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The TLR9 agonist CpG fails to enhance the acquisition of *Plasmodium falciparum*-specific memory B cells in semi-immune adults in Mali

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Abstract

Antibodies play a key role in controlling blood stage malaria infections, and an effective blood stage malaria vaccine will likely require that it induce vaccine-specific memory B cells (MBCs). Our previous studies showed that the addition of the TLR9 agonist CpG to *Plasmodium falciparum* protein subunit vaccines greatly increased their efficacy in inducing MBCs in nonimmune U.S. volunteers. Here we show that in contrast the same CpG-containing malaria vaccine did not enhance the acquisition of MBCs in semi-immune adults living in Mali. Understanding the molecular basis of this apparent refractoriness to TLR9 agonist will be of significant interest in vaccine design.

Keywords

Memory B cells; *Plasmodium falciparum*; Malaria; Toll-like receptor 9

1. Introduction

Malaria is an infectious disease for which immunity is slow to develop, incomplete and short-lived [1]. Despite hundreds of infectious bites children living in malaria endemic areas under the age of five are susceptible to severe malaria disease and death and remain susceptible to mild disease until approximately age 10. It is not until the early teens that children become

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protected from symptomatic malaria but they rarely develop resistance to infection. Even this immunity appears to be rapidly lost in individuals who leave endemic areas. The difficulty in establishing long-lived effective immunity through natural infection suggests the possibility that infection with the malaria parasite may interfere with immune mechanisms necessary for the generation and maintenance of immunological memory.

For the blood stage of infection, parasite-specific antibodies have been shown to play a key role in controlling both parasite numbers in infected children and the high fevers that accompany infection [2]. Given the importance of antibody in controlling this stage of the infection, an effective blood stage malaria vaccine will likely need to induce robust humoral memory. Current evidence from studies of a variety of infectious diseases in humans indicate that long-lived humoral memory is encoded in both memory B cells (MBCs) and long-lived plasma cells (LLPC) that are generated during primary responses to antigenic challenge, either through natural infection or vaccination [3-5]. MBCs and LLPCs differentiate from naïve B cells in specialized lymphoid tissue microenvironments termed germinal centers [6]. LLPCs are terminally differentiated cells that reside in specialized niches in the bone marrow and contribute to the long-term maintenance of serum antibody levels [7]. MBCs express isotype switched somatically mutated Igs and respond to secondary antigenic challenge by rapidly proliferating and differentiating into plasma cells, accounting for the characteristic rapid, high affinity memory antibody responses [3-5]. MBCs and LLPC generated by a single antigen exposure have been shown to persist for tens of years in the absence of subsequent antigenic challenge [8,9].

Although the importance of sustaining antibody immunity is clear, at present our understanding of the cellular and molecular mechanisms underlying the generation and maintenance of MBCs and LLPC is only partial. We recently investigated the effect of activation of the innate immune system through Toll-like receptor 9 (TLR9) on the antigen-driven generation of MBCs in the context of two separate phase 1 clinical trials of candidate malaria subunit protein vaccines formulated on alum [10]. TLR9 detects microbial DNA containing hypomethylated CpG motifs and in humans is primarily expressed in B cells and plasmacytoid dendritic cells [11]. TLR9 activation results in TH1 cell differentiation and the consequent production of IgG antibodies. In humans, TLR9 is preferentially expressed in MBCs rather than in naïve B cells and CpG has been shown to stimulate MBCs to differentiate into LLPC *in vitro* providing a possible mechanism for replenishment of LLPCs during infections [12]. The results of our studies showed that inclusion of CpG in two different malaria vaccines composed of the *Plasmodium falciparum (Pf)* recombinant apical membrane antigen 1 (AMA1) or merozoite surface protein 1 (MSP1) formulated on alum (AMA1-C1/Alhydrogel and $MSP1_{42}$-C1/Alhydrogel) greatly augmented the vaccine's ability to induce antigen-specific MBCs in nonimmune adults in the U.S. [10]. Following immunization with CpG-containing vaccines as compared to vaccines that did not contain CpG, MBCs appeared sooner, in greater numbers and persisted in circulation for longer. At steady state there was a positive correlation between vaccine-specific MBCs and antibody levels suggesting that CpG also enhanced the generation of LLPCs.

Here we report that the inclusion of CpG did not enhance the generation of MBC in semi-immune adults living in Mali. Malian adults enrolled in the study had low levels of circulating AMA1-specific MBCs and these numbers increased following immunization but neither the kinetics of the appearance of MBCs nor the numbers of MBCs were influenced by vaccination with CpG. The AMA1-specific antibody titers were approximately two-fold higher in individuals receiving the CpG-containing vaccine as compared to the vaccine alone. However, we observed no correlation between the frequency of AMA1-specific MBCs and the levels of AMA-1 specific antibodies. These results suggest that adults living in malaria endemic areas are relatively refractory to CpG stimulation and suggest caution in extrapolating from the

results of vaccine trials in nonimmune adults in the U.S. to semi-immune adults living in malaria endemic areas.

2. Methods

2.1 Study population and vaccination procedure

This analysis was carried out in conjunction with a phase 1 clinical study of 24 semi-immune Malian adults (www.clinicaltrials.gov NCT00414336) who were randomized 1:1 to receive AMA1-C1/Alhydrogel ± CPG 7909 on day 0 and 28 (Sagara *et al.*, accompanying manuscript). The production and formulation of the AMA1-C1/Alhydrogel $^{+/-}$CPG 7909 vaccines have been previously described [13]. AMA1-C1 is a combination of two recombinant allelic proteins (FV0 and 3D7); the proteins are adsorbed to Alhydrogel and the vaccine is mixed with the novel adjuvant CPG 7909 (Coley Pharmaceuticals, a Pfizer Company) at the time of injection. As part of the assessment of the immune responses to vaccination, peripheral venous blood samples for peripheral blood mononuclear cells (PBMCs) were drawn from participants on day 0 prior to the first vaccination, on day 3 following the first vaccination, on day 28 prior to the second vaccination and on days 35, 90 and 210 following the first vaccination. Vaccinations were given in October and November, 2007, during the malaria transmission season in Mali. PBMCs were isolated from whole blood, cryo preserved and thawed for MBC analyses as described previously [10]. For flow cytometry phenotypic analyses, PBMCs were used before freezing.

2.2 Multiparameter flow cytometry

The preparation and labeling of PBMC with fluorescently-labeled antibodies for flow cytometric analyses has been detailed previously [10]. Briefly, PBMCs were washed in PBS and centrifuged through fetal bovine serum to remove platelets. Cells were incubated for 30 min at 4°C with fluorescently labeled antibodies specific for CD19 (PE-Cy5.5), CD27 (PE) and CD38 (APC) purchased for Caltag Laboratories and IgD (FITC) purchased from BD Bioscience. Cells were washed in PBS, fixed with 1% paraformaldehyde and analyzed on a FACSCaliber. Data were analyzed using FlowJo software.

2.3 Memory B cell assay and ELISA

MBCs were quantified from thawed PBMCs by the method of Crotty *et al.* [14] as previously detailed [10]. Briefly, PBMCs were cultured in complete media containing a cocktail of polyclonal activators including CpG-2006, SAC and pokeweed mitogen at 37°C for 5 days. Cells were washed and placed in 96-well ELISPOT plates coated with either AMA1 to detect AMA1-specific antibody secreting cells or goat antibodies specific for human IgG to detect all IgG secreting antibody producing cells and incubated at 37°C for 5 h. The plates were washed and bound antibodies were detected using goat antibodies specific for human IgG Fc conjugated with alkaline phosphatase developed using BC11/NBT. Spots were counted using an ImmunoSpot series 4 analyzer. Antigen-specific MBC data are expressed as the number of Ag-specific MBC per 10^6 PBMC after the 5 day culture divided by the number of total IgG-secreting MBC per 10^6 PBMC after the 5 day culture times 100.

The ELISA protocol for measuring AMA1-specific antibodies has been previously described [15]. An ELISA unit value of a standard was assigned as the reciprocal of the dilution giving an OD405 of 1 in a standardized assay. The optical density of individual test samples was converted into ELISA units using a standard curve generated by serially diluting the standard in the same plate: ELISA units of the standard were fixed once assigned, regardless of actual OD405 value of a standard curve in a plate.

Data Analysis

The Wilcoxon signed-rank test for matched pairs was used to compare continuous outcomes at different time points. The Wilcoxon rank-sum test was used to compare continuous outcomes of different groups at the same time point. The correlation between different continuous measures was determined using the Spearman correlation coefficient. All P values were two-sided, and P values of less than 0.05 were considered to be statistically significant. Data analyses were performed with STATA, version 10.0 (StataCorp LP) and GraphPad Prism version 5.01 for Windows (GraphPad Software).

3. Results

3.1 The acquisition of vaccine-specific MBCs in semi-immune adults following vaccination

To determine the kinetics of appearance and numbers of AMA1-specific MBCs induced by vaccination with AMA1 and the impact of TLR9 activation on this process in malaria semi-immune adults, we determined the frequency of AMA1-specific MBCs per total MBCs in the peripheral blood of 20 Malian adults enrolled in a phase 1 clinical trial of the candidate vaccine AMA1-C1/Alhydrogel $^{+/-}$ CpG. Individuals were vaccinated twice, on day 0 and day 28 and peripheral blood samples were collected on days 0, 3, 28, 35, 90 and 210 as indicated in Table 1 and a portion of the cells were cryopreserved for analyses of MBCs. Frozen PBMCs were subsequently thawed and both AMA1-specific and total MBCs were quantified by the method of Crotty *et al.* [14] that relies on the selective polyclonal activation of MBCs during five days in culture to differentiate into antibody secreting cells measured in an AMA1-specific ELISPOT assay. This assay has been shown to be highly specific for $CD19^{+}$ $CD27^{+}$ MBCs as $CD19^{+}$ $CD27^{-}$ naïve cells produced no antibody secreting cells under the assay conditions [14]. We have carried out a similar analysis and confirmed that $CD19^{+}$ $CD27^{+}$ MBC yield antibody secreting cells under the assay conditions whereas $CD19^{+}$ $CD27^{-}$ naïve B cells do not (unpublished observations). For both the CpG and non-CpG groups the viability of cells after thawing was similar. On day 0, before the first vaccination individuals in both groups had low but detectable numbers of AMA1-specific B cells representing on average 0.04 percent of all MBCs (Fig. 1). The frequency of AMA1-specific MBCs increased significantly to 0.19 and 0.22 percent in CpG and non-CpG groups by day 28 after the first vaccination. Following the second vaccination the frequency of AMA1-specific MBCs rose above the day 28 frequencies in both CpG and non-CpG groups by day 90 (CpG versus non-CpG, 0.30 versus 0.27). By day 210 the frequency of AMA1-specific MBCs had decreased in both groups to levels approximately two fold above prevaccination levels (CpG versus non-CpG, 0.11 versus 0.08), although the mean frequencies were not significantly different from baseline. The peak frequencies of AMA1-specific MBCs are lower than those reported recently following immunization of U.S. volunteers with the same vaccine [10] but until the efficiencies of the MBC assays carried out in the U.S. laboratories and in the Mali laboratory are harmonized, comparisons of absolute frequencies between these different laboratories may not be meaningful. These data indicate that the TLR9 agonist CpG had no measurable effect on either the kinetics of appearance or number of MBCs induced by vaccination in semi-immune adults. These data also indicate that AMA1-specific MBCs generated in response to natural infection can be transiently expanded by vaccination.

3.2 Vaccination showed no antigen-independent effects on MBCs

We also investigated whether vaccination with CpG had an effect on the total number of MBCs, naïve B cells, or plasma cells in the peripheral blood. Assessing total MBCs by the method of Crotty *et al.* [11] using antibodies specific for human IgG to identify all antibody secreting cells in an ELISPOT assay showed that vaccination with the TLR9 agonist had no effect on the total number of MBCs in circulation (Fig. 2). The mean total MBC frequencies, ranging

between 30 to 50 per 10^6 PBMCs, remained stable over the course of the seven month trial. No significant differences were found between the CpG and non-CpG groups at any time point. The numbers of MBCs ($CD19^+$, $CD27^+$, $CD38^-$), naïve B cells ($CD19^+$, IgD^+), and plasma cells (PCs) ($CD19^+$, $CD38^{++}$) were also determined by flow cytometry. The results also showed no differences in the numbers of cells in each subpopulation in the CpG and non-CpG groups over the 210 day course of the trial (data not shown). $CD19^+$ B cells represented approximately 10% of total PBMCs and MBCs and PC represented approximately 20-30% and 2-3% of total B cells respectively. Collectively these data indicate that exposure of the immune system of semi-immune adults to the TLR9 agonist CpG has no observable effect on the circulating B cells in those individuals. This was in contrast to our observation in U.S. volunteers in which case vaccination resulted in a transient decrease in the number of total MBCs in circulation [10].

3.3 The relationship between MBCs and antibody levels

AMA1-specific IgG antibody levels measured in plasma samples by ELISA (Sagara *et al.* accompanying manuscript) and the AMA1-specific MBC frequencies showed a similar pattern of acquisition and loss. The peak antibody levels in individuals receiving the CpG-containing vaccine showed a modest increase of approximately two fold as compared to those of individuals receiving the vaccine alone. U.S. volunteers receiving the same vaccines showed 11-14 fold differences in antibody levels [10]. However, both the kinetics of appearance and persistence of antibodies and MBC appeared similar in the two Malian groups. In U.S. volunteers receiving these vaccines we not only observed that the AMA1-specific antibody responses mirrored the MBC frequencies but also found that the frequencies of AMA1-specific MBCs present at the time of reimmunization predicted the AMA1-specific antibody levels 14 days later and that at steady state a positive correlation existed between the frequencies of AMA1-specific MBCs and antibody levels. In Malian adults we observed no correlation between the frequencies of AMA1-specific MBCs at 28 days, at the time of booster vaccination, and the AMA1-specific antibody levels 14 days later (Fig. 4A, Spearman's correlation coefficient = 0.22). At steady state, at day 210 after the first vaccination we observed no correlation between the frequency of AMA1-specific MBCs and levels of AMA1-specific antibodies (Fig. 4B, Spearman's correlation coefficient = 0.04).

4. Discussion

In areas of high transmission, malaria is an infectious disease to which individuals are chronically re-exposed. In chronic viral infections including those caused by HIV and hepatitis B and C viruses current evidence indicates that chronic exposure to the viruses leads to alterations and what has been termed exhaustion in both T [16,17] and B lymphocytes [18, 19]. Perhaps of particular relevance to the findings reported here an exhausted phenotype has been described recently for MBCs in individuals with high HIV viremia [18]. Exhausted MBCs were shown to be refractory to stimulation with polyclonal B cell activators and to upregulate a number of inhibitory receptors and were implicated in the poor antibody response in HIV-infected individuals [18]. We recently observed a large expansion of an atypical MBC subpopulation that resembles exhausted MBCs in children as young as two years of age in malaria endemic areas [19]. Such studies provide evidence that chronic exposure to a pathogen can result in the alteration of both the T and B lymphocytes leading to their overall hyporesponsiveness to the infecting pathogen. Here we provide evidence that individuals living in a malaria endemic area and chronically re-infected with malaria as well as other parasites and pathogens are relatively refractory to TLR9 activation by CpG.

The TLRs have been implicated to play a role in replenishing both MBCs and LLPCs over the lifetime of an individual [12]. Our earlier results provided evidence that in nonimmune U.S.

volunteers addition of the TLR9 agonist CpG to two different malaria subunit protein vaccines resulted in a more rapid appearance of vaccine-specific MBC that reached higher peak numbers and persisted at higher frequencies [10]. In this previous study, antibody levels in the AMA1-CpG vaccinated group were 11-14 fold higher as compared to the group receiving AMA1 alone and the MBC numbers mirrored the vaccine-specific antibody levels. Vaccination also appeared to have an antigen-independent effect, reducing the number of total IgG^+ MBCs in circulation by approximately one half three days after vaccination. We concluded that this drop may reflect the migration of MBCs into lymphoid tissues for differentiation into PCs. Consistent with this possibility we observed a concurrent increase in the number of plasma cells in circulation three days after vaccination [10].

In contrast to the observed effect of CpG on the generation of MBCs in nonimmune adults in the U.S., here we report that semi-immune adults in Mali appear relatively refractory to the effects of CpG administration. Although individuals receiving AMA1-CpG had a two fold increase in antibody levels as compared to those receiving AMA1 alone, when comparing the outcomes of vaccination with or without CpG, we observed no change in the kinetics of appearance or the peak numbers of vaccine-specific MBCs or in the total numbers of naïve B cells, MBCs or PCs in circulation. Thus, the immune system of semi-immune adults appears relatively refractory to the potential effects of CpG.

In both U.S. and Malian volunteers we observed that the changes in the levels of antigen-specific antibody coincided with the changes in the frequencies of antigen-specific MBCs. However, in contrast to what was observed in vaccinated U.S. adults, we saw no correlation between the number of antigen-specific MBCs and the antigen-specific antibodies either 14 days after the second vaccination or at steady state. The interpretation of this observation at this time is not clear. At present, the relationship between antibody levels and MBC numbers is not established. In several studies of MBCs induced by vaccination and infection including in measles, mumps and rubella infections [9] and vaccinia [8] and meningococcal C glycoconjugate [20] vaccination correlations similar to the one we reported earlier [10] were observed. In other cases no correlations were observed including for HIV [21], Epstein-Barr virus and varicella-zoster infections [9] and tetanus, diphtheria and vaccinia vaccination [9]. Understanding the role that MBCs play in maintaining antibody titers and LLPC is of considerable interest but will require further study.

Recent studies have identified alterations in TLR expression and function during acute uncomplicated malaria infections. Franklin *et al.* [22] observed that peripheral blood cells from infected adults in a malaria endemic area in the Amazon basin were hyperresponsive to *in vitro* stimulation with TLR agonists. However, three to four weeks following treatment with antimalarials the responses to TLR agonists returned to baseline, but did not appear to be suppressed. Similar results were observed earlier by McCall *et al.* [23] for experimentally induced malaria infections but these increases in TLR responsiveness were not as robust as those reported by Franklin *et al.* and were not observed for all TLRs. The peripheral blood cells of asymptomatic parasitemic children in Ghana were also shown to be hyperresponsive to TLR activation [24]. Taken together these studies provide evidence for a priming effect of *Pf* on TLR responses during malaria infections. Whether such a priming effect of malaria on TLR responses during infection might relate to the presumably constitutive refractoriness to the effects of CpG described here remains to be determined.

The findings reported here raise a number of questions, perhaps the most important of which is: are the immune systems of infants and children living in endemic areas, the target population for malaria vaccines, similarly refractive to the effects of CpG? It will also be of significant interest to determine the cellular and molecular basis of the refractoriness of semi-immune adults to CpG. TLR9 is expressed in human MBCs and the effect of CpG on MBC generation

could be due to hyporesponsiveness of MBCs to CpG. Alternatively, the refractoriness to CpG could be indirect through TLR9-expressing dendritic cells. Clearly, understanding the impact of chronic malaria reinfections on the immune system will aid in the rational design of effective malaria vaccines.

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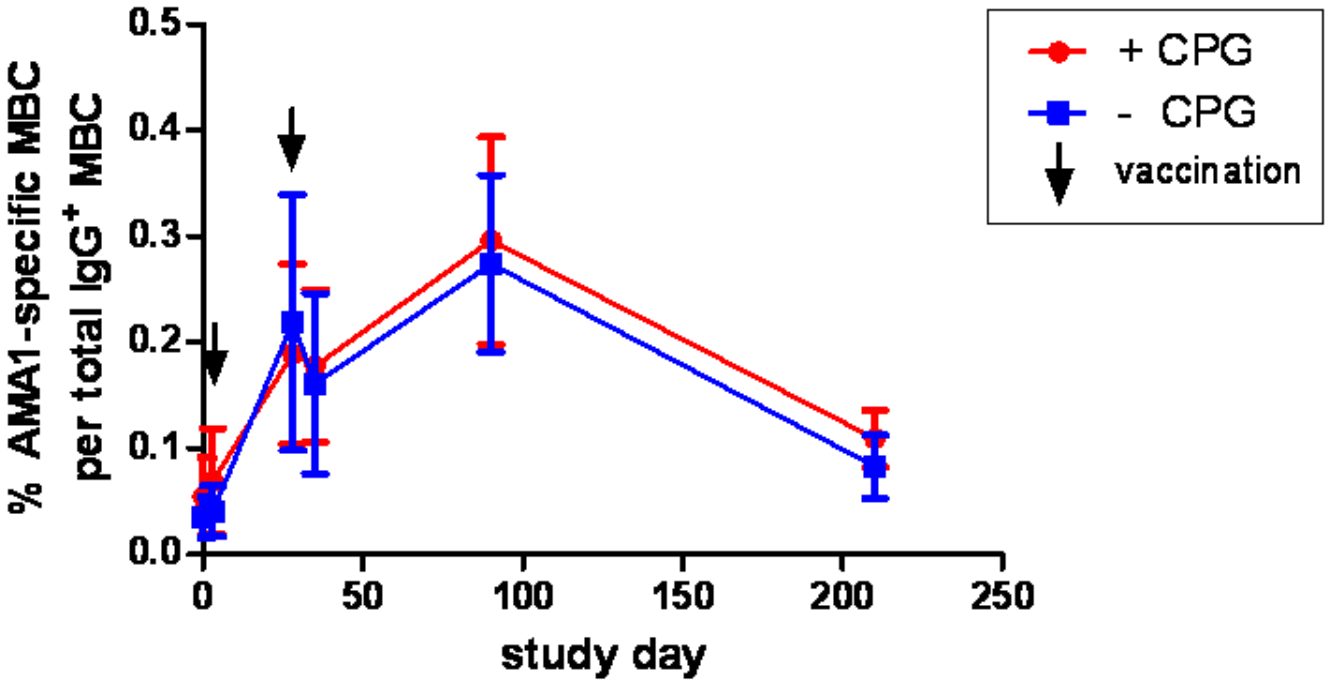

Fig. 1. The acquisition of AMA1-specific MBCs following vaccination with AMA1-C1/Alhydrogel $^{+/-}$ CpG

AMA1-specific MBCs were quantified by the method of Crotty *et al*. [14] using AMA1-coated ELISPOT plates. Shown are the average percent of AMA1-specific MBCs per total MBCs $^{+/-}$ SEM for individuals receiving AMA1-C1/Alhydrogel with (red) and without (blue) CpG7909 at various times after vaccinations on day 0 and day 28 (indicated by arrows).

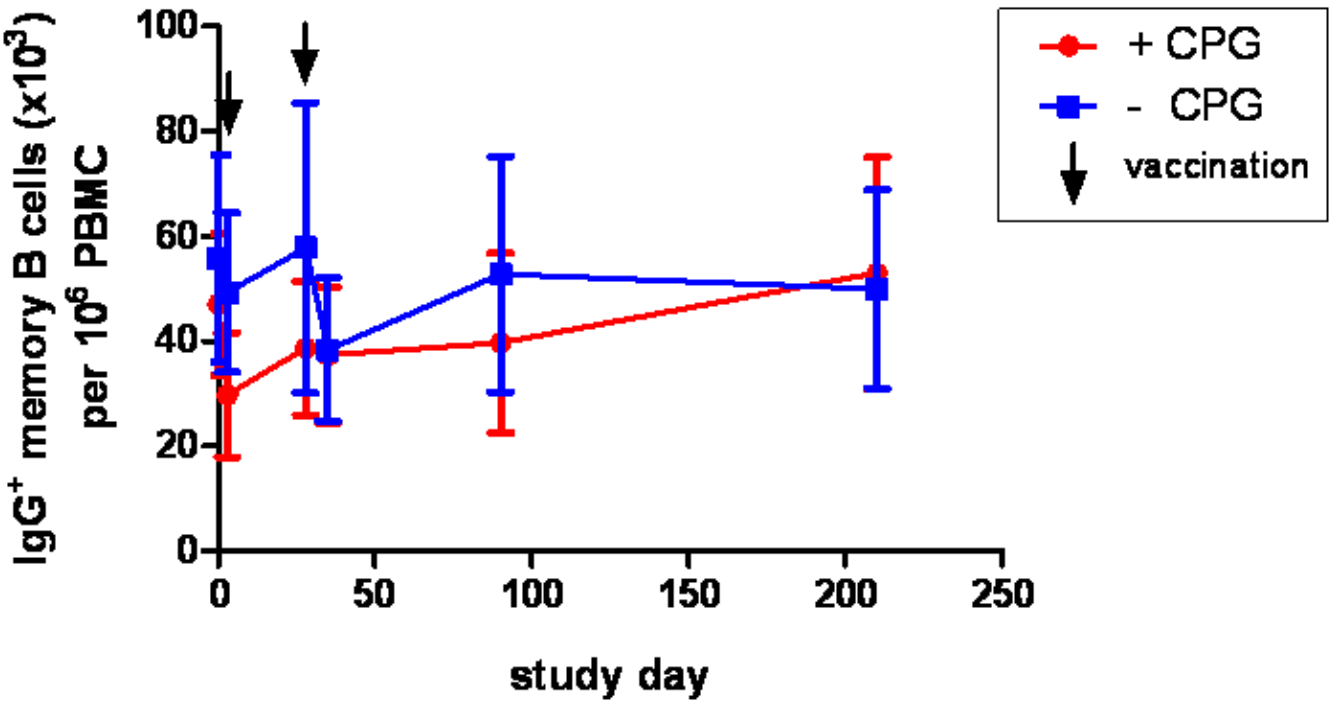

Fig. 2. CpG-containing vaccines showed no antigen-independent effects on the total number of MBCs

The total number of MBCs were quantified by the method of Crotty *et al.* [14] using ELISPOT plates coated with antibodies specific for human IgG. Given is the mean number of MBCs per 10^6 PBMC $^{+/-}$ SEM at the end of the five-day culture.

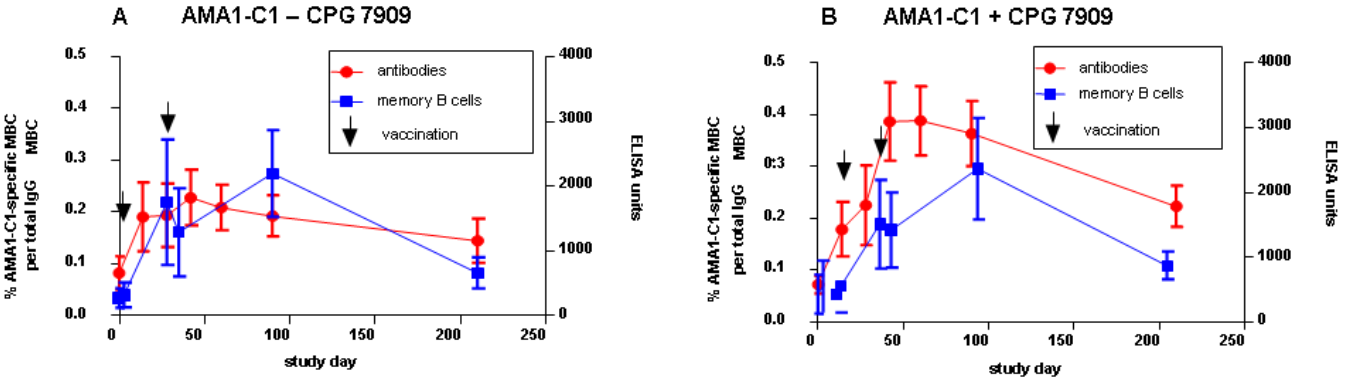

Fig. 3. A comparison of the levels of AMA1-specific antibodies and the frequencies of AMA1-specific MBCs with time after vaccination

The AMA1-specific antibody levels (red circles) were determined by ELISA for individuals immunized with (A) AMA1-C1/Alhydrogel without CPG 7909 or (B) with CPG 7909. The frequencies of AMA1-specific MBCs (blue squares) are given for comparison. Data are given as the mean percentage AMA1-specific MBC per total IgG MBC $^{+/-}$ SEM or for AMA1-specific antibody the mean ELISA units $^{+/-}$ SEM. The sample sizes for each MBC point are given in Table 1 and for each antibody time point in Sagara *et al.* (accompanying manuscript).

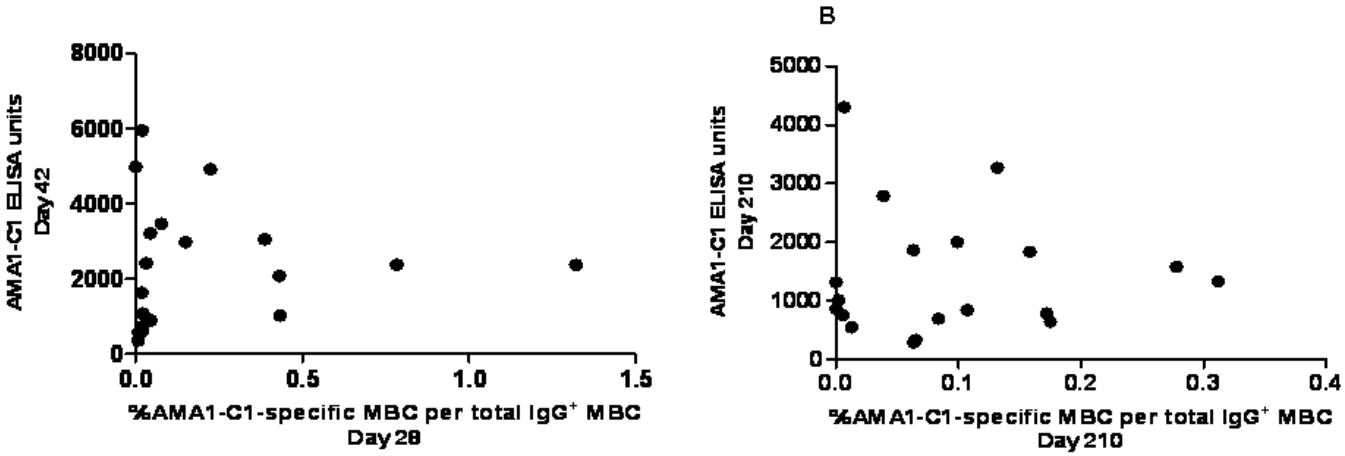

Fig. 4. The relationship between the frequencies of AMA1-specific MBC and AMA1-specific antibodies

(A) The level of AMA1-specific MBCs at the time of the second vaccination (day 28) did not predict the antibody response 14 days later ($r = 0.22$, $p = 0.36$). (B) The correlation between AMA1-specific MBCs and antibody levels at steady-state was determined using the last time point for which corresponding ELISPOT and ELISA data (day 210 after first vaccination) were available (19 individuals). The data was analyzed in cross-section and did not show a correlation between AMA1-specific MBCs and antibody levels ($r = 0.04$, $p = 0.89$).

Table 1

Sample size and mean AMA1-C1 specific MBC percentage by CpG group and study day.[a]

Study day	WithoutCPG 7909		With CPG 7909	
	n	% MBC (SD)	n	% MBC (SD)
0[b]	11	0.03 (0.06)	9	0.05 (0.11)
3	11	0.04 (0.08)	9	0.07 (0.15)
28[b]	11	0.22[c] (0.40)	9	0.19[c] (0.26)
35	11	0.16[c] (0.28)	9	0.18[c] (0.22)
90	11	0.27[c] (0.28)	9	0.30[c] (0.29)
210	11	0.08 (0.10)	9	0.11 (0.08)

[a] There was no difference in the AMA1-C1-specific MBC response between the CpG and non-CpG group at any time point ($p > 0.05$ for all comparisons).

[b] Vaccination day.

[c] $p < 0.05$ vs study day 0 using the Wilcoxon signed rank test for matched pairs.