l/dose (16 μ g of rMSP1/dose), and for the i.m. route was 30 μ l/dose (5 μ g/dose). Mice were immunized 3 times at 21 days intervals. Mice were also immunized via i.p. with rMSP1 emulsified in either CFA/IFA or Montanide ISA51. The first immunization consisted of a sub-optimal dose of 2 μ g antigen, followed by two booster injections at 21 days interval with an optimal dose of 5 μ g [43]. Sera were obtained through tail bleeds on the 14th day after each immunization.

MSP1-specific Antibody Assays

Mouse sera were assayed for anti-MSP1 antibodies (MSP1-19 specific) by direct binding ELISA as previously described [44]. The MSP1-19 used for coating ELISA plates was obtained from a previous study [45]. Plates were coated with MSP1-19 at a concentration of 0.4 μ g/ml. Mouse sera were serially diluted in 1% yeast extract, 0.5% BSA in Borate Buffer Saline (BBS). Horseradish peroxidase conjugated goat anti-mouse antibodies (H & L chain specific) (Kirkgaard and Perry Laboratories, Gaithersburg, MD) were used as the secondary conjugate at a dilution of 1:2000. Color development was produced by using the peroxidase substrates, H_2O_2 and 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)/ABTS (Kirkgaard and Perry Laboratories, Gaithersburg, MD). Optical density (O.D.) was determined at 405 nm. End point titers were calculated using the serum dilutions that gave an O.D. reading of 0.2, which is greater than 4-fold of the background absorbance using pre-immune mouse serum samples.

Antigenicity of rMSP1 conjugated to QD nanoparticles as determined by ELISA

Following the same ELISA procedures described in the previous section, serial dilutions of rMSP1-QD and unconjugated QD nanoparticles were made and used for coating ELISA plates. The coated ELISA plates were incubated with mAb 5.2 [46] at a concentration of 0.2 μ g/ μ l in 1% yeast extract, 0.5% BSA in BBS, followed by incubation with horseradish peroxidase conjugated goat anti-mouse antibodies. The O.D. readings for each serial dilution of rMSP1-QD and unconjugated QD were plotted and the levels of reactivity were compared to the standard ELISA reactivity of mAb 5.2 against unconjugated rMSP1.

Isotype-specific ELISAs

The immunoglobulin isotypes of the anti-MSP1-19 specific antibodies were determined by isotype specific ELISAs as previously described [47]. Goat anti-mouse-IgG1 and IgG2a (Southern Biotechnology, Birmingham, AL) were used at a dilution of 1:4000. Optical density was determined at 405 nm and the O.D. ratios of IgG1/IgG2a were calculated.

IFN- γ and IL-4 ELISPOT Assays

ELISPOT assays of splenocytes from immunized mice were performed according to methods previously described [48]. Briefly, ninety-six well PVDF plates (Millipore Inc., Bedford, MA) were coated with 10 μ g/ml of the monoclonal antibody (mAb) against IFN- γ (R4-642) and 5 μ g/ml of mAb against IL-4 (11B11) (BD Biosciences, San Diego, CA), and incubated overnight at room temperature. Plates were washed with Phosphate Buffered Saline (PBS) and blocked with 10% fetal bovine serum in DMEM for 60 minutes. Mouse spleens were harvested and single cell suspensions of splenocytes were prepared as previously described [48]. Purified splenocytes were plated at 0.5×10^6 , 0.25×10^6 , and 0.125×10^6 cells per well and rMSP1 (4 μ g/ml) was added to each well as the stimulating antigen. Positive control wells were incubated with 5 ng/ml of phorbol myristate acetate (PMA) and 1 ng/ml of ionomycin. Plates were incubated at 37° C in 5% CO₂ for 48 hours. Wells were washed and incubated with biotinylated mAb against IFN- γ at 2 μ g/ml (XMGI.2), or mAbs against IL-4 at 1 μ g/ml (BVD6-24G2) (BD, Biosciences, San Diego, CA), followed by the addition of peroxidase conjugated streptavidin (Kirkgaard and Perry Laboratories, Gaithersburg, MD) at a dilution of 1:800. Spots were developed with a solution consisting of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich St. Louis, MO, 1mg/ml) and 30% H₂O₂ (Sigma-Aldrich St. Louis, MO) and enumerated microscopically. Data were presented as spot-forming units (SFU) per million of plated splenocytes.

In vitro Parasite Growth Inhibition Assay with Purified Mouse Serum Samples

The ability of mouse sera generated from SW mice immunized with different rMSP1 formulations to inhibit parasite growth was determined using an *in vitro* assay [5, 45, 49, 50]. Immunoglobulins from pooled mouse serum samples from each group were purified as previously described [47] with modifications. Briefly, antibodies were purified by ammonium sulfate precipitation and followed by dialysis using an Amicon Ultra-10 Centrifugal Filter (Millipore, Billerica, MA) with a molecular weight cut off of 100 kDa. Purified antibodies were reconstituted to original serum volume with RPMI 1640. Inhibition assays were performed using sorbitol synchronized parasite cultures (3D7 strain) as described [45]. Synchronized parasite cultures at a starting parasitemia of 0.2% and 0.8% hematocrit were incubated in purified mouse antibodies at an equivalent of 20% serum concentration. Cultures were then incubated for 72 hours with periodic mixing. Parasitemias of the parasite cultures were determined microscopically by Giemsa staining of thin blood smears. The degree of parasite growth inhibition was determined by comparing the parasitemias of cultures incubated in pre-immune antibodies as previously described [45, 49, 50].

Dendritic Cell Isolation and QD Uptake Assay

Immature bone marrow dendritic cells (BMDC) were isolated from 12–14 week old C57Bl/6 mice as described [51]. Stromal cells were purified by passage through a cell strainer to remove bone and debris. Red blood cells were lysed using a RBC lysis buffer consisting of 0.15M NH₄Cl, 10mM KHCO₃, and 0.1mM EDTA. After washings, BMDCs were plated in 6-well plates (Cell Star, Monroe, NC) at a density of 10^6 cells/ml together with GM-CSF (Peprotech Inc, Rocky Hill, NJ) at a concentration of 20 ng/ml. After 24 hours, cell cultures

were further incubated in RPMI 1640 with GM-CSF (20 ng/ml) for an additional 7 days. On day 7, BMDCs in suspension were removed and transferred to a new plate and these cells were used as the cell source for all subsequent experimentations [52].

Unconjugated QD nanoparticles were introduced at a final concentration of 4 μ M to the 7-day old BMDC cultures, and incubated for 24 hours at 37°C. Cells were fixed with 1% paraformaldehyde (PFA) and labeled with goat anti-CD11c-PE antibodies (eBioscience, San Diego, CA), at a dilution of 1:2000, for identification and purity assessment. The cells were imaged using a fluorescent microscope (Olympus ix71) with a fluorescent cube containing the following filters: V-N41004 (ex560 and em585) and V-N41001 (ex480 and em535).

Dendritic Cell Activation by QDs

Unconjugated QD nanoparticles (4 μ M) were introduced to 7-day old BMDCs [52] for 24 hours at 37°C. The cells were harvested and washed twice with FACS buffer (PBS with 2% FBS), fixed with 0.25% PFA for 10 minutes on ice, and stained with monoclonal antibodies to cell surface markers: (APC)-labeled anti-CD80, (PE)-labeled anti-MHC II, (AlexaFluor488)-labeled anti-CD11c (eBiosciences, San Diego, CA), and (PE-Cy7)-labeled anti-CD86 (Invitrogen, Carlsbad, CA). Cells were analyzed using the FACS Aria flow cytometer with FACSDiva software (Becton Dickinson, San Jose, CA).

Cytokine Gene Expression by QD stimulated Dendritic Cells

RNA was extracted from BMDCs (3×10^6 cells) at 0, 3, 6, and 12 hours after the addition of unconjugated QD (4 μ M) or LPS (100 ng/ml), using the RNeasy Kit (Qiagen, Valencia, CA). RNA concentrations were measured and then the RNA samples were reverse transcribed in 50 μ l reactions using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) following manufacturer's protocol. Real-time PCR reactions using 1 μ l of cDNA and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) were run on the MyiQ Single-Color Real Time Detection System (Bio-Rad, Hercules, CA). Both forward and reverse primers for TNF- α , TGF- β , IL-12, IL-6, IFN- γ , IL-1 β were used at a 10 nM concentration (IDT, Coralville, Iowa). The primer sequences are listed in Supplemental Table 1. Analysis of gene expression was performed using the RT² Profiler PCR Array Data Analysis (SABiosciences, <http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). Briefly, each sample was normalized to an endogenous control, GAPDH, and the fold changes for each cytokine gene assayed was determined.

Multiplex Assay for Cytokines and Chemokines Detection

The presence of cytokines and chemokines in the supernatants of the BMDCs stimulated with unconjugated QD nanoparticles or with LPS over a 12-hour period were measured using the Milliplex MAP Mouse Cytokine/Chemokine 32 plex assay and Luminex 200 (Millipore Corp, Billerica, MA). The following cytokines/chemokines were simultaneously measured: Eotaxin, G-CSF, GM-CSF, IFN- γ , IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC-like, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1 α , MIP-1 β , MIP-2, RANTES, TNF- α , VEGF.

Data Handling and Statistics

Sigma Plot 10 and GraphPadPrizm 4 were used to calculate the endpoint antibody titers. The Mann-Whitney test was used to determine significant differences in antibody titers and isotype ratios among the different test groups. Cytokine responses (ELISPOT) in mice were analyzed by Logistic Regression for Repeated Measures (IBM SPSS Statistics). A $p < 0.05$ was considered statistically significant.

Results

Antigenicity of rMSP1-conjugated QD Nanoparticles

The rMSP1-conjugated QDs were tested to determine if the antigen was successfully conjugated to the nanoparticles, and whether the chemical conjugation processes affected the antigenicity of the rMSP1. Conjugated and unconjugated QDs were analyzed by agarose gel electrophoresis (Figure 1B). rMSP1-QDs (Lane 1) migrated as a single and higher molecular mass band, compared to the unconjugated QDs (Lane 2). This indicates that the conjugation process produced a homogeneous species of rMSP1-QDs. The antigenicity of rMSP1 was evaluated by examining the reactivity of the conformation dependent anti-MSP1-42 monoclonal antibody, mAb 5.2, with rMSP1-QD. The mAb 5.2 strongly recognized the rMSP1 conjugated to the QDs but not the unconjugated particles (Figure 1C). As a reference, an O.D. reading of 1.3 was observed with mAb 5.2 incubated with unconjugated rMSP1-42 at the plating concentration of 0.4 ug/ml. This suggests that the antigenicity of the rMSP1 antigen was preserved.

Immunogenicity of rMSP1-QD Nanoparticles

The efficacy of QD nanoparticles in enhancing vaccine immunogenicity was compared with that of conventional adjuvants. Three groups of outbred SW mice were immunized via i.p. with rMSP1-QDs, rMSP1 formulated with CFA, and rMSP1 with ISA51. Immune sera were tested for antibodies against MSP1-19 by ELISA. Vaccine responders were defined as having an ELISA O.D. of >0.2 at a 1:50 serum dilution. This was above the O.D. values observed for pre-immune mouse sera. As shown in Figure 2A, rMSP1-QDs induced an antibody response in all mice after two immunizations, resulting in a 100% response rate. In comparison, only five out of ten mice immunized with ISA51 had detectable antibodies, resulting in a 50% response rate (Figure 2B). All mice that received immunizations with CFA also responded, as shown in Figure 2B.

Comparison of antibody endpoint titers of the tertiary bleeds among the three vaccination groups shows that the rMSP1-QDs induced the highest mean antibody titer of 5.3×10^{-6} (Figure 2B); in contrast with the CFA formulation that induced a mean antibody titer of 2.9×10^{-4} ($p=0.012$), and with the ISA51 formulation that induced the lowest mean antibody titer of 1.9×10^{-3} ($p=0.001$). Thus, immunization of rMSP1-QDs produced antibody titers that were 2 to 3 log higher than the commonly used adjuvants, CFA and ISA51. Despite the high mean antibody titer observed with rMSP1-QD immunizations, there were high and low responders (Figure 2B) within the group of outbred mice as reflected in the broad range of endpoint titers.

Mice were also immunized with the rMSP1-QD via the i.m. and s.c. routes. Analysis of the tertiary immune sera revealed that a 100% response rate was achieved with all three immunization routes (Figure 2C). The mean antibody titers induced by s.c. immunizations (3.9×10^{-6}) were comparable to i.p. immunizations (5.3×10^{-6}), while i.m. immunizations elicited the lowest mean antibody titer of 0.96×10^{-6} (Figure 2C). However, there were no statistically significant differences in antibody titers among the three routes.

IgG Isotype Response to MSP1-19

Analyses of the MSP1-19 specific Ig sub-classes (IgG1/IgG2a ratios) in mice immunized with rMSP1-QD (i.p.), rMSP1-CFA (i.p.), and rMSP1-ISA51 (i.p.) showed no significant differences among these groups (Table 1). Comparison of mice immunized via i.p., i.m., and s.c. routes also showed no significant differences (Table 1). However, rMSP1-ISA51 induced a more polarized IgG1 response compared to other immunization groups that induced a more balanced IgG1/IgG2a response.

TH1/TH2 Response

ELISPOT analyses of mice immunized with rMSP1-QDs via the i.p., i.m., and s.c. routes showed balanced responses in terms of IL-4 (Figure 3A) and IFN- γ (Figure 3B) production. In comparison, rMSP1 formulated with CFA and ISA51 predominantly induced IL-4 (Figure 3). Analysis by logistic regression for repeated measures (SPSS) showed no significant differences across all groups.

In vitro Parasite Growth Inhibitory Activity of Recombinant Anti-MSP1-42 Antibodies

Purified mouse antibodies from all immunized groups were tested for their ability to inhibit parasite growth in vitro [47]. As shown in Table 2, the anti-MSP1-42 antibodies obtained from immunizations with rMSP1-QDs via the i.p., i.m., or s.c. route significantly inhibited parasite growth, with inhibition ranging from 73–81% (Table 2). None of the anti-MSP1-42 antibodies induced by rMSP1-CFA and rMSP1-ISA51 inhibited parasite growth by more than 50%, a level that is considered to be biologically significant [47, 53].

Dendritic Cell Uptake of QDs

To better understand the mechanisms by which QDs may enhance immune responses, we studied QD interaction with dendritic cells in vitro. QDs (emitting at 540 nm) were introduced to 7-day old BMDC cultures and assayed for nanoparticle uptake. Figure 4(A–C) shows that BMDCs (CD11c positive) actively internalized the QD nanoparticles. The portion of BMDCs with internalized QDs was approximately 92%.

Dendritic Cells are Activated by QDs

Unconjugated QD nanoparticles were introduced to immature BMDC cultures and the degree of activation was measured by MHC II, CD80, and CD86 expression using flow cytometry. Unstimulated, QD-stimulated, and LPS-stimulated (positive control) dendritic cells were first measured for CD11c positivity and then were further gated for the MHC II, CD80, and CD86 activation markers. QD-stimulated, CD11c-positive (Figure 5A, Panel iv) dendritic cells were activated and showed increased expression of MHC II (Figure 5A, Panel v), CD80, and CD86 (Figure 5A, Panel vi). QD-stimulated dendritic cells had the highest percentage (42%) of positive MHC II markers compared to unstimulated (32%) and LPS-stimulated (38%) dendritic cells; however, these levels were not statistically significant (Figure 5B). The percentage of single positive CD80 and CD86 cells were statistically higher in QD-stimulated dendritic cells than in unstimulated dendritic cells, with a p value of 0.0172 and 0.0431 respectively (Figure 5B). Double positive CD80/CD86 expression was also significantly higher than in unstimulated dendritic cells ($p = 0.0086$). QD-stimulated dendritic cells induced similar levels of MHC II and double positive CD80/CD86 expression as the LPS-stimulated dendritic cells. However, significantly higher levels of CD80 were observed in QD-stimulated dendritic cells than in LPS-stimulated cells ($p = 0.007$), indicating that the QD nanoparticles were able to induce CD80 activation more efficiently than LPS (Figure 5B). Conversely, LPS-stimulated DCs expressed significantly higher CD86 levels than QD-stimulated DCs, ($p = 0.0312$) (Figure 5B).

QD Uptake Induces Cytokine/Chemokine Production by BMDCs

Immature BMDCs exposed to unconjugated QD nanoparticles over a 12-hour period expressed cytokines vital for immune response activation/enhancement. By RT-PCR, QD nanoparticles significantly increased the production of the cytokines, TNF- α , IL-6, IFN- γ , IL-12, and TGF- β by more than twofold when compared to levels at 0 hour (Figure 6A). QDs uptake primarily led to the increased expression of pro-inflammatory cytokines, TNF- α and IL-6 indicating that immunization with QDs can induce early inflammation similar to LPS stimulation (Figure 6). On the other hand, LPS-stimulated DCs produced a broader

array of cytokines with significant fold increases in expression levels of all cytokines assayed, with the sole exception of TGF- β (Figure 6B).

To broaden our assay for cytokine/chemokine expression, a 32-plex Luminex assay was performed. BMDCs stimulated with unconjugated QD nanoparticles or with LPS secreted a number of cytokines (Figure 7) and chemokines (Figure 8) over a 12-hour period. Most notably, QD uptake/stimulation led to higher levels of production of the pro-inflammatory cytokines, IL-6, TNF- α , IL-1 β , and IL-1 α (Figure 7) in comparison to media alone. Gradual increases in the levels of these cytokines were observed over time in the QD-stimulated BMDC cultures (Figure 7). A number of chemokines were also produced in response to stimulation by unconjugated QDs (Figure 8). Among these, CCL3 and CCL4 were highly expressed and reached the same levels as LPS-stimulated BMDCs at 12 hours post-incubation.

Discussion

The main objective of this study is to investigate an alternative strategy to effectively deliver vaccines against malaria in order to enhance their immunogenicity. The secondary purpose is to show greater efficacy of the solid nanoparticles (<15 nm) over commonly used vaccine adjuvants. The results demonstrated the effectiveness of these water-soluble, inorganic nanoparticles for the delivery of a recombinant blood stage malaria vaccine.

One of the key findings from recombinant MSP1 delivered by the QD nanoparticles is the very high antibody titers it induced in comparison to CFA and ISA51. The mean titer induced by rMSP1-QDs was two to three logs higher than those induced by CFA and ISA51. Moreover, these levels of antibodies were much higher than levels with any other adjuvants we have tested in previous studies with MSP1 vaccines [47, 54]. Results from antibody subclass determination and ELISPOTs showed that QD immunizations potentiated a balanced TH1/TH2 response. While the importance of TH1 versus TH2 response in anti-MSP1-mediated immunity has yet to be established, the balanced TH1/TH2 responses mediated by QDs may be important against other infectious diseases [55, 56].

Equally significant is the ability of rMSP1-QDs to elicit a 100% response rate in outbred mice, irrespective of immunization route. This level of generalized responsiveness could be achieved only with the very potent adjuvant, CFA. The low toxicity adjuvant, ISA51 induced only a 50% response rate (Figure 2). Of note is the requirement of two immunizations to induce the high level of responsiveness observed with rMSP1-QDs in the un-optimized study. Further optimization of the nanoparticle platform in terms of particle concentration, particle size, and surface coating may lead to the potentiation of similar levels of immunogenicity with a single immunization.

Studies have shown that the levels of parasite inhibitory anti-MSP1 antibodies correlate with immunity [9–13]. In this context, the rMSP1-QD exhibited potency far superior to the rMSP1-CFA and rMSP1-ISA51 formulations. Antibodies from rMSP1-QD immunized mouse sera were highly inhibitory against parasite growth (81%), while antibodies induced by CFA and ISA51 were completely ineffective. The high levels of parasite growth inhibition observed with the rMSP1-QD immunized sera, compared to those induced by rMSP1-CFA or rMSP1-ISA51, may or may not be due to higher overall antibody titers, as we have previously shown that the ability of anti-MSP1 antibodies to inhibit parasite growth does not correlate with antibody titers [46]. It is possible that as a group the rMSP1-QD immunization induced antibodies that are more focused on parasite inhibitory epitopes despite the fact that some of the animals in this group had lower antibody titers similar to those in the rMSP1-CFA group.

The route of immunization has been shown to play a role in the outcome of immune responses [21, 57]. Our results showed that rMSP1-QD elicited similar high antibody titers and parasite inhibitory antibodies when delivered via i.p., i.m., or s.c routes. Thus, the potency of this delivery platform was independent of immunization route. Future studies will investigate its effectiveness in non-parenteral routes, i.e. intra-nasal and oral administrations.

The significance of the QDs as a delivery platform lies in its ability to induce antibody and T cell responses without the addition of other adjuvants. However, it is possible that incorporation of adjuvants such as CpG and other TLR ligands to the nanoparticle delivery system could further increase its potency, which may allow for dose sparing administration of the conjugated vaccines.

Recently, self-assembling polypeptide-based nanoparticles (SAPNs) were reported as a delivery platform for a malaria sporozoite vaccine [36]. These SAPNs are highly effective for small peptide antigens but lack the capability to incorporate large polypeptide antigens such as the MSP1-42 [36]. Another study using recombinant vault nanoparticles has shown efficacy in delivering recombinant proteins via the intra-nasal route [58]. Whether this platform is suitable for parenteral immunizations remains to be demonstrated. However, antigens bearing vault nanoparticles may break self-tolerance, producing self-reactive antibodies [58].

There has been very limited information on the effectiveness of solid inorganic nanoparticles (<15 nm) for the delivery of polypeptide antigens. An obstacle in the use of these nanoparticles as delivery platform for biomolecules is that they are too rapidly cleared from the body [59, 60]. However, at sizes <15nm, the nanoparticle suspensions behave as true solutions and thus may readily disperse and penetrate tissues to reach key immunological sites [40]. These particles can be highly effective when they are readily taken up by antigen presenting cells (APCs), as shown by our particle uptake studies with bone marrow derived dendritic cells (Figure 4). Our dendritic cell activation studies have shown further that these nanoparticles had the ability to upregulate the key activation marker, CD80 on BMDCs (Figure 5). Additionally, nanoparticle-stimulated DCs expressed and secreted markedly high levels of pro-inflammatory cytokines (IL-6, IL-1 α , TNF- α) and chemokines (CCL3, CCL4, CXCL1) necessary for efficient immune responses, especially toward a TH1-mediated immunity (Figures 6, 7, and 8). Based on the chemokines produced, stimulation of BMDCs by QDs would enhance the migratory characteristics of these immune cells which might result in more efficient antigen presentation and/or T cell activation. QDs were also able to induce production of some chemokines (CCL3/CCL4) at levels similar to those induced by LPS-stimulated BMDCs, indicating the potential of these nanoparticles to activate a strong immune response (Figure 8).

This study provides “proof of concept” for the utilization of water-soluble, solid inorganic nanoparticles (<15nm) as a vaccine vehicle/platform to enhance the immunogenicity of antigens in adjuvant-free immunizations. These nanoparticles not only served as a delivery platform for protein antigens but could also activate key immune cells. To further increase the immunogenicity of this platform, other parameters may be studied or optimized. These include conjugation method, antigen orientation (either N-terminal or C-terminal conjugation), and antigen release characteristics. Concurrent with the present study, a toxicity evaluation was performed on the immunized mice by examining the plasma levels of Glu, BUN, Na, Cl, TCO₂, AnGap, Hct, Hb, pH, PCO₂, HCO₃, and BEecf; and by histological studies of kidney sections. Results showed no significant deviations in these laboratory values or in histological findings from non-immunized mice (data not shown). Although in vivo tests demonstrated no immediate toxic effects, these nanoparticles are not

compatible for human use due to the presence of cadmium (Cd) in the particle core. The Cd toxicity in the QDs is contained by coating the core with a shell layer of ZnS, which is further coated with an amphiphilic polymer. Because of the possibility of eventual polymer and nanoparticle degradation, attempts are currently being made to develop cadmium free QD nanoparticles. We used the CdSe/ZnS QDs in this study because the main focus was to demonstrate as proof of principle that <15 nm solid nanoparticles can be used as an effective vaccine delivery platform. Furthermore, due to their high fluorescence, we have the advantage of being able to track the fate of these nanoparticles in vivo in order to help determine the mechanisms of immune enhancement for further optimization of the delivery platform. Similarly, we can also determine the mechanisms and rate of clearance of <15 nm solid nanoparticles for future safety and toxicity evaluations. In parallel, other investigations will determine whether similarly sized and surface-modified nanoparticles having other core compositions, such as Fe₂O₃, and Au, which are biocompatible and have been used in clinical studies and applications, will have immunogenicity profiles comparable to the CdSe nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- <15nm, water soluble, inorganic nanoparticles (QDs) as a vaccine delivery platform.
- Malaria vaccine-conjugated QDs was highly immunogenic, giving 100% response rate.
- Malaria vaccine/QD formulation induced potent parasite inhibitory antibodies.
- Immune responses by adjuvant-free QDs were superior to conventional adjuvants.
- QDs activation of dendritic cells is a possible mechanism for immune enhancement.

Figure 1. Purification, conjugation, and antigenicity analysis of rMSP1 protein to nanoparticles. Panel A, SDS-PAGE of purified recombinant C terminus MSP1 protein. Lane 1: Molecular Marker, Lane 2: Purified recombinant MSP1 (rMSP1). Panel B, 1% agarose gel electrophoresis of rMSP1 conjugated (Lane 1) and unconjugated QDs (Lane 2). Panel C, antigenicity of rMSP1 conjugated nanoparticles. ELISA titration curves of rMSP1 conjugated nanoparticles (open circles) and unconjugated nanoparticles (filled circles) against MSP1-42 specific monoclonal antibody, mAb 5.2. Straight line represents OD reading of mAb 5.2 binding to native MSP1-42 at coating concentration of 0.4 ug/ml.

Figure 2.

ELISA antibody response against MSP1-19 in SW mice immunized with recombinant MSP1. Panel A, antibody titers of mice vaccinated (IP) with rMSP1-QD. Results of primary, secondary, and tertiary bleeds are shown. Panel B, antibody titers of mice vaccinated with different adjuvant/delivery platforms (rMSP1-QD, rMSP1-CFA, and rMSP1-ISA51). Results of tertiary bleeds are shown. Panel C, antibody response in mice vaccinated with rMSP1-QD via different routes (i.p., i.m., and s.c.). Results of tertiary bleeds are shown. Horizontal bars indicate mean antibody titers. Significant differences in ELISA titers among vaccination groups are indicated with p-values (Mann-Whitney test).

Figure 3. MSP1-specific IL-4 and IFN- γ responses induced by rMSP1-QD and other adjuvants. MSP1-specific IL-4 (Panel A) and IFN- γ (Panel B) responses, as determined by ELISPOT, in SW mice immunized with rMSP1 in five different adjuvant/delivery platforms. Horizontal bars indicate mean SFU. Mouse splenocytes were harvested 21 days after the last immunization.

Figure 4. Uptake of QD nanoparticles by bone marrow derived dendritic cells (BMDC). Panel A, localization of QD particles (green) in BMDC cultured cells. Panel B, surface staining of the same BMDC cells with PE conjugated goat anti-mouse CD11c (red). Panel C, merged image of Panel A + B.

B.

Figure 5.

Activation of BMDCs by QD nanoparticles. Panel A, representative of one experiment in which BMDCs (1×10^6 cells) were incubated with media alone, QD nanoparticles, or LPS at 37°C for 24 hours. Cells were stained for surface markers: CD11c-FITC, MHC II-PE, CD80-APC, and CD86-PE-Cy7 and analyzed using flow cytometry. Panel i. gated for CD11c+ BMDCs, Panel ii. & iii. are live gates of Panel i. and stained for MHC II, CD80, and CD86. Similarly, Panel v. and vi. are live gates of Panel iv.; and Panel viii and ix are live gates of Panel vii. Panel B, summary of three BMDC activation experiments. Significant differences were observed between unstimulated and QD-stimulated DCs (*) for the expression of CD86+ ($p = 0.0431$), CD80+ ($p = 0.0172$), and CD80+/CD86+ ($p = 0.0086$). Significant differences were also observed between QD-stimulated DCs and LPS-stimulated DCs (**) for the expression of CD86+ ($p = 0.0312$) and CD80+ ($p = 0.0007$).

Figure 6.

Cytokine expression by stimulated BMDCs. RT-PCR quantification of expression of six different cytokine genes in QD-stimulated dendritic cells (Panel A) and LPS-stimulated dendritic cells (Panel B) over a 12-hour period. Data were first normalized to GAPDH and fold changes were calculated based on “0 hour” samples.

Figure 7.

Cytokines production by stimulated BMDCs. BMDCs (1×10^6 cells) were incubated with media alone (open squares), QD nanoparticles (4uM) (open circles), or LPS (100 ng/ml) (open triangles). Culture supernatants were collected at 0, 3, 6, and 12 hours and cytokines IL-6 (A), IL-1b (B), TNF- α (C), and IL-1a (D) were measured by Luminex using the Milliplex MAP Mouse Cytokine/Chemokine 32 plex assay. Only the cytokines with highest expression detected are depicted here. Cell supernatants were measured in triplicates.

Figure 8.

Chemokines production by stimulated BMDCs. BMDCs (1×10^6 cells) were incubated with media alone (open squares), QD nanoparticles (4 μ M) (open circles), or LPS (100 ng/ml) (open triangles). Culture supernatants were collected at 0, 3, 6, and 12 hours and chemokines CCL3 (A), CCL4 (B), CXCL1 (C), CXCL2 (D), CXCL10 (E), and CCL2 (F) were measured by Luminex using the Milliplex MAP Mouse Cytokine/Chemokine 32 plex assay. Only the chemokines with highest expression detected are depicted here. Cell supernatants were measured in triplicates.

Table 1

Immunoglobulin Isotype Specific Antibodies Against MSP1-19 in Mice Immunized with rMSP1 in Different Adjuvant/Delivery System[@]

Immunogen	IgG1	IgG2a	IgG1/IgG2a ^{†*}
rMSP1-QD (i.p.)	1.567±0.342	0.499±0.132	4.147±1.561
rMSP1-QD (i.m.)	1.431±0.114	0.667±0.217	3.161±0.882
rMSP1-QD (s.c.)	1.399±0.132	0.579±0.190	4.487±1.492
rMSP1-ISA51 (i.p.)	1.363±0.344	0.028±0.009	101.8±51.88
rMSP1-CFA (i.p.)	1.239±0.320	0.721±0.314	2.989±1.148

[@] Mean O.D.±SD are shown for IgG1 and IgG2a

[†] Mean mean ratio of O.Ds IgG1/IgG2a ±SD

^{*} Unpaired t test performed. Significantly different from the rest of the groups

Table 2

In vitro Parasite Growth Inhibition by Ig of Mouse Immunized with Various MSP1 Formulations

Pooled Mouse Purified Antibody (Tertiary Bleeds)	% Parasite growth inhibition [@]
rMSP1-QD (i.p.)	81%
rMSP1-QD (i.m.)	73%
rMSP1-QD (s.c.)	78%
rMSP1-CFA (i.p.)	17%
rMSP1-ISA51 (i.p.)	0%

[@] Mean of two growth inhibition assays