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Immunogenicity of synthetic peptide constructs based on PvMSP9$_{E795-A808}$, a linear B-cell epitope of the *P. vivax* Merozoite Surface Protein-9

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ABSTRACT

Plasmodium vivax Merozoite Surface Protein-9 (PvMSP-9) is a malaria vaccine candidate naturally immunogenic in humans and able to induce high antibody titers in animals when delivered as a recombinant protein. Recently, we identified the sequence EAAPENAEPVHENA (PvMSP9$_{E795-A808}$) as the main linear B-cell epitope in naturally exposed individuals. However, the potential of PvMSP9$_{E795-A808}$ as an immunogen in experimental animal models remained unexplored. Here we assess the immunogenicity of PvMSP9$_{E795-A808}$ using synthetic peptides. The peptides tested in BALB/c mice include two repeats of the sequence EAAPENAEPVHENA tested alone (peptide RII), or linked to an autologous (PvMSP9 peptide pL; pLRII) or heterologous (p2 tetanus toxin universal T cell epitope; TTRII) T cell epitope. Immune responses were evaluated by ELISA, FLUOROSPOT, and indirect immunofluorescence. We show that all of the peptide constructs tested were immunogenic eliciting specific IgG antibodies at different levels, with a prevalence of IgG1 and IgG2. Animals immunized with synthetic peptides containing T cell epitopes (pLRII or TTRII) had more efficient antibody responses that resulted in higher antibody titers able to recognize the native protein by immunofluorescence. Relevantly, the frequency of IFN-γ secreting SFC elicited by immunization with TTRII synthetic peptide was comparable to that reported to the PvMSP9-Nt recombinant protein. Taken together, our study indicates that PvMSP9$_{E795-A808}$ is highly immunogenic in mice and further studies to evaluate its value as promising vaccine target are warranted. Moreover, our study supports the critical role of CD4 T cell epitopes to enhance humoral responses induced by subunit based vaccines.

1. Introduction

After more than a century of basic research on malaria, this vector-borne disease continues to be a global health threat. Although *Plasmodium falciparum* continues to cause the greatest morbidity and lethality among the five species of *Plasmodium* that infect humans, an increasing number of severe cases caused by *P. vivax* have been reported. In 2016, *P. vivax* was responsible for about 40% of malaria cases outside of Africa, representing 64% of malaria cases in the Americas, above 30% in Southeast Asia and 40% in Eastern Mediterranean regions [1]. Several factors are involved in the high transmissibility and spread of *P. vivax* and include: early and continuous production of gametocytes during the erythrocytic cycle [2,3], shorter development cycle in the vector compared to other *Plasmodium* spp. [4], and ability to relapse

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from long-lasting dormant liver stages (hypnozoites) [5,6]. These biological features along with the enormous socioeconomic impact caused by *P. vivax* [7], the report of severe and lethal *P. vivax* malaria cases [8–10] and the emergence of chloroquine [11–13] and primaquine resistant strains [14–16] make the development of a safe and affordable vaccine a critical component in *P. vivax* control strategies.

The identification and validation of potential vaccine targets against *P. vivax* have been delayed, in part due to difficulties associated with the absence of a continuous, long-term *in vitro* culture of this parasite, but also due to limited of investment in available tools and methods [17–19]. Regardless, peptide constructs, containing B and T-cell epitopes, have been considered in strategies for developing vaccines for *P. vivax* and this direction has advanced for several known target antigens. This vaccine platform based on the design of minimal subunits, using synthetic peptides, has the potential to deliver precisely defined epitopes that can be produced at large-scale, high yield and relatively low cost [20,21]. Synthetic peptides are also stable in the absence of proteases, do not have contamination with biological agents, and can be produced in a fast and reproducible manner [22]. Furthermore, peptide vaccines allow the conjugation of multiple epitopes in a single construct representing a promising approach against genetic variants of vaccine candidates that are involved in parasite escape mechanisms [20,23] and a good strategy to develop multi-stage or multi-specific vaccines. Unfortunately, synthetic peptides have overall poor immunogenicity [24,25]. Because this, several alternative approaches have been used to overcome this barrier, like the use of virus-like particles and the conjugation of B-cell linear epitopes to T-cell epitopes or lipid moieties [26].

T cell-independent immune responses induced in the absence of T cell help are weaker, uneven and have impaired memory responses in comparison to those elicited by T cell-dependent antigens. T-helper cells play a crucial role in linking innate and adaptive immunity and they become critical components of peptide-based vaccines [27,28]. A challenge for testing subunit vaccines in preclinical trials is that individual epitopes could not be recognized by the experimental animal models used [29]. Recently, bioinformatics tools have been introduced for the successful *in silico* identification of potential epitopes on vaccine candidates against several pathogens [30–33]. However, the number of predicted and validated epitopes within *P. vivax* antigens, as well as the knowledge on protective efficacy is still limited.

Merozoite Surface Protein 9 (MSP9) is a conserved protein among *Plasmodium* species infecting humans, rodents and primates [34–36], which is expressed on the merozoite surface during schizont development and segmentation [37]. Antibodies produced against *P. cynomolgi* and *P. knowlesi* MSP9 homologs inhibit merozoite invasion of erythrocytes [36] and the immunization with MSP9 from *P. berghei* protected BALB/c mice from challenge with homologous parasite [38]. *P. vivax* MSP9 (PvMSP9) is also immunogenic in animal models [39] and naturally exposed individuals [40]. This experimental evidence supports the research and development of a *P. vivax* vaccine based on MSP9. Structurally, PvMSP9 contains a long non-repetitive conserved N-terminal domain, with five promiscuous CD4 T cell epitopes (pE, pJ, pK, pH and pL) [41,42] and a C-terminal domain, that contains two blocks of tandem repeats, described as the main target of the humoral response in adults living in endemic areas [40]. Recently, using a combination of *in silico* tools we identified the sequence EAAPENAEPVHENA ($PvMSP9_{E795\text{-}A808}$) as a minimal linear B-cell epitope. The native PvMSP9 includes five $PvMSP9_{E795\text{-}A808}$ tandem repeats, corresponding to 29% of the PvMSP9's two blocks of repeats. Furthermore, the potential role of $PvMSP9_{E795\text{-}A808}$ in the acquisition of protective immunity has been reported [43]. Based on this observation, we aimed to assess the value of $PvMSP9_{E795\text{-}A808}$ for the development of a subunit-based *P. vivax* vaccine by characterizing the immunogenicity of this epitope in animal models. Here we characterize the immune responses elicited by immunization with peptide-based immunogens that incorporate PvMSP-$9_{E795\text{-}A808}$. Peptides were synthetized representing the B cell epitope alone or conjugated to well-characterized CD4 T cell epitopes. Our data add further support for the development of vaccines based on linear synthetic-peptides and epitope mapping strategies of *P. vivax* proteins.

2. Material and methods

2.1. Peptide synthesis

Three epitopes were selected to design the peptide constructs: (a) the sequence EAAPENAEPVHENAEAAPENAEPVHENA (Peptide RII), consists of two repeats of the identified B-cell linear epitope PvMSP-$9_{E795\text{-}A808}$ arrayed in tandem conformation [43]. (b) The sequence ASIDSMIDEIDFYEK (PvMSP-$9_{A443\text{-}K456}$, Peptide pL), a well-defined promiscuous and naturally immunogenic CD4 T cell epitope [42], and (c) the sequence QYIKANSKFIGITE (Peptide TT), an epitope that has been studied for immune reactivity in 35 publication(s), tested in 52 T cell assays, 4 B cell assays and 57 MHC ligand assays (epitope ID 52929; http://www.iedb.org/epId/52929) and is considered a well-known epitope to peptide vaccines in murine models, human and primates [44–46]. All peptides were synthesized by fluorenylmethoxycarbonyl (F-moc) solid-phase chemistry [47] (GenOne Biotechnologies, Brazil) as single peptides (RII, pL, TT) and as hybrid peptides, containing a combination of a B-cell epitope and a T-cell epitope (pLRII and TTRII) (Table 1). Synthetic peptides containing PvMSP-$9_{E795\text{-}A808}$ were flanked by cysteine residues at N- and C-terminal regions, which allows spontaneous polymerization, a strategy that has been used to enhance immunogenicity [48–50]. Analytical chromatography of the peptide demonstrated a purity of >95% and mass spectrometric analysis also indicated an estimated mass corresponding to the mass of the peptides.

2.2. Immunization of mice with synthetic peptides

Female BALB/c mice of 6–8 weeks of age were obtained from the Institute of Science and Technologies in Biomodels (ICTB)/FIOCRUZ. Groups of 21 mice were immunized subcutaneously (s.c) three times at 3-week intervals (days 0, 21 and 42) at the base of the tail with 50 μg of one of the synthetic peptides (RII, pL, TT, pLRII and TTRII) emulsified in 150 μL of Montanide ISI 51 (SEPPIC, France). Controls received only PBS emulsified in the same adjuvant. Mice were bled at days 0, 11, 21, 33, 42, 63, 84 and 132, and the sera samples were tested by enzyme-linked immunosorbent assay (ELISA) for antibody responses. On day 63 three mice in each group were sacrificed, and splenocytes were harvested to evaluate cellular immune responses using IFN-γ and IL-5 Fluorospot assays. Twenty-one non-immunized animals were bled and sacrificed at each time point to serve as additional control group.

All the animal studies were performed at the animal facilities of Oswaldo Cruz Foundation in accordance with guidelines and protocols approved by the Ethics Committee for Animal Experimentation of the Oswaldo Cruz Foundation CEUA-FIOCRUZ (Protocol N° LW-12/14).

2.3. Recombinant PvMSP9-RIRII

The recombinant protein PvMSP9-RIRII, containing the C-terminal blocks of tandem repetitions, was expressed as a GST fusion protein, as described [40] and were used in Absorption ELISA tests.

Table 1
Design of synthetic peptides used in this study. Five peptides were synthesized based on three selected epitopes. The single peptides – RII, representing two repeats of the B cell epitope PvMSP9$_{(E795\text{-}A808)2}$; Peptide pL representing the T cell epitope PvMSP9$_{(A443\text{-}K456)}$ identified within the N-terminal region of PvMSP9; Peptide TT, Tetanus toxin$_{(Q830\text{-}E843)}$ corresponding to a well-defined CD4 T cell epitope. To induce spontaneous polymerization, the peptides used for immunization were synthesized with flanked cysteine residues.

Nomenclature	Topology	Amino acid sequence	MW (Da)
RII	cys-PvMSP9$_{(E795\text{-}A808)2}$-cys	CEAAPENAEPVHENAEAAPENAEPVHENAC	3143.25
pL	PvMSP9$_{(A443\text{-}K456)}$	ASIDSMIDEIDFYEK	1775.93
TT	Tetanus toxin$_{(Q830\text{-}E843)}$	QYIKANSKFIGITE	1611.84
pLRII	cys-PvMSP9$_{(A443\text{-}K456)}$PvMSP9$_{(E795\text{-}A808)2}$-cys	CASIDSMIDEIDFYEKEAAPENAEPVHENAEAAPENAEPVHENAC	4901.17
TTRII	cys-Tetanus toxin$_{(Q830\text{-}E843)}$PvMSP9$_{(E795\text{-}A808)2}$-cys	CQYIKANSKFIGITEEAAPENAEPVHENAEAAPENAEPVHENAC	4737.07

2.4. Antibody assays

The presence and levels of specific antibodies against the synthetic peptides in sera of mice were evaluated by Enzyme-linked immunosorbent assay (ELISA). Briefly, 96- microwell plates (Nunc-Maxisorb) were coated with 5 μg/mL of synthetic peptides (RII, TT, and pL). After overnight incubation at 4 °C, the plates were washed with PBS and blocked with PBS-0.05% Tween 20 containing 5% non-fat dry milk (PBS-Tween-M 5%) for 1 h at 37 °C. Individual mice serum samples at two-fold serial dilutions in PBS-Tween-M 2.5% were added to duplicate wells, and the plates were incubated at 37 °C for 2 h. After three washes with PBS-Tween, bonded antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (Southern Biotech) followed by o-phenylenediamine and hydrogen peroxide. The absorbance was read at 492 nm using an ELISA reader (Spectramax 250, Molecular Devices, Sunnyvale, CA). The end-point titers in the mice sera were determined as the highest dilution at which immunized mice sera had optical density (OD) value three times higher than sera from control mice (the OD values in the control mice were about 0.045, 0.053 and 0.054 for peptides RII, TT, and pL, respectively). The determination of IgG subclass profile against peptide RII was also performed as described above, except that the secondary antibodies used were goat anti-mouse monoclonal antibodies specific for mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech). Moreover, to confirm the reactivity of induced antibodies against PvMSP9, an ELISA was performed following the same methodology above described, except that plates were coated with 2 μg/mL of recombinant protein PvMSP9-RIRII.

2.5. Absorption ELISA

The absorption ELISAs were performed as previously described [51]. Briefly, 96-microwell plates (NUNC-Maxisorp) were coated overnight with 5 μg/mL of the peptide RII or 2 μg/mL of PvMSP9-RIRII recombinant protein, then washed, and blocked as described. Sera were added to the plates at end-point titers and incubated for two hours at 37 °C. After incubation, sera were transferred to plates coated overnight with PvMSP9-RIRII (2 μg/mL) after appropriate washing and blocking, and the ELISAs were performed as described. After the read of absorbance, the OD values against PvMSP9-RIRII of serum before and after absorption were compared.

2.6. Indirect immunofluorescence assays

The specificity of the antibody response elicited by immunization was tested by immunofluorescence assays (IFA) using air-dried thin films of erythrocytes infected with *P. vivax* schizonts as described previously [39]. Surface expression was detected using sera from mice immunized with synthetic peptides RII, pLRII and TTRII and affinity-purified goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma, St. Louis). Pools of sera from animals of each group (RII, TTRII, pLRII) collected on days 63 and 84 were tested at 1:50 dilution. DAPI (4′,6-Diamidine-2′-p henylindole dihydrochloride) (SIGMA, St. Loius) was used to confirm the presence of DNA. Serum of an individual from Brazilian Amazon, who presented high antibody titers against *P. vivax* merozoite proteins was used as positive control.

2.7. Fluorospot

The relative number of mouse antigen-specific T-cells secreting IFN-γ and IL-5 was determined by FluoroSpot (FluoroSpot kit for mouse IFN-γ/IL-5; MabTech). Briefly, Fluorospot plates were pre-wetted with 15 μL 35% ethanol for 1 min, immediately followed by washing with sterile water (200 μL/well). 100 μL of anti-mouse IFN-γ (AN18) and anti-mouse IL-5 (TRFK5) antibodies, both diluted to 15 μg/mL in sterile PBS, were added to each well. After overnight incubation at 4 °C, plates were washed with sterile PBS (200 μL/well), and blocked with 200 μL/well with cell culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 1 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 0.5 mM HEPES) for at least 30 min at room temperature. The blocking medium was removed and fresh medium with or without one of the stimulants (ConA, RII, TTRII, pLRII, pL, and TT) combined with anti-CD28 mAb at 0.2 μg/mL. Splenocytes of immunized mice were added to each well (250,000 cells/well) in duplicate and incubated for 30 h at 37 °C and 5% CO_2. Cells were removed by washing the plates with PBS (200 μL/well), and 100 μL of monoclonal antibodies anti-IFN-γ (R4-6A2-BAM; 1:200) and anti-IL-5 (TRFK4-biotin; 2 μg/mL) in PBS with 0.1% bovine serum albumin (PBS/BSA) were added to each well. Plates were incubated at room temperature for 2 h, followed by washing as described above. Secondary detection reagents (anti-BAM-490, and SA-550) were diluted 1:200 in PBS/BSA and 100 μL added to each well for 1 h at room temperature. Plates were washed as above and 50 μL fluorescence enhancer added to each well for 15 min. The enhancer was discarded thoroughly, the plate underdrain removed, and the plates left to dry protected from light. IFN-γ and IL-5 secreting cells were counted with an Immunospot reader S6UV ultra (Cellular Technology Ltd, Cleveland, OH). The number of IFN- γ, and IL-5 secreting cells per 10^6 spleen cells was expressed as the mean number of spots induced by antigen subtracted by the number of spots induced by PBS.

2.8. Statistical methods

GraphPad Prism version 5 (GraphPad Software, Inc, La Jolla, CA, USA) was used for statistical analysis. Statistical difference in categorical variables between the two defined groups was determined using Fischer exact test while Mann-Whitney *U* test was used to determine differences in continuous variables. P values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Synthetic peptides containing the epitope PvMSP9$_{E795\text{-}A808}$ are immunogenic in BALB/c mice and showed enhanced activity when linked to a T helper epitope

To evaluate the immunogenicity of the linear B-cell epitope PvMSP9$_{E795\text{-}A808}$, we determined antibody end-point titers elicited

by immunization against peptide RII, a synthetic peptide that includes two repeats of the PvMSP9$_{E795-A808}$ epitope. Plasma samples from mice immunized with different synthetic peptides (RII, pLRII, TTRII, pL and TT) were collected at different time points and antibody titers determined by ELISA (Fig. 1). Firstly, we confirmed that epitope PvMSP9$_{E795-A808}$ was immunogenic in BALB/c, once that all groups immunized with synthetic peptides containing the peptide RII (RII, pLRII and TTRII) presented specific IgG antibodies against this peptide. Animals immunized with single peptide RII had a detectable level of antibodies at day 42 after the first immunization and reached its maximum antibody level (1:6400) on day 84. Moreover, the immunization with peptides linked to T cell epitopes (pLRII and TTRII) elicited earlier and higher IgG antibody titers against peptide RII. Mice immunized with pLRII had detectable levels of antibodies (1:800) 33 days after the first immunization and presented the maximum titers of antibodies (1:12,800) at day 63. Besides, animals immunized with TTRII presented a detectable level of antibodies (1:100) 21 days after the first immunization, even before the second immunization, and also reached its maximum level of antibodies (1:25,600) at day 63. In groups RII, pLRII and TTRII, the maximum antibodies titers against RII were maintained since than reached until the kinetic last time point.

No cross-reactions were observed between antibodies specific to peptide RII and the T cell epitopes (pL and TT), since plasma collected from mice immunized with the single peptide RII, did not develop specific antibodies against peptides pL or TT (Supplementary Fig. 1a and 1b, respectively). Mice immunized with a single T cell epitope (pL or TT) did not elicit specific antibodies against RII (Fig. 1). Moreover, synthetic peptides containing T cell epitopes (pL, pLRII, TT, and TTRII) elicited low IgG specific responses against the T cell epitopes, 32 days after the first immunization (Supplementary Fig. 1). A specific response against peptide pL was observed in plasma of animals immunized with the single peptide pL or the hybrid peptide pLRII (Supplementary Fig. 1a). In the same way, anti-peptide TT IgG antibodies were identified in samples collected from mice immunized with the single peptide TT or the hybrid TTRII peptides (Supplementary Fig. 1b).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.10.016.

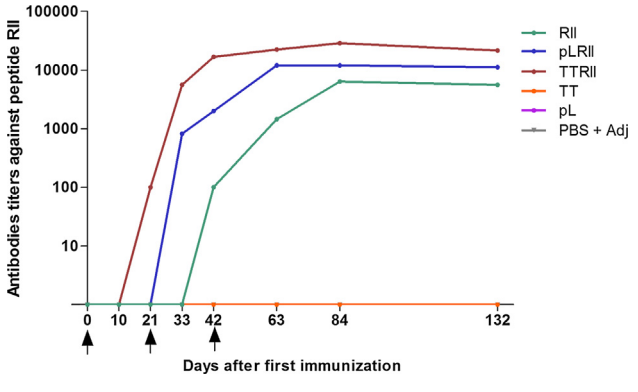

Fig. 1. Endpoint anti-RII antibody titers in mice immunized with synthetic peptides. The figure summarize the results of two different experiments and the values represent the mean of six animals in each point of both experiments (three animals per time point, in each experiment). Lines indicate the variation of antibody titer along experimental kinetic in immunized groups: RII (green), pLRII (blue), TTRII (red), pL (purple), TT (orange) and PBS (gray). All immunogens were formulated in adjuvant Montanide ISA51. Arrows indicate the immunization times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. IgG1 and IgG2 are the predominant isotypes against RII

To evaluate the profile of anti-RII IgG subclass induced by synthetic peptides, we determined the final IgG1, IgG2a, IgG2b and IgG3 antibody titers in plasma collected at day 63. In all studied groups, a similar subclass profile was observed with no differences between induced titers of IgG1, IgG2a, and IgG2b (Fig. 2). Moreover, the absence of detectable levels of IgG3 was a common finding in all groups. No significant changes in IgG isotype patterns were observed in the course of the follow-up after each immunization (Data not shown).

3.3. Anti-RII antibodies elicited by immunization with synthetic peptides recognized the PvMSP9-RIRII recombinant protein

To confirm the specificity of anti-RII antibodies elicited by immunization, we tested by ELISA the plasma of immunized mice against the recombinant protein PvMSP9-RIRII that represents the two blocks of repeats on the C-terminal region of PvMSP9. Plasma samples collected from mice immunized with the synthetic peptides constructs containing the peptide RII (RII, pLRII and TTRII) were able to recognize the recombinant protein at 1:100 dilution (Fig. 3). The O.D. mean values were higher in groups immunized with pLRII or TTRII than in group immunized with RII ($p = 0.038$ and $p = 0.031$; respectively). Moreover, mice immunized with peptide TT, pL or with PBS formulated in adjuvant presented similarly low ODs than groups immunized with RII, pLRII, and TTRII ($p < 0.0001$).

3.4. The anti-RII antibodies were able to recognize specifically the MSP9 native protein

After having demonstrated that anti-RII antibodies recognized the recombinant protein representing the two blocks of repeats within PvMSP9, we investigated whether these antibodies recognize the native protein expressed during the blood stage of the parasite life cycle. To accomplish this, pool of sera collected from mice immunized with different synthetic peptides were tested for reactivity using IFA, at dilution of 1:50. Plasma from mice immunized with peptides containing the sequence PvMSP9$_{E795-A808}$ (RII, pLRII and TTRII) recognized the native MSP9 with a fluorescent pattern

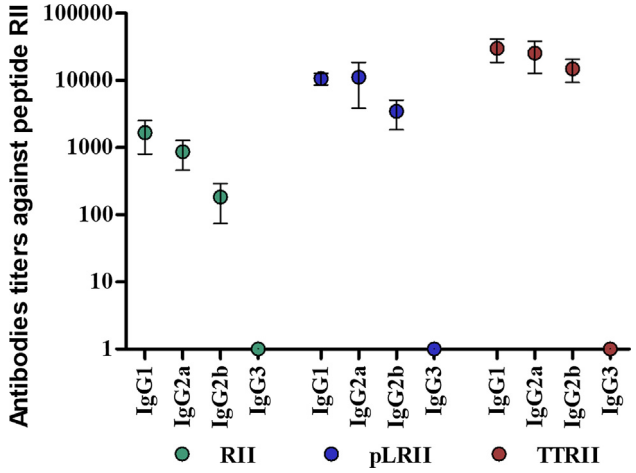

Fig. 2. IgG subclass profile against peptide RII induced by immunization with synthetic peptides. Each point represents end-point mean values of IgG1, IgG2a, IgG2b and IgG3 antibody titers ± SEM. RII (green), pLRII (blue), TTRII (red). Samples were collected three weeks after the last immunization (day 63) from six mice per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

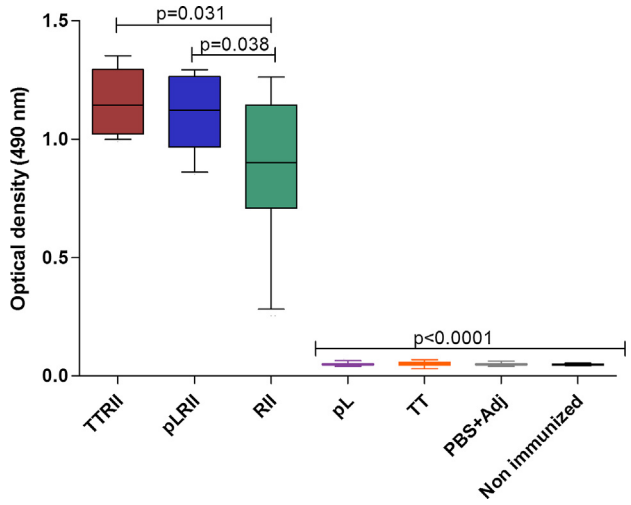

Fig. 3. Evaluation of reactivity of anti-RII IgG against the recombinant protein PvMSP9-RIRII. Optical densities of antibodies elicited by immunization with synthetic peptides TTRII (red box), pLRII (blue box), RII (green box), pL (purple box) or TT (orange box) against rPvMSP9-RIRII. The antibody responses are significantly higher in comparison to control mice immunized with adjuvant alone ($p < 0.0001$). Mice immunized with peptides containing linked T and B cell epitopes (pLRII and TTRII) presented higher optical densities than those immunized with the single peptide RII. Animals immunized with synthetic peptides representing T cell epitopes (TT and pL) or PBS formulated in adjuvant were not able to recognize the recombinant protein. Data is presented as Box and Whiskers plots with lines representing 10–90 percentile and p values included. Each column represents the optical densities of animals from each group of samples collected 42, 63, 84 and 132 days after the first immunization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

consistent with surface staining. No reactivity was observed in sera from naive mice, mice immunized with peptides pL or TT or mice immunized with PBS formulated in adjuvant. Representative results are shown in Fig. 4.

3.5. The promiscuous T-cell epitope TT was critical to induce IFN-γ secreting T cells

Once observed that T cell epitopes enhanced the antibody responses, we explored the effect of these epitopes on cellular immune responses. To determine the number of IFN-γ and IL-5-Spots Forming Cells (SFC) induced by immunization with synthetic peptides, we used the Fluorospot. Splenocytes derived from mice immunized with the single peptide RII and hybrid peptides (pLRII and TTRII) were collected 3 weeks after the third immunization (day 63) and were stimulated ex vivo using each peptide used for immunization (pL, TT, RII, pLRII and TTRII). Mice immunized with TTRII showed an increased number of IFN-γ-SFC, when stimulated with peptides TT or TTRII, compared to the number of SFC induced by the same peptides using splenocytes collected from mice immunized with other synthetic peptides ($p = 0.024$). Moreover, no significant number of IL-5 secreting cells were observed (Fig. 5). All cells stimulated with ConA have high numbers of IFN-γ and IL-5-secreting cells (Data not showed).

4. Discussion

Synthetic peptides represent a promising approach for the development of subunit vaccines [52,53], providing a safe and inexpensive alternative to the conventional vaccine platforms. This approach can be even more effective by targeting both B and T cell epitopes known to be involved in protective efficacy aiming to induce a balanced immune response [54–57]. However, constructs containing linear B-cell epitopes from *Plasmodium* antigens have not always met with their expected success [58–60]. Both antibody-dependent and -independent T-cell-mediated protective immune mechanisms are operative at different stages of the parasite life cycle [61–65], so the ideal vaccine should combine epitopes identified as strong inducers of both antibody and cell-mediated immunity. In this study, we used synthetic peptides

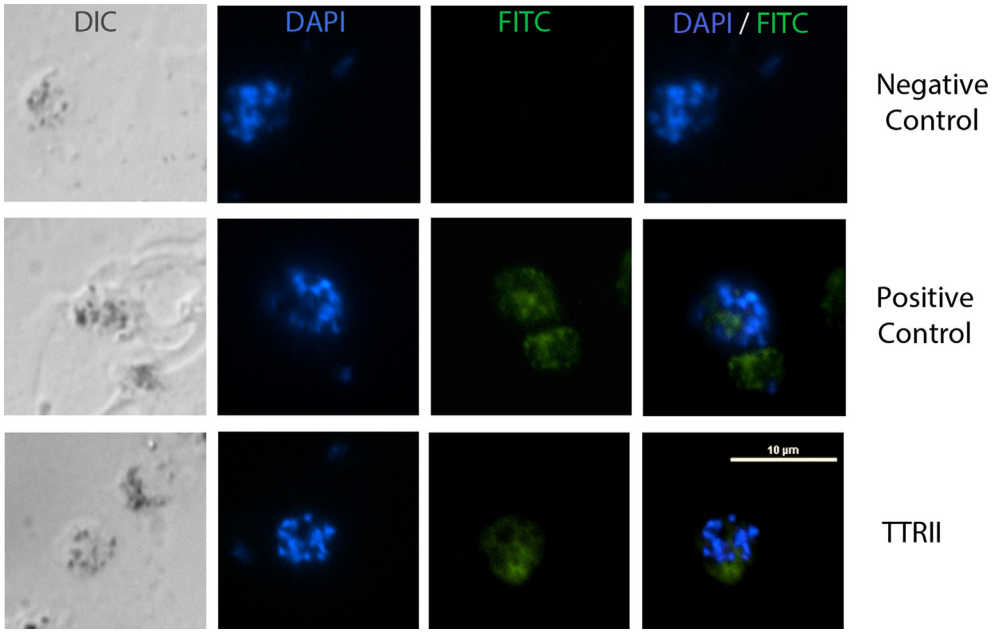

Fig. 4. RII-specific antibodies induced by synthetic peptides recognize the native PvMSP9. Binding of RII-specific IgG using a pool of sera from BALB/c mice immunized with the synthetic peptide (TTRII) and erythrocytes infected with *P. vivax* schizonts. Images were taken at 100-fold magnification. Left panels show the Differential Interference Contrast (DIC), followed by cell DNA stained with DAPI (4′,6-diamidino-2-phenylindole), the fluorescein isothiocyanate (FITC) fluorescence indicating the reactivity of anti-RII IgG against the parasite, and the right panels show the merged images (DAPI + FITC). Scale bar, 10 µm length. The sera of an individual from Brazil endemic area, who presented high response to *P. vivax* MSP-9 recombinant antigen was as used as positive control.

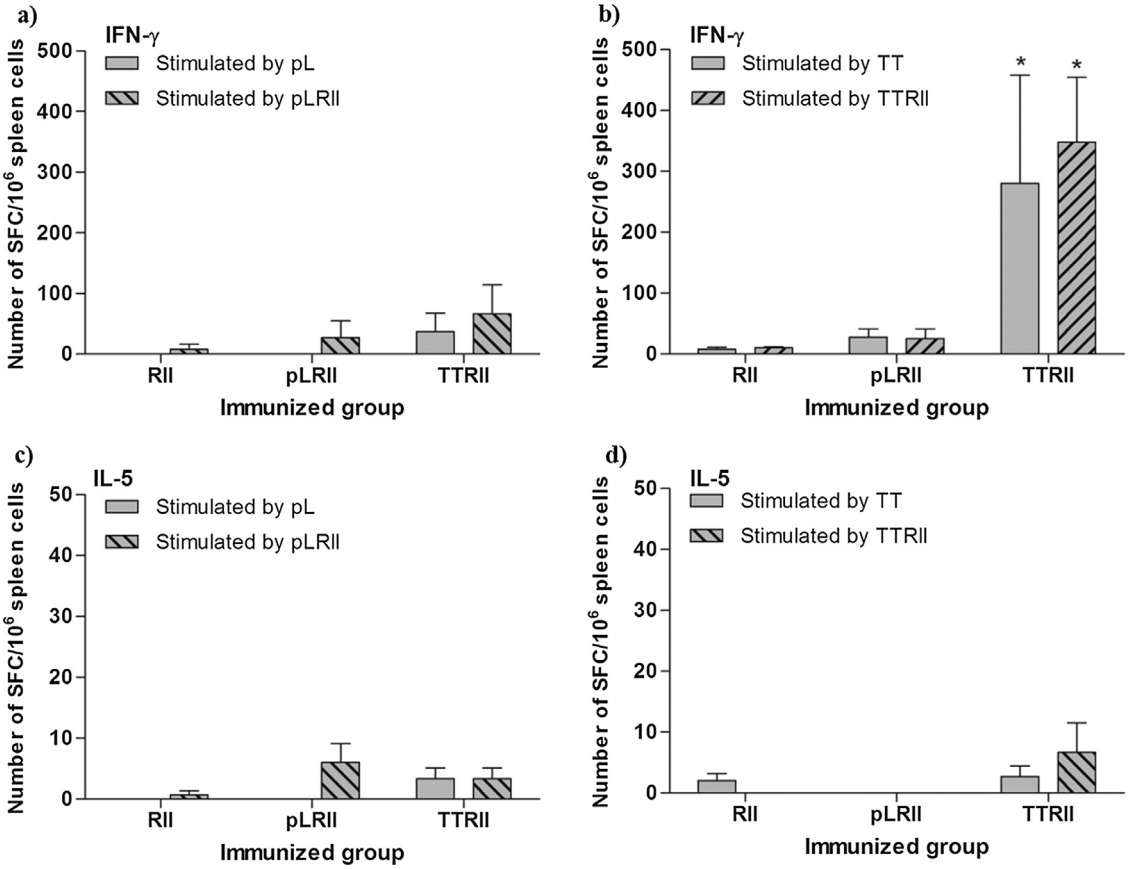

Fig. 5. Detection of IFN-γ and IL-5 secreting cells from mice immunized with synthetic peptides collected three weeks after the last immunization (day 63). Results are express as the mean values of duplicate assays using three different animals from each group individually analyzed. Cells of each animal were individually stimulated with each peptide. (a) Number of IFN-γ SFC in 10^6 spleen cells after stimulation with peptide pL (gray bars) or pLRII (striped bar). (b) Number of IFN-γ SFC in 10^6 spleen cells when stimulated with peptide TT (gray bars) or TTRII (striped bar). (c) Number of IL-5 SFC in 10^6 spleen cells when stimulated with peptide pL (gray bars) or pLRII (striped bar). (d) Number of IL-5 SFC in 10^6 spleen cells when stimulated with peptide TT (gray bars) or TTRII (striped bar). The corresponding immunized groups (RII, pLRII and TTRII) are presented in the X-axis. The bars represent the mean number of SFC stimulated by synthetic peptides and lines indicate the respective Standard Error Mean. The SFC values were subtracted from the SFC values obtained with the control group.

to immunize BALB/c mice and to verify the immunogenicity of two known epitopes described within PvMSP9, a potential vaccine candidate.

Firstly, we observed that the B cell epitope $PvMSP9_{E795-A808}$ was immunogenic and confirmed that hybrid peptides synthesized by linking the sequence to T cell epitopes exhibit enhanced antibody responses. All the peptides tested that include such a sequence (RII, pLRII, and TTRII) induced specific antibodies against RII and the antibody levels were maintained up to three months after the last immunization. Relevantly, both peptides containing B and T cell epitopes, pLRII and TTRII, elicited earlier and higher antibody titers (1:12,800 and 1:25,600, respectively) than the RII synthetic peptide, that includes only the B cell epitope (1:6400). The enhanced level of antibodies induced by synthetic peptides containing a T helper epitope, pLRII, and TTRII, are in agreement to previous studies [49,50], which demonstrated the enhancement of a specific response induced by the insertion of a T helper epitope and generated a specific level of antibodies similar to our study. Moreover, although this enhanced humoral response was expected to peptide TT (Tetanus $Toxin_{(Q830-E843)}$) [44–46], we present here the first evidence that the T cell epitope $PvMSP9_{A443-K456}$ has potential as a T helper epitope. The kinetics of the antibody responses elicited by immunization with the synthetic peptides that included the $PvMSP9_{E795-A808}$ epitope reported here are similar to those described for immunization with the recombinant PvMSP9 proteins. However, consistent with the lower immunogenicity of linear peptides, higher levels of antibodies were induced by the recombinant proteins [39].

Despite differences in total IgG levels between immunized groups, similar IgG isotype patterns were elicited with similar titers of IgG1, IgG2a, and IgG2b. Interestingly, the same subclass profiles were observed when BALB/c mice were immunized with PvMSP9 recombinant proteins, formulated in Montanide ISA51 [39]. This effect could be attributed to the adjuvant effect given the fact that adjuvants enhance and modulate the magnitude of adaptive immune responses to co-administered antigens, impacting longevity, antigen avidity, and modulation of isotype and IgG subclass switches [66,67]. On the other hand, based on the scarce knowledge about the role of each IgG subclass on protection against murine malaria, we cannot determine how effective the induced profile could be in a protective response. To date, amongst mouse IgG subclasses, IgG2a and IgG2b are considered to be the most potent activators of complement and most used in passive transfer experiments in murine infections (including malaria) [68,69]. Besides, IgG1 is believed not to be a potent complement activator [70]; to be poor at killing tumors [71]; but plays an important role in controlling gastrointestinal parasites [72].

Despite early studies already demonstrating that antibodies against recombinant MSP-9 were able to block merozoite invasion *in vitro* [36] and naturally acquired antibodies correlate with exposure/protection in Brazilian Amazon [40] and Southeast Asia [73], the functionality of these induced antibodies against our synthetic

constructs and their role on parasite recognition remained unknown. Therefore, we first observed that the induced IgG anti-RII were able to recognize the recombinant protein representing the PvMSP9-RIRII. This result was consistent with our previous study, in which we observed that specific antibodies against RII of naturally exposed individuals corresponded to 30% of antibodies against the PvMSP9-RIRII (Data not showed) [43]. Moreover, specific antibodies against peptide RII were also able to recognize the native protein on the surface of merozoites and schizonts in immunofluorescence assays. Unfortunately, we could not carry out an inhibition assay or challenge of immunized mice to verify the protective potential of the anti-RII antibodies. However, considering that monoclonal antibodies against PvMSP9 were able to inhibit the invasion of erythrocytes by *P. vivax* merozoites [36], that two blocks of repeats are the most immunogenic regions of the protein [40], the ability of anti-RII antibodies to recognize the recombinant protein PvMSP9-RIRII and the native protein reinforce the potential of $PvMSP9_{E795\text{-}A808}$ as a vaccine target for novel synthetic constructions.

Interestingly, even with the enhancement in humoral responses elicited by immunization with the synthetic peptide representing the B cell epitope linked to T cell epitopes, only the peptide TTRII was able to induce IFN-γ secreting cells. In our point of view, this finding could be associated with differences on presentation of T cell epitopes by MHC of different models, once for efficient induction of either B-cell or cytotoxic T cell responses, the induction of a robust T helper cell responses is crucial [74,75]. The use of promiscuous or universal T helper epitopes, which bind several or most MHC class II molecules, respectively, offer a good alternative to design subunit vaccines able to induce a robust immune response regardless of the MHC makeup [76,77]. Unfortunately, the evident bias on MHC presentation of synthetic peptides in humans and animal models limit several applications. For example, whereas the universal T cell epitope PADRE binds many human HLA-DR molecules with high affinity, they only show strong binding to H2I-Ab in mice [78]. Here, although the epitope $PvMSP9_{A443\text{-}K456}$ (peptide pL) was described as a promiscuous T helper epitope in humans [42], this was the first work using this as a T helper epitope in mice. Moreover, the evaluation of their prediction data suggested no binding of this peptide by mice MHC (H-2-Ib, H-2-Id, and H-2-Ed) (data not showed), whereas peptide TT (Tetanus $Toxin_{(Q830\text{-}E843)}$) was described as a universal epitope in human and mice [79]. On the other hand, the induction of IFN-γ secreting cells by TTRII was comparable to the number of secreting cells induced by recombinant protein PvMSP9 [39], supporting that the adequate choices of T helper epitopes could potentiate the immunogenicity of synthetic peptides.

In conclusion, this was the first work to evaluate the immunogenicity of the B-cell epitope $PvMSP9_{E795\text{-}A808}$ and the T helper epitope $PvMSP9_{A443\text{-}K456}$, using synthetic peptides as a vaccine platform. The B cell epitope $PvMSP9_{E795\text{-}A808}$ was immunogenic in BALB/c mice, and specific antibodies to this epitope were able to recognize the native parasite protein. Moreover, we confirmed that a Tetanus Toxin derived T-cell epitope enhanced the humoral immune response when conjugated to B cell epitope RII, once TTRII elicited an earlier and higher humoral response than a single peptide RII. Besides, our data suggest that epitope $PvMSP9_{A443\text{-}K456}$ was not a potential T helper in mice, disagreeing with the described promiscuity in binding to several MHC alleles in humans. Our data reinforces the importance of $PvMSP9_{E795\text{-}A808}$ as a potential epitope to be included in a subunit malaria vaccine against *P. vivax*.

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