Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Supporting Information

A Mimotope Attached to an ITIM-SHP-1 Interaction Inhibitory Peptide Boosts Immune Response and Efficacy

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Score = 559 bits (1441), Expect = 4e-157, Identities = 271/525 (51%), Positives = 364/525 (69%), Gaps = 10/525 (1%)

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Figure S1: Sequence alignments AMA1$_{PY}$ and AMA1$_{PF}$: The red color amino acids represent sequence of PY-AMA1 and blue color amino acids represent sequence of PF-AMA1. 191-203 of PF-AMA1 is highlighted in yellow and 136-148 of PY-AMA1 is highlighted in yellow. The highlighted sequences of PF-AMA1 and PY-AMA1 show significant similarity.

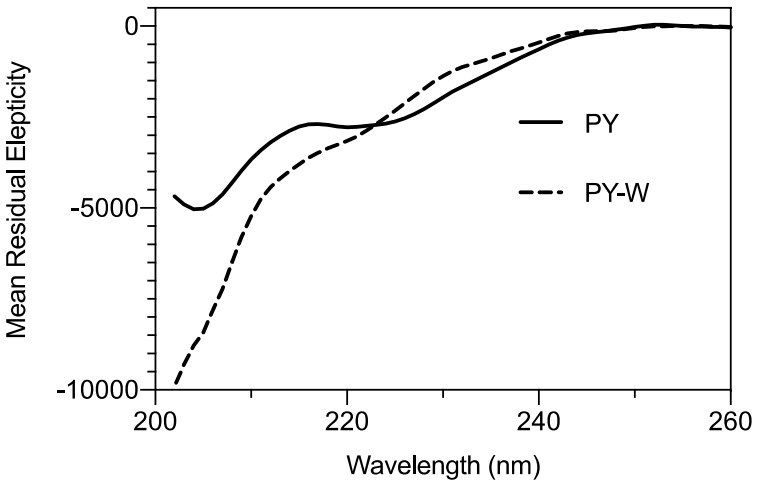

Figure S2: Circular Dichroism spectra of Wild type PY peptide (PY-W) and constrained PY peptide (PY) in PBS. 5 μM peptide was used and the representative curve are average of 10 scans. The data are plotted as Mean residue ellipticity as described by Majumder et al.,[1].

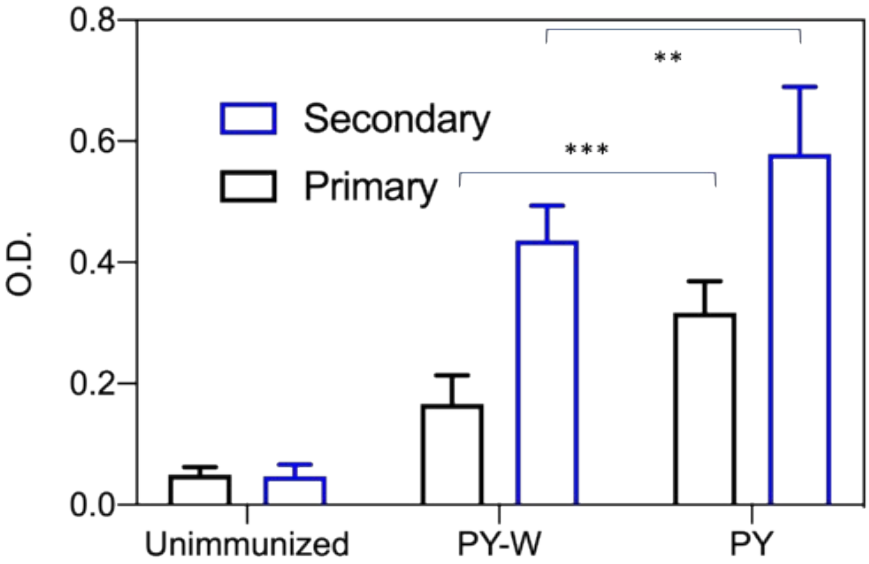

Figure S3: Measurement of anti-PY antibody (IgG) following immunisation with PY-W, and PY peptide. IgG was measured in the serum 21 days after primary immunization and 7 days after booster immunization. IgG was measured at 1:100 serum dilution by ELISA and expressed in OD (arbitrary unit). Unimmunized mice were used as a control to detect basal anti-PY IgG in the no immunization condition. Statistical significance was judged by One-way ANOVA, followed by post-hoc Tukey-Kramer test.

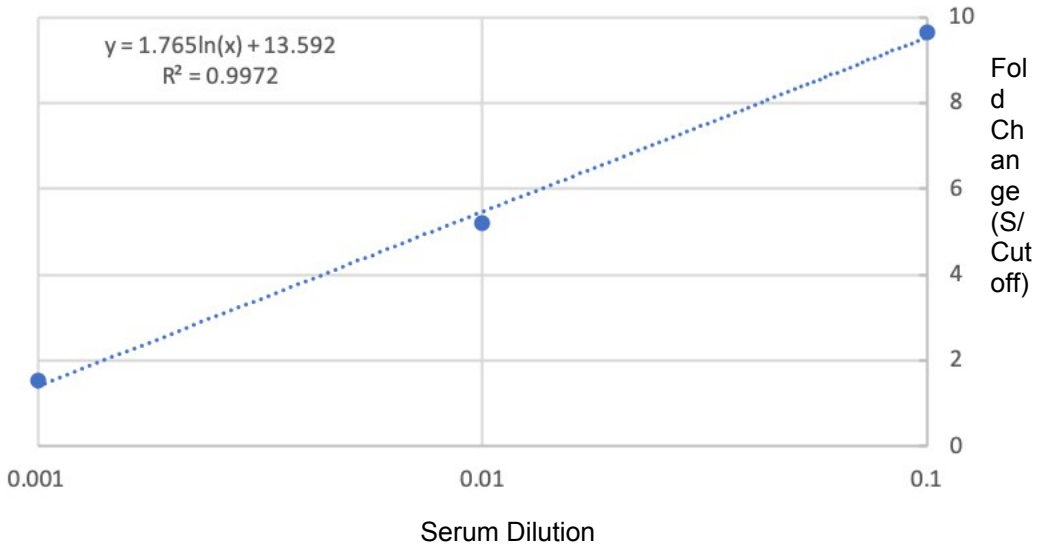

Figure S4: Representative figure of calculating end point titer by extrapolation method. The fold change O.D. value was plotted with serum (1:1000, 1:100 and 1:10) dilution of ITIMi-OVA-PY immunization. X-axis serum dilution (log scale) and Y-axis fold change (S/Cutoff). Estimated endpoint titers were determine using the straight line through the fold change and serum dilution to find at which the fold change is equal 2.

Table S1: List of peptides used in this study

Peptide	**Sequence**
OVA	ISQAVHAAHAEINEAGR
PY-wild type (PY-W)	SPITITNLKTMYK
PY-constrained (PY)	SPITBTNLBTMBK
OVA-PY	ISQAVHAAHAEINEAGRKKSPITBTNLBTMBK
ITIMi-OVA-PY	VY^{P}SEIKKISQAVHAAHAEINEAGRKKSPITBTNLBTMBK

B is α-aminoisobutyric acid. Y^{P} is phosphonomethyl phenylalanine.

Table S2: Estimated antibody titer of PY peptide (arbitrary unit)

Immunization	PY	OVA-PY	ITIMi-OVA-PY
Primary	10 ± 14	373 ± 92	617 ± 142
Secondary	19 ± 22	852 ± 154	997 ± 141

Table S3: Estimated antibody titer of parasite lysate (arbitrary unit)

Immunization	*Plasmodium yoelii*	*Leishmania donovani*
OVA-PY	12 ± 7	0.006 ± 0.008

Supplementary Materials and Methods.

Ethics statement

The use of mice was approved by the Institutional Animal Ethics Committee of the Indian Institute of Chemical Biology, India. All animal experimentations were performed according to the National Regulatory Guidelines issued by CPSEA (Committee for the Purpose of Supervision of Experiments on Animals), Ministry of Environment and Forest, Govt. of India. The protocol number is SDR/SYR/2007.

Antibodies and Other Reagents

FBS was purchased from Invitrogen. Penicillin-streptomycin, sodium bicarbonate, HEPES, 2-mercapto-ethanol, starch, BSA, RPMI-1640, 3,3',5,5'-Tetramethylbenzidine (TMB), and Complete Freund's adjuvant (CFA) were purchased from Sigma Aldrich (St. Louis, MO). Anti-mouse IgG-HRP, anti-mouse IgG1-HRP, anti-mouse IgG2a- HRP, anti-mouse IgG2b-HRP and ELISA assay kit for IL-2 assay was purchased from BD Bioscience (San Diego, CA). All the amino acids were purchased from Merck Germany.

Peptide synthesis and purification

The peptides were synthesized on Rink Amide MBHA resin using PS3 peptide synthesizer (Protein Technologies Inc, USA) and standard solid-phase Fmoc chemistry incorporating a capping step with 5% acetic anhydride and 5% lutidine in DMF after each coupling. Fmoc- amino acids were activated with HBTU in presence of HOBt and DIEA. Peptides were cleaved from the resin and side-chain protecting groups were removed by incubating with 94% TFA, 2.5% EDT, 1.5% thioanisole, 1.5% water, 0.5% TIS for 3 h at room temperature and peptides were precipitated with ice-cold diethyl ether. Peptides were then purified by HPLC (Waters, USA) on a reverse-phase μ-bondapak C-18 column using 0-80% acetonitrile in 0.01% TFA and molecular weight determined by MALDI-TOF/TOF analyzer (Applied Biosystem, USA). The sequence of the peptide used in the study is shown in Table S1.

CD Spectroscopy

Far-UV CD spectra were collected on JASCO 500 CD spectrometer with a 1 mm pathlength quartz cuvette at 25°C. Averages of 10 independent scans were taken. CD spectra were collected in PBS with a final protein concentration 5 μM[1].

Immunization and parasite challenge

BALB/C mice were immunized subcutaneously in the footpad with 50 μg of PY or OVA- PY or ITIM-OVA-PY peptides were emulsified with CFA[2] and were kept for 21 days. Secondary immunization followed and the blood was collected on day 7 of secondary immunization. Blood was again collected on day 28. 10 days after the booster dose, animals were challenged intra-peritoneally with $5x10^5$ parasitized RBC. Thin smears of tail blood stained with Giemsa's reagent were examined daily after day 2 post-challenge. The parasitemia was scored each day. The percentage parasitemia was determined by counting 500 cells per slide.

Coupling of the peptide to BSA by EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide]

2 mg BSA was dissolved in 200 μl conjugation buffer (phosphate buffer, pH 4.5) and 2 mg PY peptide was dissolved in 500 μl conjugation buffer. 10 mg EDC was dissolved in 1 ml water. Then 200 μl of peptide solution was added in 200 μl BSA solutions followed by addition of 50 μl EDC and resulting mixture was kept room temperature with continuous string. The BSA-PY conjugate purified using desalting column.

Assessment of anti IgG antibody response

A 96 well plate was coated overnight with 100 µl of BSA-PY conjugate (2 µg/ ml in PBS) at 4°C. The plate was washed with PBS followed by the addition of 200 µl 5% FBS containing PBS (assay buffer) for 1 h at room temperature. Serum was diluted in assay buffer in 1: 100 ratios. 100 µl of the diluted serum was added to each well. After 2 h, the plate was washed thrice with PBS followed by the addition of 100 µl of anti-mouse IgG HRP (1: 5000 dilution). The plate was washed thrice with PBS after 1 h and followed by the addition of 100 µl TMB solution (2.5 mg TMB dissolved in DMSO and added in 10 ml citrate-acetate buffer pH 4.5 with 0.1 % H_2O_2). After 20 min, the reaction was stopped with 50 µl of 2 (N) H2SO4. The resulting O.D. was measured at 405 nm.

Preparation of parasite lysate

P. yoelii parasite lysate was prepared as described in Narum *et al*[3]. Blood was collected from *P. yoelii* infected mice with 50 to 60% average parasitemia; the cells were washed in PBS and then stored at -70°C. 10 volumes of buffer containing 1% Nonidet P-40 (NP-40) was added in parasitized RBC and incubated for 1 h in ice. Then the extract was centrifuged at 10,000 g (20 min at 10°C), and then the supernatant was centrifuged again (10 min, 10,000g, 10°C) and the protein concentration was measured by BCA protein assay kit.

Estimation of antibody titer

Step 1: Measurement serial dilution: The O.D. was measured at serial dilution (1:10, 1:100 and 1:1000) for immunized and unimmunized serum. The mean O.D. and standard deviation (SD) were determined at 1:10 dilution of unimmunized serum to determine cutoff as described by Hackett et al[4].

Step 2: Determination of cutoff O.D and fold change: The mean O.D. of unimmunized serum at 1:10 dilution was measured. 2. The cutoff was determined at 95% significance level by the following equation $Cutoff = Mean\ O.D. + SD\ t\sqrt{1 + (1/n)}$ as described by Frey et al.,[5]. The fold change was determined by the O.D./ cutoff as described by Long et al.,[6].

Step 3: Estimation of end point titer (Figure S4): Estimated endpoint titers were determine using the straight line through the fold change and serum dilution to find at which the fold change is equal 2 similarly described by Hacket et al[4]. The linear correlation between the fold and the serum dilution were determine by correlation coefficient (R^2) using linear regression.

Statistical methods

Each experiment was performed three to five times and the results are either expressed as means ± standard deviation (SD). Shapiro-Wilk test was performed to confirm that data were from a normally distributed population (https://www.real-statistics.com/tests-normality-and-symmetry/statistical-tests-normality-symmetry/shapiro-wilk-test/#comments). Student's t-test for significance was performed using Graphpad Prism software Version 5. For data involving comparison of multiple groups, one-way ANOVA was performed in MS-Excel. This was followed by Tukey-Kramer post-hoc analysis to detect statistical significance between groups (https://www.statology.org/tukey-kramer-post-hoc-test-excel/; Abdi & Williams, in Neil Salkind (Ed.), Encyclopedia of Research Design. Thousand Oaks, CA: Sage. 2010.)

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