



## Enhanced humoral and mucosal immune responses after intranasal immunization with chimeric multiple antigen peptide of LcrV antigen epitopes of *Yersinia pestis* coupled to palmitate in mice

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### ABSTRACT

*Yersinia pestis* is the causative agent of the most deadly disease plague. F1 and V antigens are the major vaccine candidates. Six protective epitopes of V antigen of varying length (15–25aa) were assembled on a lysine backbone as multiple antigen peptide (MAP) using standard Fmoc chemistry. Palmitate was coupled at amino terminus end. Amino acid analysis, SDS-PAGE, immunoblot and immunoreactivity proved the authenticity of MAP. MAP was immunized intranasally encapsulated in PLGA (polylactide-co-glycolide) microspheres and with/without adjuvants murabutide and CpG ODN 1826 (CpG), in three strains of mice. Humoral and mucosal immune responses were studied till day 120 and memory response was checked after immunization with native V antigen on day 120. Epitope specific serum and mucosal washes IgG, IgA, IgG subclasses and specific activity were measured by indirect ELISA and sandwich ELISA, respectively. IgG and IgA peak antibody titers of all the MAP construct formulations in sera were ranging from 71,944 to 360,578 and 4493 to 28,644, respectively. MAP with CpG showed significantly high ( $p < 0.0001$ ) antibody titers ranging from 101,690 to 360,578 for IgG and 28,644 for IgA. Mucosal peak IgG and IgA titers were ranging from 1425 to 8072 and 1425 to 7183, respectively in intestinal washes and 799–4528 and 566–4027, respectively in lung washes. MAP with CpG showed significantly high ( $p < 0.001$ ) SIgA titers of 8000 in lung and 16,000 in intestinal washes. IgG isotyping revealed IgG2a/IgG1 ratio  $> 1$  with CpG. Serum and mucosal antipeptide IgG and IgA specific activities correlated well with antibody titers. All the constituent peptides contributed towards immune response. Structural analysis of MAP revealed little or no interaction between the peptides. Present study showed MAP to be highly immunogenic with high and long lasting antibody titers in serum and mucosal washes with good recall response with/without CpG as an adjuvant which can be used for vaccine development for plague.

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### 1. Introduction

*Yersinia pestis* is the etiological agent of plague. Currently available vaccines provide little or no protection against the most severe pneumonic form and there were concerns about its reactogenicity [1]. Different laboratories worldwide are working extensively towards the development of improved vaccines using diverse strategies like peptide based or recombinant immunogens [2–8]. It is implicit that using F1 or V or both will be more advantageous. Although both cellular and humoral immunity can mediate protection [9] most reports identify humoral immunity as the key implication. In several studies F1 and V antigens in combination

or alone using various routes of immunizations were shown to be protective, V antigen being the major protective antigen [10–25]. Survival against *Y. pestis* challenge was related to increased levels of IL-17 mRNA as well as protein in the lungs of challenged mice [26].

Earlier studies from our laboratory demonstrated *in vivo* protection with F1 antigen [2,27]. B- and T-cell epitopes on V antigen were mapped and their immunogenicity was studied [28,29]. The above studies proved intranasal route as the superior way in generating systemic and mucosal immune responses as it is well equipped to protect the vast mucosal surface areas with SIgA as the main protective antibody along with IgG, which is transudated to the mucosal compartment [30]. Thus, an ideal plague vaccine should contain epitopes that include protection at both mucosal and systemic compartments.

Peptide vaccines have the advantages of simplicity, safety and unambiguity over conventional approaches [31,32]. In the present study we have assembled defined epitopes on V antigen

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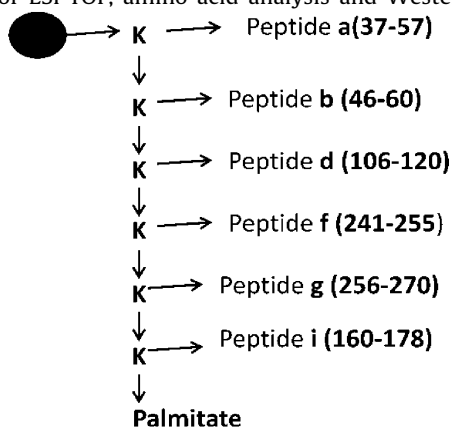
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[28,29,33] as multiple antigen peptide (MAP), which is shown to be a self adjuvanting with high immunogenicity in several studies, on a lysine backbone [34–37]. We attached a palmitate, a proven adjuvant in enhancing B-cell [38] as well as T cell [39] immune responses and also acts as a ligand for TLR-2 [40]. Its immunogenicity in humoral as well as mucosal compartments were studied in outbred and two inbred i.e., H-2<sup>d</sup> and H-2<sup>b</sup>, strains of mice. We have used murabutide (MB) which is apyrogenic [41] with no side-effects [42] and CpG ODN 1826 [43] as adjuvants. Poly(lactide-co-glycolide) (PLGA) microspheres were used as a delivery vehicle [44]. Intranasal route of administration was chosen as it is the best way of eliciting systemic and mucosal immune responses [45–48]. The present study reports chimeric MAP linked with a single palmitate to be highly immunogenic and more so with CpG ODN can be used for vaccine development.

## 2. Materials and methods

Different B- and T-cell protective epitopes of V antigen [33,49] were synthesized as MAP using Fmoc chemistry [50]. At the N-terminal end of each branch, t-Boc protected amino acid was used to prevent further chain elongation. Fmoc-Lys(ivDde)-OH (novabiochem) was incorporated at the beginning of the synthesis for each branch to serve as the branching point. Palmitate was attached at the amino terminus. The MAP was purified by HPLC, lyophilized and stored at room temperature. MAP was characterized by HPLC, MALDI-TOF or ESI-TOF, amino acid analysis and Western



blotting. MAP construct

### 2.1. Mice

Six to eight weeks old female outbred mice were procured from Experimental Animal Facility, AIIMS, New Delhi, India. BALB/c (H-2<sup>d</sup>) and C57BL/6 (H-2<sup>b</sup>) mice were procured from National Institute of Nutrition, Hyderabad, India. Each experimental group consisted of six to eight mice. All experiments were conducted in accordance with the guidelines of AIIMS ethics as well as CPCSEA, Ministry of Social Justice, Government of India.

### 2.2. Microparticle preparation, particle size and morphology

PLGA microparticles were prepared by double solvent evaporation method [29]. The percentage entrapment was found to be 50–60% as determined by bicinchoninic acid (BCA) method. The size was determined by laser diffraction (Malvern Instrument, UK) and was found to be 1–2  $\mu\text{m}$ . Morphology was studied by scanning electron microscopy and found to be spherical in shape.

### 2.3. Route of immunization and sample collection

Mice were immunized intranasally with 40  $\mu\text{g}$  of MAP with or without adjuvant (5  $\mu\text{g}$ /2.5  $\mu\text{g}$ ) under isoflurane anesthesia. Adjuvants used are CpG-ODN 1826 (CpG(5'-TCC ATG ACG TTC CTG ACG TT-3')) (Coley Pharmaceuticals, USA) and murabutide (Invivogen, USA). Bleeds were collected on days 0, 28, 42, 60, 90 and 120. The sera was separated and stored at  $-20^{\circ}\text{C}$  till use. Mucosal washes (intestinal and lung) were collected as reported [29] and stored at  $-70^{\circ}\text{C}$  till use.

### 2.4. Measurement of MAP specific serum and mucosal antibody by ELISA

MAP specific antibody titers were estimated using ELISA protocol. Briefly, 96 well plate (Immulon IIB) was coated with 100 ng/well of MAP in coating buffer and kept overnight at  $4^{\circ}\text{C}$ . After blocking, two-fold dilution of anti-sera, lung and intestinal washes were used to measure IgG, IgA and SIgA peak antibody titers. After washing, peroxidase conjugated goat-anti mouse IgG or IgA (1:1000 dilution) was added and incubated at  $37^{\circ}\text{C}$  for 1 h. Color was developed and absorbance was taken at 492 nm. Pre-immune sera/lung/intestinal washes were used as negative control. The titers were expressed as the geometric mean (geometric SE) of the reciprocal of the highest dilution giving an absorbance higher than pre-immune sera. Serum and mucosal total IgG and IgA concentrations were determined by sandwich ELISA. The IgG subclasses in sera and washes were determined using Sigma isotyping kit (ISO-2) as described earlier [29]. SIgA peak titers were assayed using anti-mouse secretory component [Genetex] antibody at a dilution of 1:1000.

To correct for variation in dilution during mucosal washes collection, the data was expressed as specific activity (antipeptide IgA/IgG titer (serum and washes)/total IgG/IgA ( $\mu\text{g}/\text{ml}$ )) [51].

### 2.5. Individual peptide reactivity

The reactivity of different individual peptide antisera and V antigen antisera towards MAP was tested by indirect ELISA. Briefly, each plate was coated with MAP (100 ng/well) and kept at  $4^{\circ}\text{C}$  overnight. Different antisera were added at a fixed dilution as well two fold serial dilution of anti-sera were incubated for 2 h at  $37^{\circ}\text{C}$ . Anti-mouse IgG-HRPO was added at a dilution of 1:1000 and incubated at  $37^{\circ}\text{C}$  for 1 h. After the color development, reaction was stopped and absorbance was taken at 492 nm.

### 2.6. Recall/memory response

Mice were immunized intranasally with 20  $\mu\text{g}$  of native V antigen in saline on day 120 and the bleeds were collected on day 135. Sera were separated. ELISA was done with MAP coated plates.

### 2.7. Structural analysis

The structural analysis of MAP was carried out using PyMOL (DeLano Scientific, LLC) based on the V antigen structure [52]. Bond distances less than 4.0 Å were taken into consideration for assessing the interactions between different amino acids. Three different sets were made depending on closeness in the LcrV antigen structure and in MAP.

### 2.8. Statistical analysis

Mean values were analyzed using one-way ANOVA and the Tukey post test for multiple comparisons. The values between the groups and between the days in all formulations were compared. When the differences between groups were statistically significant

**Table 1**  
CpG ODN 1826 enhances the humoral immune response to multiple antigen peptide vaccine of V antigen of *Y. pestis* in outbred mice.

Exp gp <sup>a</sup>	Formulations	IgG titer <sup>b</sup>									
		Days									
		30	60		90		120		135		
1	MAP in saline (40 µg)	71,944.9	(1.45)	141,808.1	(1.43)	113,818.81	(1.43)	80,954.48	(1.55)	141,941.1	(1.32)
2	MAP + mura (40 µg + 5 µg)	70,886.7	(1.42)	144,085.3	(1.46)	117,816.83	(1.43)	72,886.71	(1.43)	144,818.8	(1.43)
3	MAP + CpG (40 µg + 5 µg)	101,690.4	(1.54)	360,578.6	(1.77)	284,499.01	(1.77)	143,057.71	(1.78)	321,645.3	(1.42)
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	81,742.7	(1.45)	164,284.3	(1.46)	144,018.11	(1.68)	90,020.07	(1.46)	164,085.3	(1.46)
5	MAP + microspheres (40 µg)	80,872.9	(1.43)	181,941.1	(1.32)	204,800.00	(1.00)	102,400.00	(1.00)	228,916.8	(1.32)

<sup>a</sup> All experimental groups were immunized single time through intranasal route of the specified formulations.

<sup>b</sup> Serum IgG antibody titer is reported as the geometric mean (geometric SE). *n* is equal to 6 in each group.

further analysis was done using a Student's *t*-test (two-tailed). Tests were performed using GraphPad software. Results were considered statistically significant at the  $p < 0.05$  level.

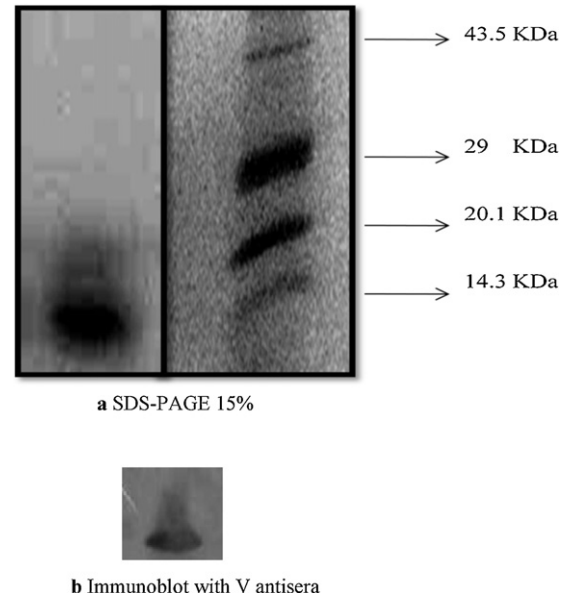
### 3. Results

#### 3.1. Molecular weight determination and immunoreactivity

SDS-PAGE showed a band corresponding to 12.5 kDa (Fig. 1a). Immunoblotting showed single band (Fig. 1b). HPLC analysis of the MAP yielded essentially a single peak indicating over 95% pure. No mass spectrum was obtained when subjected to MALDI/ESI-TOF mass spectral analysis [50]. MAP seems to be inherently difficult to ionize possibly due to its complex structure. Heterogeneity of the branches in the MAP might have further depressed any signal during mass spectral analysis. Amino acid analysis showed expected values. All the different anti-peptide antibodies as well as anti-V antibodies showed immunoreactivity with MAP.

#### 3.2. Humoral responses

IgG peak antibody titers of all the MAP construct formulations in sera of mice showed a range from 70,886 to 360,578 with predominant titers on days 60 and 90 followed by a marginal decrease on day 120 (Table 1). The MAP + CpG showed significantly high ( $p < 0.0001$ ) titers ranging from 101,690 to 360,578 in outbred mice. In inbred mice also the formulations were found to be immunogenic with peak titers ranging from 76,800 to 305,200 (data not shown). Interestingly the trends in antibody profile were similar in all the strains. The serum IgA antibody titers were also found to be significantly high ( $p < 0.001$ ) with peak titers ranging from 4493 to 28,644. The MAP + CpG formulation showed high peak titer of 28,644 on day 60 while MAP in saline showed titers ranging from 5047 to 14,319 (Table 2). Even though, there existed minor differences between the formulations, the peak titers among the groups were insignificant.



**Fig. 1.** Electrophoretic and immunoblot analysis of V antigen multiple antigenic peptide. Multiple antigen peptide (MAP) is solubilized in milliQ grade water and subjected to 15% SDS-PAGE (upper panel) and in similar conditions. Immunoblot was performed using anti-V antigen antibodies (lower panel). Coomassie brilliant blue (CBB)-stained MAP on SDS-PAGE gels are shown above the immunoblot. The experiments were repeated thrice with similar results and representative electrophoresis and immunoblot was shown. (a) SDS-PAGE 15%. (b) Immunoblot with V antisera.

Interestingly, all the formulations showed good memory response with native V antigen (Tables 1–5). Overall, the results indicated that the MAP + CpG showed significantly ( $p < 0.001$ ) high peak IgG and IgA titers in sera followed by MAP in microspheres  $\geq$  MAP with MB + CpG > MAP in saline = MAP with MB in outbred as well as H-2<sup>d</sup> and H-2<sup>b</sup> strains of mice.

**Table 2**  
CpG ODN 1826 enhances the humoral immune response to multiple antigen peptide vaccine of V antigen of *Y. pestis* in outbred mice.

Exp gp <sup>a</sup>	Formulations	IgA titer <sup>b</sup>									
		Days									
		30	60		90		120		135		
1	MAP in saline (40 µg)	5,047.41	(1.45)	10,266.65	(1.68)	11,584.68	(1.32)	6,157.70	(1.32)	11,691.84	(1.55)
2	MAP + mura (40 µg + 5 µg)	4,493.29	(1.42)	10,584.68	(1.85)	10,691.84	(1.55)	6,341.64	(1.55)	11,584.68	(1.85)
3	MAP + CpG (40 µg + 5 µg)	10,099.74	(1.43)	28,644.48	(1.32)	20,207.93	(1.43)	8,016.56	(1.43)	22,728.89	(1.32)
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	5,002.624	(1.76)	12,584.68	(1.85)	13,584.68	(1.85)	7,157.70	(1.68)	14,691.84	(1.55)
5	MAP + microspheres (40 µg)	6,283.871	(1.85)	14,196.64	(1.68)	17,991.35	(1.46)	7,991.47	(1.46)	16,039.73	(1.43)

<sup>a</sup> All experimental groups were immunized single time through intranasal route of the specified formulations.

<sup>b</sup> Serum IgA antibody titer is reported as the geometric mean (geometric SE). *n* is equal to 6 in each group.

**Table 3**  
CpG ODN 1826 enhances the mucosal (intestinal) immune response to multiple antigen peptide vaccine of V antigen of *Y. pestis* in outbred mice.

Exp gp <sup>a</sup>	Formulations	IgG titer <sup>b</sup>							
		Day							
		30		60		90		135	
<b>A</b>									
1	MAP in saline (40 µg)	1425.43	(1.42)	2851.02	(1.97)	1794.73	(1.34)	2851.02	(1.32)
2	MAP + mura (40 µg + 5 µg)	1794.73	(1.32)	2851.02	(1.97)	1632.021	(1.11)	3198.90	(1.54)
3	MAP + CpG (40 µg + 5 µg)	3589.21	(1.32)	8072.32	(1.42)	4327.16	(2.04)	8072.35	(1.42)
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	2013.72	(1.43)	3589.22	(1.97)	1894.72	(1.43)	3589.219	(1.68)
5	MAP + microspheres (40 µg)	2851.01	(1.32)	5701.65	(1.32)	2951.03	(1.23)	4528.98	(2.61)
Exp gp <sup>a</sup>	Formulations	IgA titer <sup>b</sup>							
		Day							
		30		60		90		135	
<b>B</b>									
1	MAP in saline (40 µg)	1425.43	(1.43)	2850.87	(1.32)	1794.73	(1.34)	2540.97	(1.42)
2	MAP + mura (40 µg + 5 µg)	1269.92	(1.43)	2539.84	(1.43)	1600.00	(1.00)	2539.84	(1.43)
3	MAP + CpG (40 µg + 5 µg)	3200.00	(2.92)	7183.75	(1.32)	4027.17	(2.04)	6400.00	(1.00)
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	1425.43	(1.43)	2540.97	(1.42)	1794.73	(1.43)	2539.84	(1.43)
5	MAP + microspheres (40 µg)	1795.93	(1.32)	4027.11	(1.76)	2851.01	(1.23)	2851.01	(1.33)

<sup>a</sup> All experimental groups were immunized single time through intranasal route of the specified formulations.

<sup>b</sup> Intestinal wash IgG antibody titer is reported as the geometric mean (geometric SE). *n* is equal to 6 in each group.

### 3.3. Mucosal immune responses

Peak IgG and IgA titers were ranging from 1425 to 8124 and 1425 to 7183, respectively in intestinal washes (Table 3A and B) and 799–4528 and 566–4027, respectively in lung washes in Table 4A and B. On day 60, MAP + CpG showed significantly high ( $p < 0.001$ ) SIgA titers of 8000 in lung and 16,000 in intestinal washes (Fig. 2). Similar results were obtained in inbred mice also (data not shown). Overall, in mucosal washes also MAP + CpG showed significantly high titers ( $p < 0.001$ ) in terms of IgA, IgG and SIgA when compared to control group. Interestingly, insignificant differences between other formulations were observed between outbred and inbred strains (data not shown).

### 3.4. IgG subclasses

Although IgG1 levels were slightly higher, the isotypes IgG2a and IgG2b were also found on day 60 onwards either in greater/lesser than or equal to IgG1 in all the formulations. Especially, MAP + CpG showed a Th1 directing isotypes, Ig2a/IgG2b, more than Th2 directing isotype, IgG1. The IgG2a/IgG1 ratio was  $> 1$ . The IgG2a/IgG1 ratio was  $\geq 1$  in MAP with MB + CpG and MAP in microspheres. In MAP in saline and MAP + MB, IgG2a/IgG1 ratio was  $< 1$  (Table 5). The IgG3 levels were negligible in all the bleeds and in all the formulations. The isotyping in mucosal washes (lung as well as intestinal) were also carried out which revealed similar pattern as that of sera (data not shown).

**Table 4**  
CpG ODN 1826 enhances the mucosal (Lung) immune response to multiple antigen peptide vaccine of V antigen of *Y. pestis* outbred mice.

Exp gp <sup>a</sup>	Formulations	IgG titer <sup>b</sup>							
		Day							
		30		60		90		135	
<b>A</b>									
1	MAP in saline (40 µg)	799.83	(1.42)	1600.00	(1.42)	976.53	(1.52)	1794.73	(1.46)
2	MAP + mura (40 µg + 5 µg)	897.42	(1.97)	1794.73	(1.68)	856.43	(1.87)	2013.72	(1.76)
3	MAP + CpG (40 µg + 5 µg)	2264.64	(1.42)	4027.17	(2.57)	2064.64	(1.62)	4528.97	(1.42)
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	1006.93	(1.85)	2013.72	(1.54)	809.84	(1.55)	2264.64	(1.68)
5	MAP + microspheres (40 µg)	1600.00	(1.54)	2540.97	(2.31)	1579.16	(1.64)	2540.97	(2.31)
Exp gp <sup>a</sup>	Formulations	IgA titer <sup>b</sup>							
		Day							
		30		60		90		135	
<b>B</b>									
1	MAP in saline (40 µg)	712.85	(1.97)	1599.55	(1.55)	652.85	(2.17)	1794.73	(1.55)
2	MAP + mura (40 µg + 5 µg)	566.24	(1.78)	1270.57	(1.43)	656.24	(1.58)	1425.61	(1.43)
3	MAP + CpG (40 µg + 5 µg)	1794.73	(1.97)	4027.17	(1.76)	1594.73	(1.37)	4528.97	(1.46)
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	635.33	(1.76)	1425.61	(1.68)	875.34	(1.26)	1925.12	(1.68)
5	MAP + microspheres (40 µg)	828.97	(2.04)	2013.72	(1.76)	786.98	(1.94)	2264.64	(2.34)

<sup>a</sup> All experimental groups were immunized single time through intranasal route of the specified formulations.

<sup>b</sup> Lung wash antibody titer is reported as the geometric mean (geometric SE). *n* is equal to 6 in each group.

**Table 5**  
Subclass distribution in sera. Groups of outbred mice were immunized intranasally with the MAP construct formulations and their subclass distribution was evaluated by ELISA.

Formulations	Isotypes	Days				
		30	60	90	120	135 <sup>a</sup>
MAP in saline	IgG1	1.652 ± 0.041	1.973 ± 0.056	1.715 ± 0.071	1.375 ± 0.084	1.965 ± 0.061
	IgG2a	1.772 ± 0.058	1.998 ± 0.061	1.601 ± 0.048	1.396 ± 0.062	1.927 ± 0.038
	IgG2b	1.616 ± 0.062	1.861 ± 0.058	1.607 ± 0.062	1.232 ± 0.051	1.837 ± 0.047
	IgG3	0.312 ± 0.014	0.317 ± 0.012	0.326 ± 0.014	0.306 ± 0.013	0.296 ± 0.015
MAP + mura	IgG1	1.692 ± 0.063	1.897 ± 0.072	1.701 ± 0.051	1.406 ± 0.045	1.938 ± 0.039
	IgG2a	1.764 ± 0.055	1.931 ± 0.063	1.712 ± 0.072	1.325 ± 0.064	1.935 ± 0.061
	IgG2b	1.647 ± 0.046	1.839 ± 0.084	1.518 ± 0.056	1.231 ± 0.071	1.755 ± 0.052
	IgG3	0.288 ± 0.015	0.323 ± 0.017	0.302 ± 0.014	0.314 ± 0.013	0.335 ± 0.018
MAP + CpG	IgG1	1.873 ± 0.048	1.696 ± 0.059	1.583 ± 0.051	1.313 ± 0.048	1.677 ± 0.062
	IgG2a	1.816 ± 0.057	2.511 ± 0.051	2.298 ± 0.062	1.873 ± 0.057	2.414 ± 0.046
	IgG2b	1.314 ± 0.052	2.376 ± 0.048	1.897 ± 0.081	1.421 ± 0.068	2.289 ± 0.072
	IgG3	0.311 ± 0.015	0.351 ± 0.019	0.302 ± 0.015	0.306 ± 0.013	0.316 ± 0.017
MAP + mura + CpG	IgG1	1.848 ± 0.046	2.073 ± 0.068	1.961 ± 0.071	1.524 ± 0.054	1.926 ± 0.041
	IgG2a	1.914 ± 0.054	2.117 ± 0.057	1.966 ± 0.064	1.598 ± 0.047	2.031 ± 0.063
	IgG2b	1.751 ± 0.049	2.258 ± 0.053	1.894 ± 0.082	1.516 ± 0.061	2.104 ± 0.089
	IgG3	0.346 ± 0.014	0.379 ± 0.016	0.341 ± 0.017	0.331 ± 0.012	0.338 ± 0.015
MAP + MS	IgG1	1.889 ± 0.047	2.111 ± 0.052	1.896 ± 0.042	1.508 ± 0.056	2.059 ± 0.047
	IgG2a	1.981 ± 0.053	2.197 ± 0.049	1.849 ± 0.057	1.584 ± 0.084	2.178 ± 0.063
	IgG2b	1.748 ± 0.045	1.994 ± 0.060	1.728 ± 0.061	1.412 ± 0.047	2.134 ± 0.081
	IgG3	0.361 ± 0.013	0.355 ± 0.016	0.351 ± 0.014	0.323 ± 0.012	0.321 ± 0.018

<sup>a</sup> Indicates values after immunization with native V antigen on day 120 in saline and bled on day 135. Data are representative of three independent experiments and are shown as mean ± SD. MAP: multiple antigen peptide; mura: murabutide; CpG: CpG ODN 1826; MS: microsphere.

### 3.5. Specific activity: serum and mucosal antibody production

To account for the varying rate of immunoglobulin secretion in serum and mucosal washes due to dilution or due to serum transudation, serum and mucosal anti-peptide IgG and IgA specific activities were calculated. Interestingly, a strong correlation was observed between the antibody titer and specific activity. IgG or IgA specific activity showed peak values on day 60 and were maintained till day 90 followed by a small decrease on day 120. The sera IgG specific activity values for MAP + CpG which was found to be significantly high ( $p < 0.001$ ) with 193.85 and 161.18 on days 60 and 90, respectively which declined to 117.16 on day 120. MAP + MB showed 81.86 and 91.68 on days 60 and 90, respectively. MAP in microspheres showed 116.63 and 133.59 on days 60 and 90, respectively. MAP in

saline showed 105.41 and 104.92 on days 60 and 90, respectively (Table 6).

IgA values for MAP + CpG were 40.57 on day 60 and 22.27 on day 90. MAP with MB + CpG and MAP in microspheres showed a slightly less specific activity values which was insignificant. MAP + MB and MAP in saline showed similar values. Intestinal washes showed higher specific activity as compared to lung washes. In all the formulations insignificant ( $p > 0.05$ ) differences in specific activity were observed except with MAP + CpG. In intestinal washes IgG values were 3.13–9.98 and for IgA 3.89–13.76 (Table 7). In lung washes IgG values were 2.61–4.25 and for IgA 2.17–8.27 (Table 8). The results in inbred mice were also similar with slight variations which are insignificant (data not shown).

### 3.6. Individual peptide immune reactivity

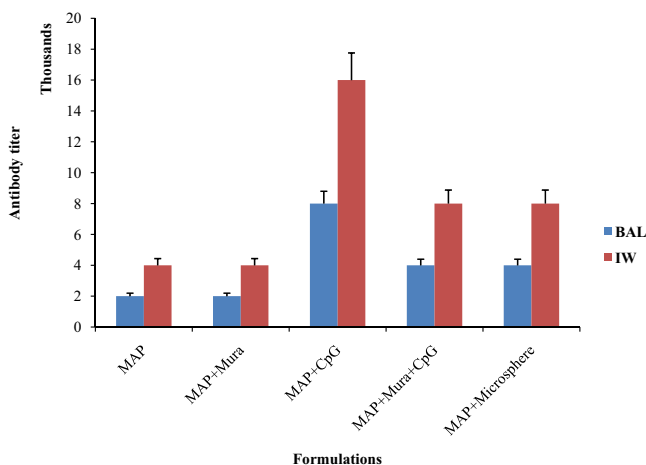
Peptides 'b', 'f' and 'i' showed a maximum titers of  $\geq 150,000$  with MAP + CpG. Interestingly, peptide 'g' showed maximum titers of  $\geq 100,000$  with MAP + CpG. Moreover, peptide 'd', and peptide 'a' which are T cell epitopes, showed maximum titers of  $\leq 100,000$  with MAP + CpG (Fig. 3).

### 3.7. MAP structural analysis

Hydrophobic interactions were present in set one, set two and set four (Table 9). In set one there were interactions between Phe118-Ile46, Leu115-Met119, Ile46-Leu115 and Ile48-Leu 115. In set two interactions were observed between Leu160-Arg246, Glu163-Arg246, Tyr167-Thr247 and Tyr167-Lys245 and in set four, interactions between Lys254-Tyr257 were present (Fig. 4).

## 4. Discussion

It was shown that immune responses can be elicited with synthetic peptides [27,29]. Usually peptides are conjugated to protein-carrier. However, the use of protein-carrier is not suitable principally because the carriers cannot be expected to



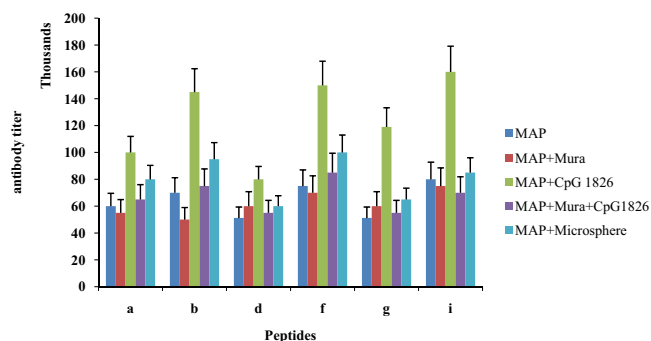
**Fig. 2.** SIgA peak titer in lung washes (BAL) and intestinal washes (IW) of outbred mice on day 60. Groups of outbred mice were immunized intranasally with the MAP construct formulations and mucosal washes were collected as per the schedule and the SIgA titer on day 60 was evaluated by ELISA. Data is representative of three independent experiments giving similar results and is shown as mean ± SD.

**Table 6**  
CpG ODN 1826 enhances the serum specific activity to multiple antigen peptide vaccine of V antigen of *Y. pestis* in outbred mice.<sup>a</sup>

Exp gp <sup>b</sup>	Formulation	Day					IgA				
		30	60	90	120	135	30	60	90	120	135
1	MAP in saline (40 µg)	61.7 ± 2.2	86.9 ± 4.8	85.6 ± 2.1	70.9 ± 2.7	87.5 ± 3.1	8.6 ± 4.7	17.3 ± 0.63	17.5 ± 0.45	7.6 ± 0.36	15.7 ± 0.75
2	MAP + mura (40 µg + 5 µg)	72.1 ± 3.3	81.6 ± 5.7	81.7 ± 2.9	72.4 ± 2.2	85.9 ± 2.9	6.3 ± 0.59	16.6 ± 0.61	15.8 ± 0.55	6.8 ± 0.57	14.9 ± 0.66
3	MAP + CpG (40 µg + 5 µg)	87.3 ± 3.6	193.8 ± 6.6	161.2 ± 6.9	117.1 ± 3.8	182.8 ± 7.2	14.3 ± 0.74	40.6 ± 3.9	22.3 ± 2.49	12.4 ± 1.1	31.9 ± 3.5
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	66.9 ± 4.4	105.4 ± 5.1	104.9 ± 4.7	81.1 ± 2.9	116.6 ± 4.9	8.6 ± 0.41	17.8 ± 0.78	14.2 ± 1.01	8.4 ± 0.83	17.3 ± 1.1
5	MAP + microspheres (40 µg)	61.2 ± 3.4	116.6 ± 3.2	133.6 ± 3.8	83.8 ± 3.1	139.6 ± 5.1	10.8 ± 0.52	23.4 ± 0.92	21 ± 1.68	11.8 ± 0.91	20.6 ± 1.89

<sup>a</sup> Specific activities were calculated by dividing the specific MAP formulation endpoint titer by the total antibody concentration (µg/ml).

<sup>b</sup> All experimental groups were immunized through intranasal route of the specified formulations. Data are representative of three independent experiments and are shown as mean ± SD.



**Fig. 3.** Peptide specific antibody titer in sera of mice. Groups of outbred mice were immunized intranasally with the MAP construct formulations, bled as per the schedule and sera were separated and the antibody titer of day 60 sera was evaluated by ELISA. Data is representative of three independent experiments giving similar results and is shown as mean ± SD.

be recalled by the native antigen. In addition to, the protein carrier may rather impair the host immune response to the peptides. Subunit immunogens should induce immunological memory that can be recalled following infection. Thus, it is necessary to use T- and B-cell epitopes derived from the same molecule. Regions inducing undesired side effects or suppressive T-cell responses can be avoided while designing peptide vaccines [53,54].

Appropriate B- and T-cell epitopes showed enhanced immune responses when included in MAP [55]. In a major advantage, the exclusively H-2<sup>b</sup> restricted antibody response was bypassed by assembling an oligopeptide in MAP, the immunogenicity of di epitopes containing B- and T-cell epitope repeats of *Plasmodium falciparum* protein induced high specificity antibody levels in mice with different genetic background [56,57]. To increase the immunogenicity of peptides incorporated into MAP and/or to overcome genetic major histocompatibility restriction, additional peptide sequences containing T cell epitopes can also be included [58]. Because of the various advantages, the MAP system is of interest for vaccine development. Our results showed that palmitoylated MAP elicited high antibody titers in humoral as well as mucosal sites following intranasal immunization in terms of IgG, IgA and SIgA. MAP alone could generate immune response and with CpG as an adjuvant, the titers increased significantly ( $p < 0.001$ ). There was insignificant difference when murabutide was added suggesting CpG to be a better adjuvant. Interestingly, very high SIgA antibody levels in mucosal secretions were observed indicating MAPs are stable in mucosal compartment and can constantly activate the immune system. Large quantities of IgA and IgG present in the secretions may be a result of the extensive transudation from the systemic compartment as well as local production.

In mice, all TLR agonists enhance the immune response [59,60]. CpG ODN exerts their adjuvant effect by increasing the survival rate of B cells as well as the output of plasmablasts. They induce B-cell proliferation, antibody secretion and enhance the antigen-specific T-cells and differentiation of naive T cells towards the Th1 phenotype [61]. After stimulation with CpG ODN, JNK2<sup>-/-</sup> B cells displayed an enhanced antibody production [62]. Moreover, CpG ODN expresses biphasic effects. The stimulatory activities of CpG ODN resulted from specific interactions with TLR-9 [63]. In the present study, MAP + CpG showed significantly high antibody levels suggesting that CpG ODN enhanced the antigen specific B and T cells and when animals were immunized with V antigen on day 120, a robust immune response was observed in the sera (day 135) thereby showing the memory or recall response. The high antibody

**Table 7**  
CpG 1826 enhances the intestinal specific activity to multiple antigen peptide vaccine of V antigen of *Y. pestis*<sup>a</sup> outbred mice.

Exp gp <sup>b</sup>	Formulations	IgG				IgA			
		Day							
		30	60	90	135	30	60	90	135
1	MAP in saline (40 µg)	3.13 ± 0.12	8.28 ± 0.35	4.95 ± 0.19	7.82 ± 0.32	4.59 ± 0.15	6.63 ± 0.35	4.77 ± 0.22	8.93 ± 0.37
2	MAP + mura (40 µg + 5 µg)	4.25 ± 0.24	8.09 ± 0.31	5.46 ± 0.21	7.46 ± 0.29	4.06 ± 0.19	6.86 ± 0.33	4.81 ± 0.21	8.81 ± 0.35
3	MAP + CpG (40 µg + 5 µg)	5.57 ± 0.31	10.62 ± 0.33	7.45 ± 0.28	10.35 ± 0.37	7.47 ± 0.24	12.05 ± 0.56	7.68 ± 0.34	13.76 ± 0.55
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	6.93 ± 0.29	8.12 ± 0.29	6.81 ± 0.23	8.59 ± 0.28	6.39 ± 0.21	9.21 ± 0.42	6.52 ± 0.32	10.76 ± 0.47
5	MAP + microspheres (40 µg)	7.03 ± 0.41	8.15 ± 0.32	6.34 ± 0.21	8.25 ± 0.29	6.69 ± 0.23	9.64 ± 0.47	6.38 ± 0.35	10.66 ± 0.49

<sup>a</sup> Specific activities were calculated by dividing the specific MAP formulation endpoint titer by the total antibody concentration (µg/ml).

<sup>b</sup> All experimental groups were immunized single time through intranasal route of the specified formulations. Data are representative of three independent experiments and are shown as mean ± SD.

**Table 8**  
CpG ODN 1826 enhances the lung specific activity to multiple antigen peptide vaccine of V antigen of *Y. pestis*<sup>a</sup> in outbred mice.

Exp gp <sup>b</sup>	Formulations	IgG				IgA			
		Day							
		30	60	90	135	30	60	90	135
1	MAP in saline (40 µg)	2.61 ± 0.34	3.89 ± 0.45	2.28 ± 0.19	3.72 ± 0.38	2.73 ± 0.29	4.74 ± 0.32	3.99 ± 0.28	5.98 ± 0.34
2	MAP + mura (40 µg + 5 µg)	2.67 ± 0.27	3.74 ± 0.37	2.43 ± 0.23	3.68 ± 0.27	2.17 ± 0.18	4.22 ± 0.21	3.15 ± 0.19	5.57 ± 0.28
3	MAP + CpG (40 µg + 5 µg)	3.85 ± 0.19	5.25 ± 0.26	4.45 ± 0.22	5.86 ± 0.25	7.06 ± 0.49	10.27 ± 0.62	9.19 ± 0.55	11.01 ± 0.68
4	MAP + mura + CpG (40 µg + 2.5 µg + 2.5 µg)	3.23 ± 0.32	4.27 ± 0.34	3.78 ± 0.29	4.65 ± 0.31	5.93 ± 0.34	8.01 ± 0.38	5.98 ± 0.31	7.47 ± 0.38
5	MAP + microspheres (40 µg)	3.41 ± 0.33	4.22 ± 0.38	3.94 ± 0.27	4.46 ± 0.29	5.29 ± 0.31	8.22 ± 0.36	5.97 ± 0.29	7.44 ± 0.36

<sup>a</sup> Specific activities were calculated by dividing the specific MAP formulation endpoint titer by the total antibody concentration (µg/ml).

<sup>b</sup> All experimental groups were immunized single time through intranasal route of the specified formulations. Data are representative of three independent experiments and are shown as mean ± SD.

levels hints towards a possibility of high T cell activation indirectly witnessed in IgG isotyping (towards IgG2a and IgG2b), which are cytophilic antibodies beneficial to the host. Thus, the MAP+CpG is able to generate both humoral as well as mucosal immune responses which will be beneficial against bacterial infections.

It was shown that intranasal administration of IL-17 plays a crucial role in inducing pIgR expression by the epithelium. Moreover, high SIgA levels could be due to the heightened production of IL-17 as well as TGF-β [64]. The high levels of SIgA suggests that CpG administration may have lead to an increase in the levels of

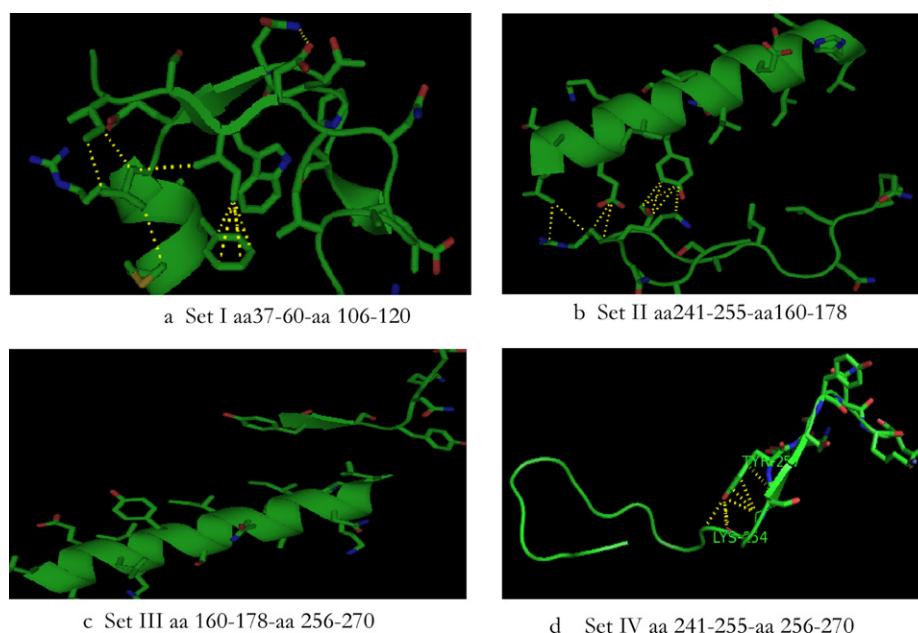
IL-17, further which might have increased the SIgA levels. Peptide 'd' and peptide 'a' which are T cell epitopes, showed maximum titers with MAP+CpG indicating that high antibody titers can be obtained with T cell epitopes when presented together with B cell epitopes as MAP. Further we observed that the responses contributed by the peptides to be independent in nature suggesting there might be synergism between the peptides as evident from the increase in titers towards MAP construct as that of individual peptides. After observing this synergism, the structural analyses were carried out where we could find little or no interaction among various peptides thus strengthening the fact that the peptides could be acting synergistically. Put together, the presence of B-and T-cell epitopes in MAP elicited both humoral and mucosal responses and when given with CpG, antibody levels were further enhanced in all three strains. Therefore, based on the immunological and structural analysis, we suggest that there might be synergism between the peptides and the immune responses contributed by the peptides are of independent in nature. Moreover, high antibody titers can be obtained with T cell epitopes when presented together with B cell epitopes in the MAP system. In conclusion, the present study reports the synthesis of highly immunogenic lipidated chimeric multiple antigen peptide in plague vaccine development. All the peptides are contributing independently towards the immune response and there might exist synergism between the peptides in enhancing the immune response in the MAP system. The synergism shown by the epitopes supports their possible co-ordinated role in effecting the virulence of LcrV antigen as suggested in our earlier studies [33]. Overall CpG ODN proved to be an effective adjuvant. MAP can be administered intranasally after entrapping in microspheres to increase its stability and duration in mucosal compartment. Presently we are studying T cell and *in vivo* protective immune responses of the MAP in murine model.

**Table 9**  
Structural analysis of MAP.

Set	Peptides	Region	Interactions (hydrophobic bond)
I	a, b and d	aa37–60 and aa106–120	(1) Phe118-Ile46 (4) (2) Leu115-Met119 (5) (3) Ile46-Leu115 (4) (4) Ile48-Leu 115 (4)
II	i and f	aa160–178 and aa241–245	(1) Leu160-Arg246 (2) (2) Glu163-Arg246 (7) (3) Tyr167-Thr247 (5) (4) Tyr167-Lys245 (1)
IV	i and g	aa160–178 and aa256–270	Nil
III	f and g	aa241–255 and aa256–270	(1) Lys 254-Tyr 257 (7)

The structural analysis of MAP was carried out using molecular visualization system, PyMOL software.

Bond distances less than 4.0 Å were taken into consideration for assessing the interactions between different residues.



**Fig. 4.** The structural analysis of MAP was carried out using molecular visualization system, PyMOL (DeLano Scientific, LLC). Bond distances less than 4.0 Å were taken into consideration for assessing the interactions between different amino acids. (a) Set I aa37–60 and aa106–120. (b) Set II aa241–255 and aa160–178. (c) Set III aa160–178 and aa256–270. (d) Set IV aa241–255 and aa256–270.

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