TLR5-dependent immunogenicity of a recombinant fusion protein containing an immunodominant epitope of malarial circumsporozoite protein and the FliC flagellin of *Salmonella* Typhimurium

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Recently, we described the improved immunogenicity of new malaria vaccine candidates based on the expression of fusion proteins containing immunodominant epitopes of merozoites and Salmonella enterica *serovar Typhimurium flagellin (FliC) protein as an innate immune agonist. Here, we tested whether a similar strategy, based on an immunodominant B-cell epitope from malaria sporozoites, could also generate immunogenic fusion polypeptides. A recombinant His6-tagged FliC protein containing the C-terminal repeat regions of the VK210 variant of* Plasmodium vivax *circumsporozoite (CS) protein was constructed. This recombinant protein was successfully expressed in* Escherichia coli *as soluble protein and was purified by affinity to Ni-agarose beads followed by ion exchange chromatography. A monoclonal antibody specific for the CS protein of* P. vivax *sporozoites (VK210) was able to recognise the purified protein. C57BL/6 mice subcutaneously immunised with the recombinant fusion protein in the absence of any conventional adjuvant developed protein-specific systemic antibody responses. However, in mice genetically deficient in expression of TLR5, this immune response was extremely low. These results extend our previous observations concerning the immunogenicity of these recombinant fusion proteins and provide evidence that the main mechanism responsible for this immune activation involves interactions with TLR5, which has not previously been demonstrated for any recombinant FliC fusion protein.*

Key words: TLR5 - flagellin - CS protein - *P. vivax*

Intravenous vaccination with radiation-attenuated sporozoites (RAS) of *Plasmodium* can provide solid protection against malaria (Nussenzweig et al. 1967). Immunity generated by vaccination with RAS sporozoites is targeted towards a dominant protective antigen, the circumsporozoite (CS) protein (Kumar et al. 2006). Nevertheless, there exist other protective antigens yet to be characterised (Kumar et al. 2006, Mauduit et al. 2009). Following immunisation with RAS, the CS protein is recognised by antibodies and $CD4^+$ and $CD8^+$ T cells that can eliminate pre-erythrocytic stages of the parasite (Nardin et al. 1989, Romero et al. 1989, Malik et al. 1991, Rénia et al. 1991, Rodrigues et al. 1991). These results led to the development of vaccine formulations aimed at eliciting high antibody titres against sporozoites and/or large numbers of $CD4^+$ and $CD8^+$ T cells specific for liver stage components of malaria (reviewed by Bargieri et al. 2011).

Confirmation that the CS protein can be used for a human vaccine came from recent studies using the deadly parasite *Plasmodium falciparum*. This vaccine formulation consisted of a large C-terminal fragment of the CS protein fused to the antigen S of hepatitis B (conventional hepatitis B vaccine, Engerix B®) and was expressed as a recombinant protein in *Saccharomyces cerevisiae*. The fusion protein, named RTS, when expressed with antigen S, naturally assembles into virus-like particles called RTS,S. The efficacy of the RTS,S formulation depended on the use of adjuvant systems (AS) consisting of two different formulations of monophosphoryl lipid A (a detoxified form of lipopolysaccharides) and QS21 (saponin purified from *Quillaja saponaria*) in either an oil-in-water emulsion (AS02) or a liposomal suspension (AS01) (Cohen et al. 2010).

Recent Phase II trials in naïve human volunteers challenged with *P. falciparum* sporozoites found efficacies ranging from 32-50%. Immunological studies performed on these vaccinated individuals indicated that protection correlated with the concentration of specific antibodies and the frequency of interferon gamma (IFN-γ)-producing cells detected by enzyme-linked immuno-

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sorbent spot (Kester et al. 2009). A number of Phase IIb trials have been completed and others continue to be carried out in the field. Although results from field trials are more difficult to interpret, studies performed in children in the endemic areas have shown between 49.5-62% efficacy in retarding the first malaria episode during the six-month period studied (Abdulla et al. 2008, Bejon et al. 2008). As protective immunity was found to be highly dependent on the adjuvants used in the formulation, these studies highlight the importance of adjuvant development for an effective malaria vaccine. Similar studies with the CS protein of *Plasmodium vivax* have been initiated but have not reported findings to date.

Based on the ability of flagellin to efficiently induce humoral and cellular immune responses against foreign antigens, we explored the use of this molecule for the generation of fusion proteins containing malarial antigens. For our initial studies, we selected the 19-kDa C-terminal region of merozoite surface protein-1 (MSP-1_{19}) from *P. vivax* or *P. faciparum*. MSP-1_{19} was fused to the C-terminal end of FliC flagellin from *Salmonella typhimurium*. This fusion protein was able to bind and activate TLR5 expressed by in vitro-cultured transfected cells and it was recognised by serum from patients with *P. vivax* malaria. Moreover, the fusion protein was recognised by monoclonal antibodies against three-dimensional structural epitopes of MSP-1_{19}, which indicated that it was correctly folded when expressed in *Escherichia coli* (Bargieri et al. 2008, 2010).

We immunised mice and rabbits with recombinant fusion protein in the absence of additional adjuvant. Immunised animals developed strong, specific and long-lasting antibody-mediated responses. Antibody titres after the second immunisation were similar to those following immunisation with recombinant MSP-1_{19} emulsified in complete/incomplete Freund's adjuvant. Additionally, the antibodies generated by these immunisations recognised *P. vivax* or *P. falciparum* merozoites as detected by immunofluorescence (Bargieri et al. 2008, 2010). Most importantly, rabbits injected with Pf-MSP-1_{19} fused to flagellin raised high antibody titres that dramatically inhibited the in vitro growth of the parasite lines 3D7, S20 and FCR3 (Bargieri et al. 2010).

In the absence of other adjuvant, the immune response following immunisation was biased toward a type 2 response with a high IgG1/2 ratio and limited amounts of IFN-γ secretion by splenic immune cells. Nevertheless, the addition of other adjuvants to the fusion protein, such as a TLR-9 agonist, modulated the immune response towards a type 1 response with a lower IgG1/2 ratio and significantly greater secretion of IFN-γ (Bargieri et al. 2008).

In this study, we have investigated the immunogenicity of a fusion polypeptide containing the immunodominant region of the CS protein from *P. vivax* and the innate immune agonist *Salmonella* Typhimurium FliC flagellin. The immunogenicity of the recombinant fusion protein was assessed by immunisation of mice with the recombinant protein alone. Additionally, we investigated whether the antibody response was dependent on expression of the TLR5 molecule.

MATERIALS AND METHODS

Generation of the recombinant protein His-$_6$FliC-PvCS-VK210 - Generation of the original plasmid has been described previously (Bargieri et al. 2008). The *MSP-1_{19}* gene was removed by treatment with the enzymes *Hind*III and *Xho*I. The synthetic gene containing the sequence of nucleotides CAAGCTTGCGCAAGCGATATGGCGAAAAAAGAAACCGTGTGGCGTCTGGAAGAATTTGGCCGTTTCGATCGTGCAGATGGTCAGCCGGCAGGTGACCGCGCCGACGGTCAGCCGGCAGGTGATCGTGCAGCCGGTCAGCCGGCGGGTGATCGTGCAGCGGGCCAGCCGGCGGGTGATCGCGCCGATGGTCAACCGGCCGGTGATGACCGCGCCGATGGCCAACCGGCCGGTGACCGCGCCGATGGTCAGCCGGCCGGCGACCGCGCCGCCGGCCAACCGGCCGGCGACCGCGCAGCCGGCCAACCGGCCGGTGATCGCGCAGATGGTCAGCCGGCCGGTGATTAACTCGAG was cloned into this vector. Regions in underlined font represent recognition sites for *Hind*III and *Xho*I. The recombinant protein was expressed and purified as described previously (Bargieri et al. 2008). Briefly, recombinant *E. coli* BL21 DE3 (Novagen) was cultivated at 37ºC in flasks containing Luria broth (LB) and kanamycin (30 µg/mL). Protein expression was induced at an OD_{600} of 0.6 with 0.1 mM IPTG (Invitrogen) for 4 h. After centrifugation, bacteria were lysed on ice with an ultrasonic processor (Sonics & Materials INC, Vibra Cell VCX 750) in a phosphate buffer with 1.0 mg/mL lysozyme (Sigma) and 1mM PMSF (Sigma). Bacterial lysates were centrifuged and the supernatant was applied to a column containing Ni^{2+}-NTA-agarose resin (Quiagen). After several washes, bound protein was eluted with 0.5 M imidazole (Sigma). Eluted protein was dialysed against 20 mM Tris-HCl (pH 8.0) and the recombinant protein was purified by ion-exchange chromatography using a Resource Q column (GE Healthcare) coupled to an FPLC system (GE Healthcare). Fractions containing highly pure recombinant protein were pooled and extensively dialysed against phosphate-buffered saline (PBS) (pH 7.2). Protein concentration was determined by the Bradford assay and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses.

FliC purification - Native *Salmonella* Typhimurium FliC was purified from the attenuated *Salmonella* Typhimurium SL3201 strain, which expresses FliC but not FljB (Bargieri et al. 2008). Briefly, bacteria were grown in LB supplemented with kanamycin (30 µg/mL) overnight at 37ºC under aeration (80 rpm). Cells were washed once with PBS and submitted to mechanical shearing during four 2 min cycles in a bench vortex mixer. The cell suspensions were centrifuged to remove cellular debris and, following acetone precipitation, the flagellar filaments were collected from the supernatant and suspended in PBS. The purity of the preparations was monitored by SDS-PAGE.

Immunisation regimens - Six-eight-week-old female C57BL/6 (H-2^b) or genetically deficient TLR5 (KO) (Feuillet et al. 2006) mice were purchased from the Fed-

eral University of São Paulo (UNIFESP), Brazil. Mice were immunised subcutaneously three times each three weeks apart. The first immunisation was given in the hind footpads with a final volume of 50 µL in each footpad and the second and third immunisations were in the base of the tail in a final volume of 100 µL. For each immunisation, 5 µg or 10 µg of fusion protein or FliC was used.

Immunological assays - For immunologic characterisation, proteins on SDS-PAGE gels were transferred electrophoretically onto nitrocellulose membranes (Millipore, CA, USA). The membranes were blocked for up to 1 h using nonfat milk in PBS (5% w/vol) and bovine serum albumin (2% w/vol) containing 0.1% Tween 20 (PBS-T). After washing with 0.05% PBS-T, the blots were incubated for 1 h at room temperature with the appropriate primary antibody diluted in PBS-T. The primary antibodies used were the following: (i) mouse monoclonal antibodies (MAb) to His_6 (Amersham/GE) diluted 1:1,000, (ii) MAb 2F2 to PvCS protein from the VK210 strain (1 µg/mL) (Nardin et al. 1982) and (iii) MAb to PvCS protein from the VK247 strain. After washing, peroxidase-labeled goat anti-mouse IgG (Sigma, St. Louis, Mo) diluted 1:1,000 was added for 1 h. The blots were then washed and developed with diaminobenzidin solution (0.285 mg/mL, Sigma).

Serum anti-PvCS antibodies were detected by enzyme linked immunosorbent assay (ELISA) as described previously (Bargieri et al. 2008). Recombinant His_6PvCS protein (200 ng/well) was used as the solid phase-bound antigen. This recombinant protein contains the entire PvCS protein, including the N and C-terminal portions and repeats (VK210) and further information will be published in detail elsewhere. Mouse sera were tested at serial dilutions starting at 1:200 and a peroxidase-conjugated goat anti-mouse IgG (Sigma) was applied at a final dilution of 1:1,000. Anti-PvCS antibody titres were determined for the highest dilution yielding an OD_{492} greater than 0.1. The results are presented as mean ± standard deviation.

Statistical analyses - The one-way analysis of variance (ANOVA), Student's *t* test, Tukey honestly significant difference test and Kruskal-Wallis ANOVA were used to compare the differences between the mean values of the tested immunisation groups.

Ethics - The use of animals and the experimental procedures, including the number of mice per experimental group, have been approved by the Ethics Committee for Animal Care of the UNIFESP (Id # CEP 0307/09).

RESULTS

Production and purification of flagellin fusion protein - We generated a recombinant fusion protein containing a hexa-histidine tag (His_6) consisting of the immunodominant region of the PvCS protein (VK210 strain) linked to the C-terminal end of FliC (His_6FliC-PvCS-VK210). The schematic representations of the recombinant polypeptide and purified protein are presented in Fig. 1A, B, respectively. The purified protein migrated under denatured and reduced condition as a main band of ~65 kDa and a larger band of ~130 kDa. Both bands were determined to be recombinant protein by the recognition of the His_6 MAb (Fig. 1B, Lanes 3, 4). The larger band most likely represents a dimeric form of the recombinant protein. The native FliC protein purified from monophasic *Salmonella* Typhimurium of strain SL3201 migrated as predicted at ~49 kDa (Fig. 1B, Lane 1) and was used as a control in immunologic assays.

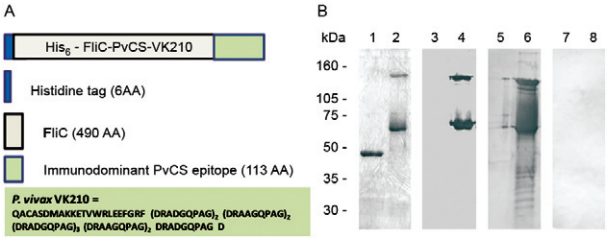

Fig. 1: generation and characterisation of recombinant *Plasmodium vivax* circumsporozoite protein VK 210 strain. A: schematic representation of the recombinant protein used in the present study; B: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of the recombinant protein. Lanes 1, 2: SDS-PAGE of purified *Salmonella* Typhimurium FliC flagellin and His_6-FliC-PvCS-VK210 proteins, respectively; 3, 4: immunoblot of the same two proteins described in Lanes 1, 2 probed with a mouse monoclonal antibodies (MAb) to His_6; 5, 6: immunoblot of the same two proteins described in Lanes 1, 2 probed with a MAb to repeats of PvCS protein VK210 strain; 7, 8: immunoblot of the same two proteins described in Lanes 1, 2 probed with a MAb to repeats of PvCS protein VK247 strain. Each Lane was loaded with approximately 1 µg of protein separated on a 15% SDS-PAGE and stained with Coomassie Blue (Lanes 1, 2).

To determine whether the fusion polypeptide retained the ability to be recognised by the MAb 2F2 specific for the immunodominant epitope of the PvCS protein of strain VK210, we performed ELISA and immunoblot assays. This MAb recognised the recombinant protein in both assays. Fig. 1B, Lane 6 shows the immunoblot confirming recognition of the recombinant protein His_6FliC-PvCS-VK210 by MAb 2F2 (Nardin et al. 1982). This epitope was not recognised by a MAb to the immunodominant epitope of the PvCS protein from strain VK247 (Fig. 1B, Lane 8). In control experiments, this MAb recognised the PvCS protein from the VK247 strain (LHT, unpublished observations).

Induction of PvCS protein-specific antibody responses in mice immunised with His_6FliC-PvCS-VK210 - The serum IgG responses to PvCS protein of the VK210 strain were determined in C57BL/6 mice immunised subcutaneously with purified His_6FliC-PvCS-VK210 protein (5 µg/dose/mouse). Mice parenterally immunised with the recombinant protein developed significant levels of specific IgG titres. Maximal IgG antibody titres were achieved after the second immunisation (Fig. 2), as a third immunising dose was not found to improve antibody titres ($p > 0.05$). These titres were maintained for 15 weeks (9 weeks after the third dose) (Fig. 2).

TLR5-dependence on PvCS protein-specific antibody responses in mice immunised with His_6FliC-PvCS-VK210 - Because FliC activates a variety of innate pattern-asso-

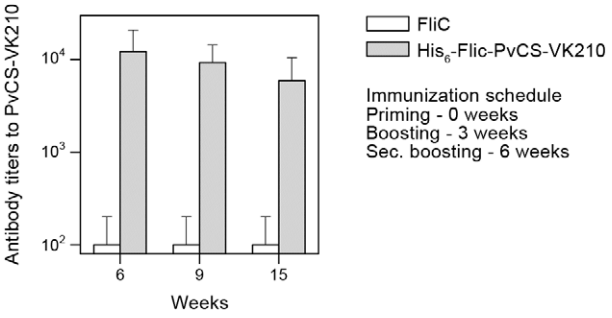

Fig. 2: detection of total IgG responses to His_6-PvCS-VK210 in serum samples of immunised mice. Female C57BL/6 mice were immunised three times with 5 µg of the purified *Salmonella* Typhimurium FliC flagellin or His_6-FliC-PvCS-VK210 proteins antibody titres were detected by conventional enzyme linked immunosorbent assay. Data are representative of two experiments using five mice per group.

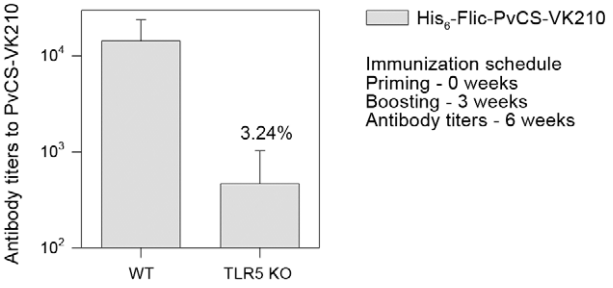

Fig. 3: TLR5-dependence of total IgG responses to His_6-PvCS-VK210 in serum samples of immunised mice. Female WT C57BL/6 or TLR5 KO mice were immunised twice with 10 µg of the purified His_6-FliC-PvCS-VK210 protein. Antibody titres were detected by conventional enzyme linked immunosorbent assay. Antibody titres of TLR5 KO were significantly lower than WT mice ($p < 0.001$). Data are representative of two experiments using five mice per group.

ciated receptors during the immune response to flagellin (Sanders et al. 2009, Mizel & Bates 2010, Vijay-Kumar et al. 2010), we sought to determine whether TLR5 was particularly important for this process in response to immunisation. To this end, we immunised WT C57BL6 mice or TLR5 KO mice with His_6FliC-PvCS-VK210. Three weeks after the second dose, we evaluated serum antibody titres in these animals. As shown in Fig. 3, the antibody titres from immunised TLR5 KO mice represented only 3.24% of the titres detected from WT immunised mice. These results unequivocally demonstrate that the main route for immune activation following immunisation with our fusion recombinant protein was via TLR5.

DISCUSSION

In the present study, we tested whether a straightforward and inexpensive fusion protein containing the prototypic innate immune activator FliC from *Salmonella* and an immunodominant epitope from the malaria sporozoite could elicit specific humoral immune responses to the CS protein, the best-studied malaria vaccine candidate. From our studies, we concluded that parenteral immunisation with the fusion protein His_6FliC-PvCS-VK210 elicited specific adaptive immune responses in vaccinated mice.

Various systems using flagellin components as carriers or adjuvants for fusion proteins containing recombinant antigens have been described in the last few years. A detailed review of these data was very recently published (Mizel & Bates 2010). Most importantly for vaccine development, the influenza vaccine candidate VAX125, which consists of an influenza antigen fused to flagellin, was given a complete Phase I clinical trial that tested safety, reactivity, immunogenicity, tolerogenicity and escalating dose-range. The results from this trial showed recombinant flagellin to be generally well-tolerated by vaccinated individuals. Importantly, 91% of individuals receiving any dose of recombinant protein developed titres of neutralising antibodies compatible with protective status against influenza infection (clinicaltrials.gov/NCT00921947) (Treanor et al. 2010).

The immunogenicity of our recombinant protein was independent of the use of conventional adjuvants, which led us to hypothesise that the main adjuvant activity seen was due to the innate immune agonist FliC. Previous studies have shown flagellin to activate multiple pattern-associated molecules such as TLR5 and NLRC4 (also known as Ipaf) (Sanders et al. 2009, Mizel & Bates 2010, Vijay-Kumar et al. 2010), which had made it difficult to determine the precise nature of the adjuvant activity using single KO mice (Sanders et al. 2009, Vijay-Kumar et al. 2010). Only very recently, by using double KO mice lacking both TLR5 and NLRC4, it was possible to demonstrate the contribution of these molecules to the adjuvant activity of flagellin when admixed to ovalbumin (Vijay-Kumar et al. 2010). We were able to decipher the main adjuvant contribution as originating at TLR5 because the TLR5 KO mice displayed significantly impaired immune responses after vaccination (Fig. 3). One likely explanation for such dominance of the TLR5 pathway comes from a very recently published article that found the adjuvant activity of flagellin to rely on the ability of TLR5 to function as an endocytic receptor and thereby enhance specific immune responses (Letran et al. 2011).

Another possible explanation for this dominance of the TLR5 pathway is that the NLRC4 activation domain of flagellin is possibly disrupted or blocked by the presence of the malarial epitope in the recombinant fusion protein. The NLRC4 activation domain is located at the D0 domain of FliC and requires the most C-terminal 35 AA of the protein. Therefore, the introduction of the malarial epitope may result in a domain that is no longer functional (Lightfield et al. 2008). Alternatively, the recombinant flagellin may not be capable of translocation to the cell cytoplasm where NLRC4 activation takes place. It will be important in the future to determine why this recombinant protein lost the ability to efficiently activate NLRC4; in the meantime, it can be a useful tool to study TLR5 activation by flagellin that is independent of the activation of NLRC4.

In summary, the present study has reproduced and extended upon our recent reports on the use of *Salmonella* flagellin as an adjuvant and antigen carrier to allow for

additional plasticity in the design and production of vaccine antigens endowed with enhanced immunogenicity for important malarial antigens. Considering the results from recent studies in humans, our findings strongly argue in favour of implementation of future clinical trials. In addition, the present study has dissected important mechanistic information on the immunostimulatory properties of these immunisations by showing that TLR5 is the primary receptor activated by our recombinant protein.

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