Surface display compared to periplasmic expression of a malarial antigen in *Salmonella typhimurium* and its implications for immunogenicity

Diana Haddad a,*, Sissela Liljeqvist b, Sanjai Kumar c, Marianne Hansson b, Stefan Ståhl b, Hedvig Perlmann a, Peter Perlmann a, Klavs Berzins a

a Department of Immunology, Stockholm University, S-106 91 Stockholm, Sweden
b Department of Biochemistry, Royal Institute of Technology, S-100 44 Stockholm, Sweden
c Laboratory of Malarial Research, National Institutes of Health, Bethesda, MD, USA

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Abstract

Two different expression systems were investigated for the production of an 80 amino acid polypeptide, M3, from the C-terminus of the *Plasmodium falciparum* blood stage antigen Pf155/RESA in an attenuated *Salmonella typhimurium* vaccine strain. Upon expression, the malarial polypeptide was targeted either to the periplasm as a soluble fusion protein containing two IgG-binding domains (ZZ) from the staphylococcal protein A or, to the bacterial surface as an insert within a chimeric outer membrane protein A (OmpA) derived from *Escherichia coli* and *Shigella dysenteriae*. Both the ZZM3 and the OmpAM3 proteins were stably expressed in the periplasm or on the surface of *Salmonella*, respectively. The ZZ expression system yielded 10-100 times more malarial immunogen than did the OmpA system. Live recombinant *Salmonella* expressing ZZM3 or OmpAM3 were used to immunize mice intraperitoneally. Both the ZZM3 and OmpAM3 genes persisted for up to three weeks in bacteria isolated from different lymphoid organs. Bacteria expressing ZZM3 induced antibodies to M3, ZZ and to the Pf155/RESA antigen whereas, bacteria producing OmpAM3 induced similar levels of antibodies reactive with M3 but not with Pf155/RESA. Both recombinants induced a memory response of antibodies reactive with both M3 and Pf155/RESA. The high levels of M3 produced by the ZZ expression system make it suitable for the expression of heterologous antigens in *Salmonella*. Nevertheless, in spite of the quantitative difference in M3 expression, the ZZ and OmpA constructs elicited comparable immune responses to M3.

Keywords: *Salmonella typhimurium*; Staphylococcal protein A; Outer membrane protein A; *Plasmodium falciparum*; Pf155/RESA

1. Introduction

Attenuated *Salmonella typhimurium* vaccine strains expressing heterologous antigens are able to induce both humoral and cellular immunity against these antigens [1]. With regard to cell-mediated immunity, both class I and II MHC-restricted cytotoxic T-cells directed against heterologous antigens have been obtained [2-4]. Furthermore, in animal models, protective immunity has been achieved upon oral immunization with recombinant *Salmonella* strains.
against bacterial, viral and protozoan infections [4–6].
When administered orally or intraperitoneally, Salmonella reaches and colonizes different lymphoid organs, thus being a useful tool for delivery of immunologically relevant antigens. However, important considerations for recombinant Salmonella vaccine development are the cellular location of the T- or B-cell epitopes for efficient presentation and the production of sufficiently high antigen levels. Within this context, targeting of foreign antigenic determinants to outer cellular locations, i.e. cell surface or periplasmic space, has been investigated in Salmonella [7–11].

In this study, a chimeric outer membrane protein A (OmpA) derived from Escherichia coli and Shigella dysenteriae [12], was used for surface display of a foreign antigen in S. typhimurium. The OmpA is one of the most abundant proteins in the outer membrane of Gram-negative bacteria, traversing the membrane eight times with four external loops [12]. Foreign antigenic determinants have been genetically introduced in the second, third and fourth loops [7,12,13], and have been demonstrated to be exposed on the bacterial surface. Upon immunization with live S. typhimurium, humoral responses to the inserted epitopes have been reported [7,14].

An expression system has been designed for production of the target gene as a fusion to a divalent synthetic version (ZZ) [15] of the IgG-binding domains of staphylococcal protein A (SpA) [16]. Upon expression in E. coli, such fusion proteins can be efficiently secreted to the periplasm and in some cases to the culture medium [17,18]. This expression system has been used for the production of vaccine immunogens [17], but has never been investigated in Salmonella. Here, we have compared the antibody responses elicited in mice by recombinant Salmonella to an antigen expressed in two different cellular compartments. In the periplasm, taking advantage of the ZZ expression system or, inserted into the bacterial outer membrane, using the recombinant OmpA expression system. The target antigen (M3) corresponds to the major repeat motifs from the C-terminal region of Plasmodium falciparum blood stage antigen Pf155/RESA, comprising 5 and 10 copies of the octamer EENVEHDA and tetramer EENV, respectively [19]. It contains important epitopes recognized by human B- and T-cells [20–22] and recombinant proteins representing these sequences are immunogenic in animal models [23]. The expression and localization of this polypeptide in Salmonella and its immunogenicity were investigated.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strain RR1ΔM15 [24] was used as bacterial host during the plasmid constructions. The restriction negative Salmonella typhimurium LB5000 [25], was used as an intermediate transformation recipient of the plasmid DNA. The S. typhimurium aroA strain SLΔ33-2, used for immunization experiments and as host for production of the fusion proteins ZZM3 and BBM3, was created by Drs. Jo Ann Flynn and Maggie So, Scripps Clinic, La Jolla, CA. Plasmid pHS164-L [7] containing the OmpA-construct was kindly provided by Prof. G. Hobom, Justus Liebig Universität, Giessen, Germany.

2.2. Construction of expression vectors

The construction of pEZZM3 has previously been described [19]. Briefly, the repetitive M3 gene was constructed using a two-step polymerization strategy based on unidirectional insertion of DNA fragments. The assembled gene fragment encoded the peptide (VEHDAEEN5 (VEEN)10, denoted M3, derived from the C-terminal region of antigen Pf155/RESA. The M3 gene was cloned into the staphylococcal protein A derived (ZZ) expression vector and the resulting plasmid pEZZM3 encodes the ZZM3 fusion protein. For the construction of the expression vector pOmpAM3, a DNA fragment encoding the repetitive P. falciparum antigen M3 was amplified according to a standard polymerase chain reaction protocol. Plasmid pEZZM3 served as template for the PCR, in which an oligonucleotide (5’-AAAGTAGACCGGAATTCC-3’), was used as upstream primer, together with oligonucleotide (5’-CAAGTCGACCCAGTGCCAACCTCAC-3’) as downstream primer. The amplified gene fragment was digested with restriction enzymes NsiI and SalI.
and ligated into similarly digested plasmid pH164-L.

The resulting expression vector, pOmpAM3, encodes the *P. falciparum* antigen M3, inserted at a position corresponding to the third extracellular loop of OmpA.

### 2.3. Preparation of recombinant bacteria for immunizations of mice and characterization of the gene fusion products

Bacteria were grown overnight at 37°C in Luria broth with ampicillin (100 µg ml⁻¹). Cultures were then diluted to 1:10 in fresh medium and pOmpAM3-transformed *S. typhimurium* SLΔ33-2 cells (SL-ΩmpAM3) with protein expression under control of the inducible tac-promoter [12], were grown to an absorbance of 0.5–0.8 when expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Cells were grown for another 3 h before they were harvested. The *S. typhimurium* SLΔ33-2 cells transformed with plasmid pZZM3, hereafter denoted SL-ZZM3, which express the ZZM3 fusion protein constitutively, were grown for approximately 6 h until late logarithmic growth phase.

To characterize the localization and production of the malarial protein, equal amounts (1 x 10⁸ cells ml⁻¹) of bacteria were fractionated into periplasmic or membrane compartments by osmotic shock [27] or spheroblast disruption respectively [12]. Whole cell lysates were prepared by pelleting equal amounts (1 x 10⁸ cells) of each recombinant bacteria and resuspending in 1 ml Laemmli reducing buffer [28].

### 2.4. Western blot analysis

The expression levels of the malarial protein were assessed by immunoblotting. The amount of bacterial extract loaded on the SDS-PAGE corresponded to approximately 1 x 10⁶ bacteria, which represented the dose of immunization. The protein standard used for this analysis was purified ZZM3 fusion protein recovered from the periplasm of *S. typhimurium* SLΔ33-2 by affinity chromatography on IgG-Sepharose (Pharmacia Biotech, Uppsala, Sweden) [29]. The purified protein was lyophilized, weighed and dissolved in Laemmli buffer and aliquoted before storage at −80°C. All bacterial preparations were suspended to 1 ml with Laemmli buffer and boiled for 5 min. Samples were separated in 10% SDS-PAGE homogeneous gels and transferred to nitrocellulose membranes by electroblotting. Following an overnight blocking with 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.5, the blots were further incubated for 2 h at room temperature with 1% human IgG (hIgG) in 0.5% BSA and 0.05% Tween-20 in PBS. This was done to avoid Fc-mediated binding of mouse or rabbit IgG to the protein A-derived ZZ part. For the same reason, 0.1% hIgG was included in the incubation buffer (PBS containing 0.5% BSA and 0.05% Tween-20). The fusion products were analysed by incubating for 5 h at room temperature with a polyclonal rabbit antiserum directed against M3, followed by another 5 h incubation with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Mabtech AB, Stockholm, Sweden) and developed with 1-naphthylphosphate (Sigma, St. Louis, MO).

The M3-reactive antiserum was obtained after intramuscular immunization of a New Zealand white rabbit with two doses of 0.1 mg BBM3 fusion protein (see below). Freund’s complete adjuvant (FCA) was used for the first injection, while Freund’s incomplete adjuvant was used for the second injection, given four weeks later. The reactivity to parasite proteins was also assessed by immunoblotting following essentially the same procedure described above using *P. falciparum* schizont extracts [21,30].

### 2.5. Immunofluorescence

Immunofluorescence analysis of membrane-associated fusion proteins was performed essentially as described by Schorr and coworkers [7]. Briefly, 1 ml of an induced bacterial culture in late logarithmic phase, was pelleted, washed twice with 500 µl of PBS pH 7.5 and resuspended in 200 µl of the same buffer. The rabbit anti-M3 polyclonal serum used in Western blot or an antiserum raised in rabbits against *S. typhimurium* lipopolysaccharide (LPS) (Sigma), was added at a final dilution of 1:100 or 1:1000 respectively, and the mixture was incubated overnight at 4°C under gentle agitation. Cells were collected by centrifugation, washed as above and resuspended in...
200 μl of PBS containing 5 μg of biotinylated goat anti-rabbit IgG (Vector, Burlingam, CA). Following incubation for 2 h at room temperature, the cells were washed and resuspended in 200 μl of PBS containing 5 μg of fluorescein avidin D (Vector). After incubation for 30 min at room temperature, cells were washed and resuspended in 50 μl PBS. One drop of the sample was placed on a slide and visualized with a coverslip under UV light. Photographs were taken on Fuji T-160 Ektachrome di-afilm with a Leica MPS 52 microscope camera mounted on a Leica microscope.

2.6. Fusion proteins

The expression vectors used for the production of the fusion proteins ZZM3 and BBM3 have been described previously [19]. The fusion proteins contain a divalent synthetic IgG-binding domain (ZZ) from SpA [15] or a serum albumin binding region (BB) from streptococcal protein G [31] fused to the malarial M3 polypeptide. Upon expression in S. typhimurium SL Δ33-2, the fusion proteins were targeted to the periplasm by the secretion signal of the SpA operon and could be purified by affinity chromatography on IgG (ZZM3) or human serum albumin (BBM3) Sepharose, as previously described [17].

2.7. Immunization procedure

Inbred female CBA/J mice 6 to 8 weeks old used in this study, were obtained from the Stockholm University breeding facilities. All injections were performed intraperitoneally (i.p.). Groups of three mice were immunized with approximately 1 × 10^6 live recombinant SL-ZZM3 or SL-OmpAM3 in 100 μl PBS, pH 7.5. Each group received three booster injections with the same amount of bacteria at 3-week intervals. The inoculum doses were checked by viable counts on Luria broth agar and by monitoring the absorbance at 600 nm. A final booster injection consisting of 50 μg of affinity purified ZZM3 fusion protein in 100 μl of PBS was given 24 weeks after the last immunization. Mice from each group were bled at various times after immunization by retroorbital plexus puncture and the sera were pooled and stored at −20°C.

2.8. Plasmid stability and bacterial persistence

Clearance of bacteria and the stability in vivo of the recombinant plasmids were assessed for the two types of constructs in the spleen, liver and a pool of periaortic and inguinal lymph nodes after one injection with 1 × 10^8 bacteria. Organs were aseptically removed from two mice, pooled separately and homogenized with a tissue grinder (Thomas, Philadelphia, PA) in 2 ml of Luria broth. Several tenfold dilutions were prepared and plated on solid medium with or without ampicillin.

2.9. Determination of serum antibodies

The mouse sera were analysed for antibodies reactive to M3, ZZ or S. typhimurium LPS by ELISA. Reagents for the ELISA were obtained from Sigma. Microtiter plates (Costar, Cambridge, MA) were coated with BBM3 fusion protein or ZZ at 2 μg/ml in carbonate buffer, pH 9.8, overnight at 4°C. When coating the wells with LPS at 2 μg/ml, PBS, pH 7.5, was used as coating buffer. All plates were blocked for 2 h at 37°C with 1% BSA in PBS. Following the blocking step, microtiter wells coated with ZZ were incubated for 1 h at room temperature with 1% hIgG in PBS, which was also included in the diluent at 0.1% (0.1% BSA, 0.05% Tween-20 in PBS). All plates were incubated overnight at 4°C with pooled sera serially diluted. Isotypes of the reactive antibodies were determined by incubating for 1.5 h at 37°C with 1% BSA in PBS. Following the blocking step, microtiter wells coated with ZZ were incubated for 1 h at room temperature with 1% hIgG in PBS, which was also included in the diluent at 0.1% (0.1% BSA, 0.05% Tween-20 in PBS). All plates were incubated overnight at 4°C with pooled sera serially diluted. Isotypes of the reactive antibodies were determined by incubating for 1.5 h at 37°C with alkaline phosphatase-conjugated goat IgG specific for mouse γ- or μ-chains. Substrate (p-nitrophenyl phosphate, Sigma) was added and the absorbance at 405 nm was measured when a standard control mouse IgG or IgM antiserum, specific for M3 or ZZ, reached an absorbance of approximately 1.0. All serum samples were run in duplicates.

3. Results

3.1. The expression systems

Two different expression systems were investigated for expression of the 80 amino acid M3
polypeptide (Fig. 1A), derived from the C-terminal repeat region of the P. falciparum malaria blood-stage antigen Pf155/RESA. The M3 polypeptide was targeted to the periplasm or to the outer surface of Salmonella typhimurium SLΔ33-2 cells. The staphylococcal protein A derived expression system was evaluated for the first time in Salmonella for the production and periplasmic secretion of the fusion protein ZZM3 (Fig. 1B). A chimeric OmpA, consisting of regions both from E. coli and Shigella dysenteriae OmpA [12], was used for targeting of M3 to the outer cell surface of S. typhimurium. The gene fragment encoding M3 [19] was inserted into a polylinker site engineered at a position corresponding to the third external loop of OmpA (Fig. 1C). The resulting outer membrane protein was denoted OmpAM3.

3.2. Analysis and quantification of the recombinant protein

We have expressed the M3 polypeptide representing malarial repeat sequences derived from P. falciparum Pf155/RESA in S. typhimurium as a periplasmic ZZM3 fusion protein or as a part of a hybrid outer membrane protein A (OmpAM3) (Fig. 1). The localization of the two recombinant proteins containing M3 was evaluated by fractionation of the different cellular compartments of S. typhimurium followed by protein analysis by SDS-PAGE and immunoblotting. Particularly, we have assessed the amount of immunogen in each cellular compartment by comparing it to a protein standard (purified ZZM3 loaded at 1.6 ng to 5 μg, the detection limit of this system being approximately 10 ng). Although the M3 yields varied in different immunoblot titration experiments, this assay indicated that the dose of bacteria used for immunization (approximately 1 × 10⁸) delivers 1 μg of M3 when expressed as a periplasmic ZZM3 (25 kDa) fusion protein (Fig. 2). This is between 10-100 times more than when delivered as a surface-expressed 45 kDa OmpAM3 hybrid protein. The apparent size of OmpAM3 exceeded the predicted size of 45 kDa but such anomalous migration has been demonstrated earlier [29]. This analysis also demonstrates that the two gene products were efficiently targeted to the expected cellular compartments; ZZM3 to the periplasm (Fig. 2, lanes 1 and 2) and OmpAM3 to the outer membrane (Fig. 2, lanes 3 and 4). The OmpAM3 fusion protein appeared to be more sensitive to degradation. This might be due to proteolysis during cultivation but could also result from the extraction and sonication procedure used to recover the membrane fractions.

3.3. Surface exposure of the inserted polypeptide

To further investigate the surface display of the malarial antigen, immunofluorescence studies of intact S. typhimurium cells expressing the OmpAM3 fusion protein were performed. Only a minor fraction of the cells (approximately 15%) expressed the recombinant protein in an accessible form on the surface (Fig. 3A). As expected, when the recombinant bacteria were tested with antiserum to S. typhimurium LPS, a strong fluorescence staining of all bacteria was observed (Fig. 3B). No staining could be demonstrated with preimmune serum or with control cells carrying the OmpA-plasmid without the
Fig. 2. Detection and quantification of M3 fusion proteins in *S. typhimurium* by immunoblotting. Equal amounts of bacteria ($1 \times 10^6$) were analysed for the presence of expressed M3 polypeptide in the periplasm, the outer membrane fraction or whole cell lysates by subjecting the samples to a 10% SDS-PAGE gel and transferring the proteins to nitrocellulose membranes. M3 was probed using a rabbit antiserum to M3. Lanes 1 and 2, periplasmic and whole cell lysate from SL-ZZM3; lanes 3 and 4, membrane fraction and whole cell lysate from SL-OmpAM3; lanes 5–10, affinity purified ZZM3 standard: 1.6 ng; 8 ng; 40 ng; 200 ng; 1 μg and 5 μg.

M3 insert (data not shown). Furthermore, when induced SL-ZZM3 cells were tested under the same conditions, no fluorescence staining was detected (Fig. 3C), indicating the specificity of this technique for cell surface exposed epitopes.

3.4. *In vivo* stability of the ZZM3 or OmpAM3 constructs

The ability of recombinant *Salmonella* to invade and persist in mouse lymphoid tissues and to maintain the constructs encoding the malarial fusion proteins was investigated by a bacterial colonization and persistence assay. Recombinant *S. typhimurium* harboring plasmids pZZM3 or pOmpAM3, maintained the gene constructs for up to three weeks in the lymphoid organs colonized (Table 1). This assay also indicated that recombinant bacteria which had lost their plasmids, persisted for longer periods in the lymphoid organs. The plasmid loss for both constructs occurred earlier in the spleen. The maintenance of the intact gene constructs by the bacterial isolates was further evaluated by isolating the plasmids from the bacteria. Plasmid mapping using restriction endonucleases and protein expression analysis in retransformed bacteria followed by Western blot, strongly suggest that the plasmids were intact (data not shown).

3.5. Immunogenicity of the hybrid proteins expressed by *S. typhimurium*

Strong antibody responses to M3 in inbred mice immunized with the fusion protein ZZM3 in FCA

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<tr>
<th>Week after immunization</th>
<th>Spleen</th>
<th>Liver</th>
<th>Lymph nodes</th>
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<tr>
<td></td>
<td>SL-ZZM3</td>
<td>SL-OmpAM3</td>
<td>SL-ZZM3</td>
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<td>4</td>
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Table 1

Plasmid stability in mice immunized with SL-ZZM3 or SL-OmpAM3 as determined by number of colony forming units per organ.

- LB amp.: Luria-broth with ampicillin.
- n.d.: not done.

*a* Number of colonies found on LB plates with or without ampicillin.
have earlier been shown to be linked to expression of the I-Ak allele [23]. Thus, our immunization experiments were all done in the CBA/J mouse strain.

In order to assess the immunogenicity of M3 when expressed by Salmonella in the periplasm as a ZZ fusion or on the outer bacterial surface as an OmpA hybrid, and to establish the efficacy of the delivery system in evoking a memory response to the M3 antigen, mice were immunized with SL-ZZM3 or SL-OmpAM3. Twenty-four weeks after the last immunization with bacteria, both groups of mice received a final i.p. injection with the purified ZZM3 protein in PBS. Blood samples were taken, and pooled sera were tested for the presence of M3, ZZ or S. typhimurium LPS-specific IgG and in certain cases also for IgM reactivity (Figs. 4 and 5).

Both constructs induced comparably low levels of IgM antibodies (titers < 200) to M3 (data not shown). Moreover, the IgG reactivity elicited to this polypeptide by SL-ZZM3 and SL-OmpAM3, was also comparable and followed similar kinetics: appearing and increasing after one booster injection, peaking after the second boost and declining thereafter (Fig. 4). When antisera from SL-ZZM3 immunized mice were analysed for their reactivity with ZZ, high levels of IgG (< 6400) but not IgM (< 200) antibodies were detected (Fig. 5A). The IgG response against ZZ in this immunization group did not parallel that elicited to M3, the latter being weaker and of significantly shorter duration (Figs. 4 and 5A). The injection with ZZM3 fusion protein administered to both groups of mice 24 weeks after the third boosting with live recombinant Salmonella,
resulted in a rapid and comparable increase of specific IgG antibodies reactive with the M3 polypeptide. Mice receiving SL-ZZM3 in the primary immunizations, developed similar M3-specific IgG levels (< 800) to those developed by mice initially receiving SL-OmpAM3 (< 400) (Fig. 4). The final injection also restored the already existing IgG antibodies to ZZ, which had not decreased completely even at 24 weeks after the last immunization with bacteria (Fig. 5A). Primary immunizations with the ZZM3 fusion protein did not induce antibodies to either M3 or to ZZ (data not shown) indicating that the booster effect observed with ZZM3 was due to a memory response elicited by the recombinant Salmonella immunizations. The antibody responses to S. typhimurium LPS in mice immunized with SL-ZZM3 or SL-OmpAM3 (> 10000) were strong, rapid and long lasting (Fig. 5B).

In immunoblotting of parasite extracts, analysis of the specificity of the antibody responses in antisera
obtained from mice after immunization with recombinant *Salmonella*, indicated that mice receiving SL-ZZM3 recognized predominantly a polypeptide of $M_\text{r}$ 155,000, corresponding to Pf155/RESA (Fig. 6). The immunization group receiving SL-OmpAM3 required an injection with the ZZM3 fusion protein in order to react with the 155 kDa antigen (Fig. 6).

4. Discussion

In the present study two different expression systems have been used to target a *P. falciparum* antigenic determinant to two separate compartments of an attenuated *Salmonella typhimurium* vaccine strain used for the expression and in vivo delivery. The staphylococcal protein A (ZZ) expression system, was used to target the antigen to the bacterial periplasm, while the outer membrane protein A (OmpA) system, was used to expose the antigen on the bacterial surface.

Our results show the presence of 10–100-fold more malarial antigen in the periplasm as a ZZ fusion than on the bacterial surface inserted into OmpA. Although this result is evidently reassured in vitro, it is not known if more protein is produced by the bacteria after immunization. According to previous studies on antigen expression by bacterial vaccine vectors and the implications for immunogenicity [32], it was demonstrated that the initial antigen dose delivered by the recombinant bacteria is the most relevant one in priming the immune system. However, it is likely that protein production is continued from the constitutive protein A promoter as opposed to the inducible tac-promoter regulating the OmpAM3 expression, since it requires IPTG or a lactose analogue for efficient transcription. Hence, the amount of antigen expressed by the recombinant bacteria, as detected by the immunoblot experiments, may not reflect entirely the dose produced and delivered in vivo.

Although the levels of M3 produced by the OmpA system in *Salmonella* were comparable with antigen amounts yielded by other surface expression systems in Gram-negative bacteria [10,33–35], the use of such systems may pose some problems which could account for low expression levels or surface inaccessibility. The inherent properties of the target protein may lead to incorrect protein folding during translocation through the cytoplasmic membrane or inappropriate integration into the outer membrane [36]. The low number of cells where M3 could be detected on the surface could also be due to inaccessibility to antibodies or conformational constraints on the epitope. In any event, it has been proposed that epitopes exposed at the cell surface of recombinant bacteria could induce antibody responses in a T-cell independent manner, by direct activation of B-cells [37]. Additionally, the outer membrane lipopolysaccharides may serve as adjuvants for surface anchored polypeptides. In contrast, antigens expressed in a soluble form in the periplasm of recombinant bacteria would most likely generate a T-cell dependent antibody response [37], requiring cell lysis by an antigen presenting cell (APC), processing and surface presentation to T-lymphocytes. The antibody responses induced herein by either the periplasmic or the surface constructs in *Salmonella* are consistent with a T cell-dependent response; these antibodies were essentially IgG and required at least one boost before significant titers were seen. The rapid anti-M3 IgG decline occurring after the second injection with both bacterial constructs is difficult to interpret. One explanation could be that the antibodies elicited to M3 are absorbed by the bacteria and are therefore not found in circulation. The reason why this antibody decline is not seen with ZZ or *S. typhimurium* LPS is probably due to the presence of higher antibody concentrations. This result and the fact that those antibodies can be sustained for a long period of time, indicate that the ZZ carrier is a strong immunogen. Further studies are needed to determine the contribution of ZZ in immune responses with regard to T-cell help or epitope suppression. Both the recognition of the native Pf155/RESA protein and the rapid appearance of an IgG response to M3 following a booster injection with M3 fused to an unrelated carrier, reflect the induction of immunological memory to the malarial antigen by both SL-OmpAM3 and SL-ZZM3. The lack of reactivity to Pf155/RESA protein in antisera from the SL-OmpAM3 immunizations, reflects weak anti-malarial antibody responses elicited by this construct in the primary immunizations.

In summary, the high levels of M3 produced by the ZZ system make it suitable for the expression of
heterologous antigens in Salmonella. It is interesting to note that, in spite of the quantitative difference in M3 expression, both the ZZ and OmpA constructs elicited comparable immune responses, indicating that the display of M3 on the surface of Salmonella was more efficient than the periplasmic presentation in priming the immune system. Although a number of parameters beyond antigen expression levels or cellular location and the effect on the antibody responses need to be further evaluated when using live bacterial vaccines, this study indicates that (i) surface display requires less antigen amounts than the intracellular location to elicit immune responses, and (ii) the structure of the fusion partner affects the magnitude of the immune responses.

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