

# A single injection of 19 kDa carboxy-terminal fragment of *Plasmodium yoelii* merozoite surface protein 1 (PyMSP1<sub>19</sub>) formulated with Montanide ISA and CpG ODN induces protective immune response in mice

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## Summary

**Objective:** To investigate the efficacy of a vaccine formulation of the 19 kDa conserved carboxyl-terminal fragment of *Plasmodium yoelii* merozoite surface protein-1 (PyMSP1<sub>19</sub>) formulated with CpG ODN 1826 and Montanide ISA51 or ISA720 when used to immunize mice by a single injection.

**Methods:** Groups of BALB/c mice were immunized parenterally with one, two or four injections with PBS or PyMSP1<sub>19</sub> formulated with CpG ODN in ISA51 or ISA720. Sera were collected weekly and assessed for total IgG and IgG subclass titers. Protection was tested by challenge infection with *P. yoelii* YM.

**Results:** Interestingly, single injection immunization showed the same kinetics of antibody responses as two- or four-injection immunization. However, the peak antibody response induced by PyMSP1<sub>19</sub> in CpG ODN and ISA51 appeared earlier than that induced by PyMSP1<sub>19</sub> in CpG ODN and ISA720 (28 days vs 41 days). At day 63 after the first injection, the PyMSP1<sub>19</sub>-specific IgG antibody levels by single injection and four-

injection immunizations were not different. However, the levels of the IgG2a antibody subclass were significantly lower by single injection immunization with PyMSP1<sub>19</sub> in CpG ODN and ISA720. The antibodies were sustained at high levels for at least 20 weeks. After challenge infection, all mice immunized by a single injection of PyMSP1<sub>19</sub> in CpG ODN and ISA51 survived with low-grade parasitemia, while 50% of mice immunized with PyMSP1<sub>19</sub> in CpG ODN and ISA720 died with high levels of parasitemia.

**Conclusion:** These findings suggest that MSP1<sub>19</sub> immunization by a single injection can induce protective immunity, particularly when formulated with an appropriate strong adjuvant. (*Asian Pac J Allergy Immunol* 2011;29:252-9)

**Key words:** immunization, MSP1, malaria, *Plasmodium yoelii*

## Introduction

Malaria remains a major public health problem, causing severe morbidity and mortality. Each year more than 2 billion people are at risk of malaria. More than 500 million people become severely ill with malaria and more than 1 million people die from it, with most of the deaths being in children under 5 years of age in sub-Saharan Africa<sup>1,2</sup>. Although a malaria vaccine is not currently available, many malaria vaccine candidates have been clinically tested. The 19-kDa carboxyl-terminus of merozoite surface protein 1 (MSP1<sub>19</sub>) is a leading malaria vaccine candidate, as it can induce protective immunity in monkeys and mice<sup>3,4,7</sup>.

MSP1<sub>19</sub> is a highly conserved protein, composed of two epidermal growth factor (EGF)-like domains which contain protective epitopes<sup>3,8</sup>. It is a final product of proteolytic cleaving of MSP1 during schizogony and merozoite maturation and is carried into newly uninfected erythrocytes<sup>9</sup>. Immunization

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with MSP1<sub>19</sub> emulsified with Freund's adjuvant confer protection in conjunction with MSP1<sub>19</sub>-specific antibodies, but not with effector T cells nor other accessory factors associated with cell-mediated immunity<sup>4</sup>. However, the T cell epitopes of MSP1<sub>19</sub> help to provide a more rapid antibody response after secondary exposure, which suggests a role for Th cells<sup>10</sup>. Passive transfer of MSP1<sub>19</sub>-immune serum can control parasitemia in recipient mice, but an active immune response post infection is also acquired for protection against lethal malaria<sup>11</sup>, and its specificity for MSP1<sub>19</sub> is not required for protection<sup>12</sup>.

An adjuvant is essential for promoting immune responses during immunization with non-living, i.e., killed or sub-unit vaccines. Bacterial CpG DNA or synthetic CpG ODNs have been recognized as strong immuno-stimulatory agents with an extensive ability to stimulate innate immune activation, which is followed by adaptive immune responses<sup>13</sup>. After exposure to B cells or plasmacytoid dendritic cells, the CpG ODN enters the endosomal compartment to trigger the toll-like receptor 9 (TLR9)<sup>14</sup>, leading to the cell activation through cell signaling pathways which results in an up-regulation of co-stimulatory molecule expression, resistance to apoptosis, chemokine receptor CCR7 expression and secreting Th1-promoting cytokines and chemokines<sup>13</sup>. Montanide (e.g. ISA51 or ISA720) is a more purified oil-based adjuvant than Freund's adjuvant and has proved efficient in clinical trials<sup>15-17</sup>. Our previous studies have shown that the combination of CpG ODN and ISA51 or ISA720 as an adjuvant for PyMSP1<sub>19</sub> immunization for mice by a standard of four injections can induce a stronger IgG antibody response and, compared to Freund's adjuvant, gives complete protection from lethal malaria infection<sup>18</sup>. Interestingly, the IgG2a antibody response is much greater in immunization using the combination of CpG ODN and ISA51 or CpG ODN and ISA720 as an adjuvant than when only using ISA51 or ISA720, or even CFA/IFA<sup>18</sup>. A more recent study on the longevity of antibody responses has shown that IgG2a, and not IgG1, persisted constantly for longer than 12 months<sup>19</sup>. As it is such a strong adjuvant, we then asked: if the number of immunization injections were reduced to just a single injection, could a protective immune response still be effectively elicited?

## Methods

### *Mice and parasites*

Female BALB/c mice, 6–8 weeks of age at the beginning of experiments, were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Prathom, Thailand. The animals were maintained under specific pathogen-free conditions. These studies have been reviewed and approved by the Faculty of Tropical Medicine-Animal Care and Use Committee (FTM-ACUC), Mahidol University. *Plasmodium yoelii* YM, a lethal murine malaria parasite, was maintained in our laboratory and used for challenge infection.

### *Recombinant MSP1<sub>19</sub> protein*

Recombinant MSP1<sub>19</sub> protein of *P. yoelii* YM was produced as a FLAG-fusion protein (PyMSP1<sub>19</sub>) from *Saccharomyces cerevisiae*, according to the instructions from the manufacturer, Eastman Kodak, Scientific Imaging Systems. The recombinant protein was purified using an anti-FLAG M1 antibody gel column (Sigma) and the purity was demonstrated with the use of an SDS-PAGE showing the protein to be of a single band<sup>18</sup>.

### *Adjuvants*

The CpG ODN 1826 (TCCATGACGTTCC TGACGTT) used in this study was kindly provided by AM. Krieg, the Coley Pharmaceutical Group, USA. The Montanide ISA51 and ISA720 were a kind gift from the SEPPIC company, France.

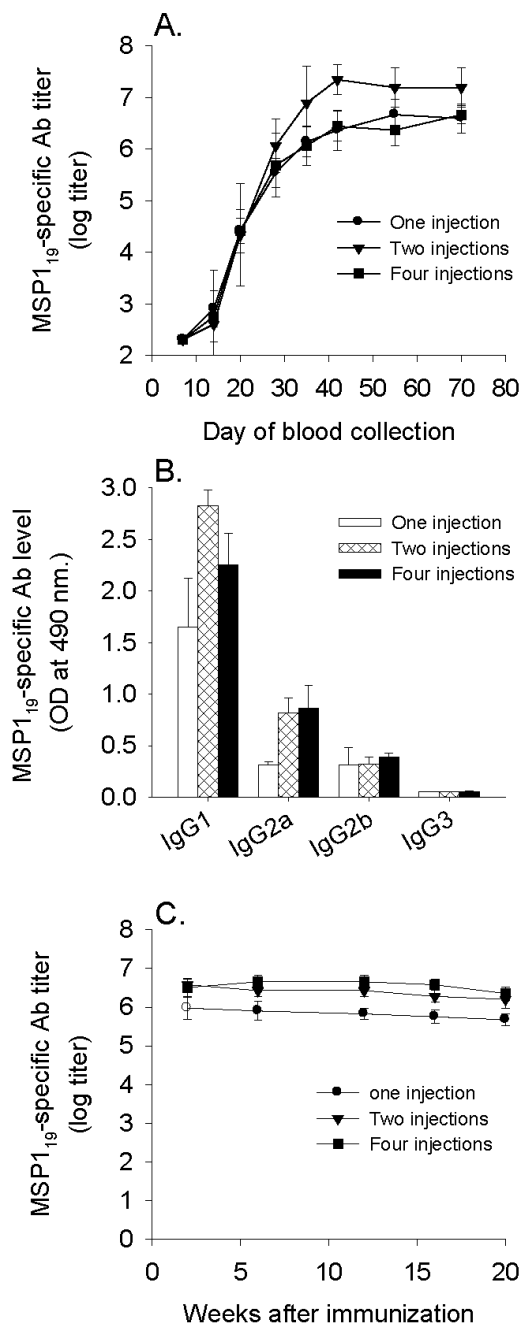
### *Immunization protocol*

For single injection immunization, groups of four mice were injected subcutaneously (s.c.) once with an emulsion of the mixture of one part of PBS or 20 µg of PyMSP1<sub>19</sub> plus 50 µg CpG ODN and one part of ISA51, or three parts of PBS or 20 µg of PyMSP1<sub>19</sub> plus 50 µg CpG ODN, and seven parts of ISA720. For two- and four-injection immunizations, mice were injected twice or four times with the same vaccine regimen as for the single injection protocol on days 0 and 21, and days 0, 21, 42, and 56, respectively.

### *Plasma collection and antibody assays*

Ten microliters of plasma was collected at different time points as indicated in the results, and placed into 90 µl of PBS which contained heparin. The mixture was centrifuged and the diluted plasma was transferred into a new tube and kept at -20°C until assayed. The assessments of PyMSP1<sub>19</sub>-specific IgG antibody levels and antibody subclasses were conducted by enzyme-linked immunosorbent





**Figure 1.** Antibody responses to PyMSP1<sub>19</sub> after immunization with one, two, and four injections of PyMSP1<sub>19</sub> formulated with CpG ODN and ISA720. BALB/c mice were immunized with PyMSP1<sub>19</sub> formulated with CpG ODN in ISA720 by one injection at day 0, by two injections at days 0 and 21, and by four injections at days 0, 21, 42, and 56. The kinetics of the antibody responses were monitored in each immunization protocol (A). The antibody isotypes of 1/2,000 diluted plasma at day 72 of the course of immunization were determined (B). The longevity of the antibody response was monitored for 20 weeks after the last immunization day of the four-injection protocol (C). The data show mean  $\pm$  S.E. Four mice were used in each group.

assay (ELISA) as described previously<sup>18</sup>. The antibody titers were determined based on the optical density (O.D.) of pre-immunized plasma.

#### Challenge infection

Mice were challenged intravenously (i.v.) with  $1 \times 10^4$  live *P. yoelii* YM-parasitized red blood cells (pRBC). Parasitemia was monitored daily by microscopic examinations of dip-quick stained blood films; counting to at least 10,000 RBC was carried out before declaring a slide negative.

#### Statistical analyses

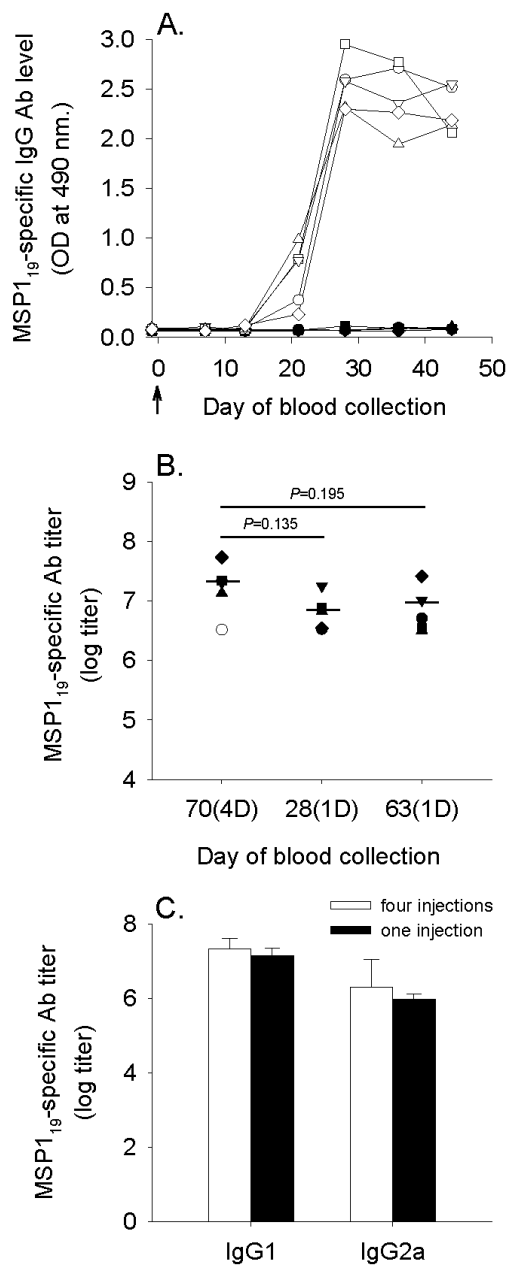
The significance of differences between the mean values of the tested immunization groups was determined by Student's *t* test. Significant was considered at  $P < 0.05$ .

#### Results

##### Antibody responses after immunization with one, two and four injections of PyMSP1<sub>19</sub> formulated with CpG ODN and ISA720

After immunization by one, two, or four injections of PyMSP1<sub>19</sub>/CpG ODN/ISA720 at day 0 only, or at days 0 and 21, or at days 0, 21, 42 and 56, respectively, plasma was collected and assayed for the PyMSP1<sub>19</sub>-specific IgG antibody by ELISA. The results showed that the kinetics of the total IgG antibody response after immunization either by one, two, or four injections had the same profile. The antibodies exponentially rose to maximum levels at day 41 and were stable thereafter (Figure 1A.). However, the antibody levels of the mice immunized by two injections were significantly higher than those of the other two groups ( $P < 0.01$ , as compared at day 41).

PyMSP1<sub>19</sub>-specific IgG antibody subclasses in mice immunized with one, two, or four injections of PyMSP1<sub>19</sub>/CpG ODN/ISA720 were determined at day 70 by ELISA. The results showed that in all three groups of mice, the major subclass was IgG1, followed by IgG2a, and then IgG2b. The IgG3 antibody was rarely detected. Mice immunized by two injections gave the highest IgG1 antibody response, followed by mice receiving four or one injections, respectively (Figure 1B.), which correlated with the total IgG antibody levels. Even though the total IgG levels of mice immunized by one injection or four injections were comparable, the level of IgG2a antibody of the former group was much lower than that of the latter one.



**Figure 2.** Antibody responses to PyMSP1<sub>19</sub> after immunization by one injection of PyMSP1<sub>19</sub> formulated with CpG ODN and ISA51. BALB/c mice were immunized by one injection of PyMSP1<sub>19</sub> formulated with CpG ODN and ISA51. Plasma was collected for analysis of the kinetics of the antibody response (A), the antibody level compared to the four injection immunization (B) and IgG1 and IgG2a antibody responses after one and four injection immunizations (C). Each symbol represents an individual mouse. Open symbols in (A) represent mice immunized with PyMSP1<sub>19</sub> formulated with CpG ODN and ISA51 and closed symbols represent mice immunized with PBS formulated with CpG ODN and ISA51. The data in (B) show the geometric mean of each group and the data in (C) show the mean  $\pm$  S.E.

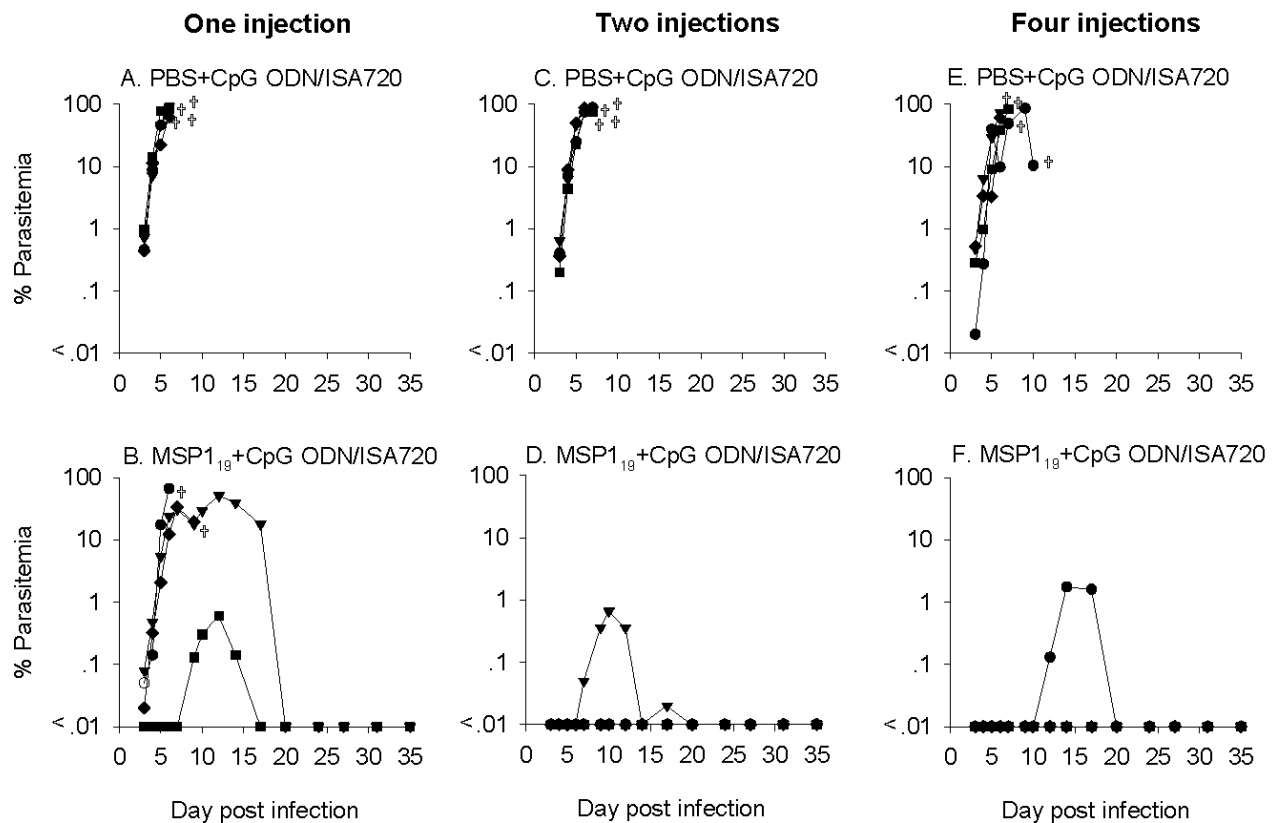
The longevity of antibody response of mice immunized by one injection was also determined and compared with that of mice immunized by two or four injections. This was done by monitoring their antibody levels for 20 weeks, starting at day 70, which is equal to the two week period after the fourth injection time point of the four injection protocol. The results showed that the IgG antibody levels of all three groups were still stable over the period of observation ( $P = 0.114$  for one injection,  $P = 0.040$  for two injections, and  $P = 0.356$  for four injections when comparing week 2 and week 20) (Figure 1C.).

#### *Antibody responses after immunization by single injection of PyMSP1<sub>19</sub> formulated with CpG ODN and ISA51*

In order to explore the potent adjuvancy of the combination of CpG ODN and ISA51 in the induction of an immune response to PyMSP1<sub>19</sub><sup>10</sup>, we examined whether one injection of PyMSP1<sub>19</sub> in combination with this adjuvant could induce a high antibody response. In the kinetics study, the total IgG antibody levels rose to their peak at day 28 (Figure 2A.). The total IgG antibody titers of mice immunized by one injection at days 28 and 63 were not significantly different from the total IgG antibody titers of the mice immunized by four injections at day 70 (Figure 2B.). The IgG1 and IgG2a antibody subclasses of mice immunized by one injection, as determined at day 70, were slightly lower than those of the mice immunized by four injections at day 70 (Figure 2C.).

#### *Protective response after immunization by single injection of PyMSP1<sub>19</sub> formulated with CpG ODN and ISA51 or ISA720*

Having demonstrated the kinetics of the antibody response, which is that a single injection immunization induces a high PyMSP1<sub>19</sub>-specific IgG antibody response comparable to a four-injection immunization, we then examined the efficacy of the antibody response in protection against malarial challenge infection. Mice were i.v. inoculated with  $1 \times 10^4$  *P. yoelii* YM-pRBC and the parasitemia was monitored daily. The results showed that all control mice immunized by one, two, or four injections of PBS/CpG ODN/ISA720 died with high parasitemia within 10 days (Figure 3A., C., and E.). Conversely, two of four (50%) mice immunized by one injection of PyMSP1<sub>19</sub>/CpG ODN/ISA720 survived infection even though they experienced some parasitemia. The peak parasitemia



**Figure 3.** Parasitemia post challenge infection with *P.yoelii* YM-pRBC in mice immunized with PyMSP<sub>19</sub> formulated with CpG ODN and ISA720. Mice were immunized with PBS (A,C,E) or PyMSP<sub>19</sub> (B,D,F) formulated with CpG ODN and ISA720 by one injection at day 0, or by two injections at days 0 and 21, or by four injections at days 0, 21, 42, and 56. Twenty weeks after the last immunization of the four-injection protocol, mice were i.v. challenged with  $1 \times 10^4$  pRBC and parasitemia was monitored. Each symbol in each figure represents the parasitemia of an individual mouse. † = death.

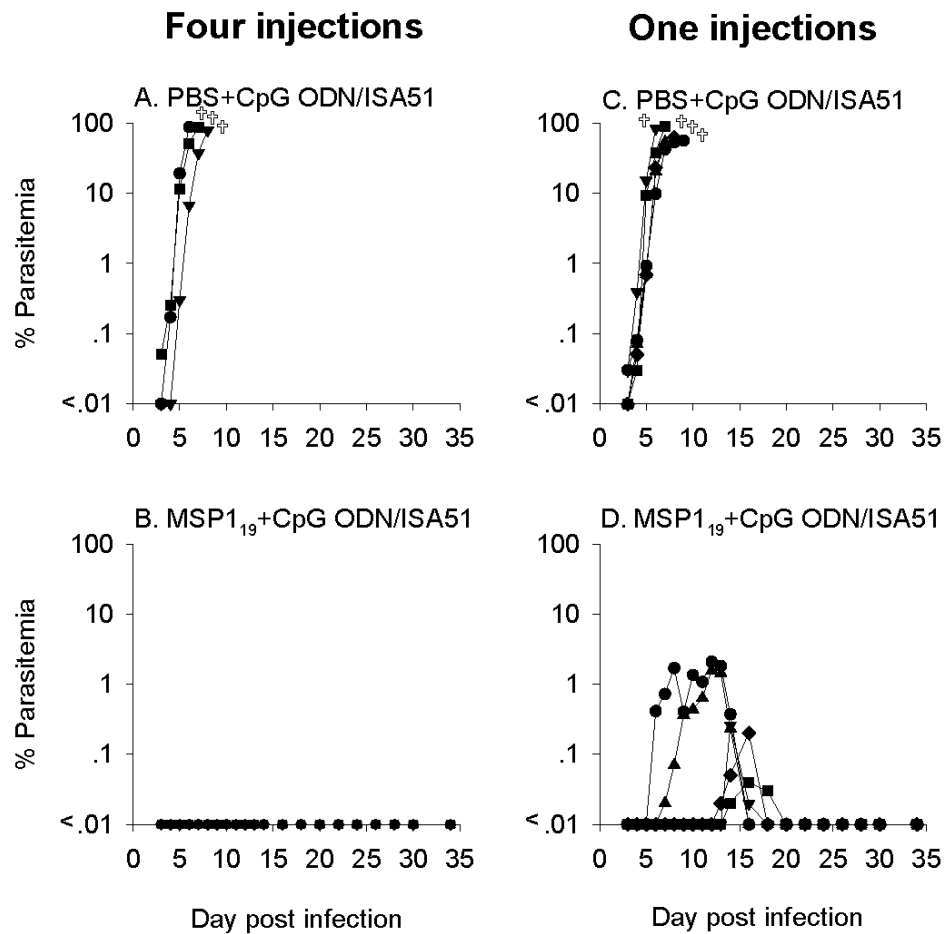
of 0.6 and 51% both occurred at day 12. All mice immunized by two or four injections with PyMSP<sub>19</sub>/CpG ODN/ISA720 survived the infection, with one mouse from each group experiencing about 1-2% parasitemia (Figure 3B., D., and F.).

In contrast, all mice immunized by one injection of PyMSP<sub>19</sub>/CpG ODN/ISA51 survived the subsequent challenge with the pRBC and experienced <math><2\%</math> parasitemia which was cleared by day 20 (Figure 4D.). The mice subjected to four injections of PyMSP<sub>19</sub>/CpG ODN/ISA51 survived infection without detectable parasitemia during 35 days of observation (Figure 4B.). However, all control mice immunized either by one or four injections with PBS/CpG ODN/ISA51 died within 8 days with high parasitemia (Figure 4A. and C.).

## Discussion

The efficacy of immune responses post vaccination with an antigen depends on its immunogenicity and the adjuvant used, as well as recipient factors, such as genetic background and immune status. Our previous studies have shown that MSP<sub>19</sub> is a vaccine candidate with great potential, since immunization of mice with a four-injection protocol of MSP<sub>19</sub> formulated with Freund's adjuvant induces complete protection against a lethal blood-stage infection<sup>4</sup>. The protection is associated with high MSP<sub>19</sub>-specific antibody titer just before the infection challenge, but is not related to the role of effector CD4<sup>+</sup> T cells. Passive transfer of the MSP<sub>19</sub> immune serum can control parasitemia and so the recipient mice survive





**Figure 4.** Parasitemia post challenge infection with *P.yoelii* YM-pRBC in mice immunized with PyMSP1<sub>19</sub> formulated with CpG ODN and ISA51. Mice were immunized with PBS (A and C) or PyMSP1<sub>19</sub> (B and D) formulated with CpG ODN and ISA51 by one injection at day 0, or by four injections at days 0, 21, 42, and 56. Twenty-two weeks after the last immunization of the four-injection protocol, mice were i.v. challenged with  $1 \times 10^4$  pRBC and parasitemia was monitored. Each symbol in each figure represents the parasitemia of an individual mouse. †= death.

infection post challenge by an active immune response<sup>11</sup>. In addition, our recent studies have shown that MSP1<sub>19</sub> in combination with CpG ODN and ISA51 or ISA720 confers complete protection and the degree of antibody response is about ten times higher than the combination of MSP1<sub>19</sub> and Freund's adjuvant<sup>18-19</sup>. This has led us to hypothesize that minimizing the number of immunizations to a single injection may be sufficient to induce the same protective immune response. Here we demonstrated that immunization with a single injection of MSP1<sub>19</sub> formulated with CpG ODN and ISA51 or ISA720 could induce protective immunity against a lethal *P. yoelii* infection, even though the latter adjuvant was less efficacious.

The kinetics of MSP1<sub>19</sub>-specific antibody responses following immunization by a single injection of MSP1<sub>19</sub> formulated with CpG ODN and ISA720 was shown to have the same profile (with the maximum point of antibody response being at day 42) as those of the immunizations by two or four injections of the same regimen, suggesting that the immune response by single-injection immunization can be continuously boosted. The emulsion of Montanide ISA, an oil-based agent, resulted in a gradual release of the vaccine. However, the MSP1<sub>19</sub>-specific antibody titers derived from a single-injection immunization were significantly lower than those from a two-injection immunization ( $P < 0.01$  as compared at day 42 since the initial injection), but were comparable to those

for four-injection immunization with MSP1<sub>19</sub> formulated with CpG ODN and ISA720. In this case, the findings suggest that multiple injections of the vaccine containing MSP1<sub>19</sub>, CpG ODN and ISA720 do not accelerate the boosting effect but provide a higher magnitude of antibody response. However, too many injections (e.g. four injections), may diminish the outcome.

In contrast to immunization with MSP1<sub>19</sub> formulated with CpG ODN and ISA720, a single-injection immunization with MSP1<sub>19</sub> formulated with CpG ODN and ISA51 conferred a more rapid and higher magnitude of MSP1<sub>19</sub>-specific antibody response. The antibody response reached its maximum level by day 28 post initial immunization, at which point the antibody titers were not significantly different from either those at day 63 of the same immunization, nor of those at day 70 of the four-injection immunization regime (Figure 2B.). In addition, after the challenge infection with *P. yoelii*, a single-injection immunization with MSP1<sub>19</sub> formulated with CpG ODN and ISA51 was more protective than the immunization with MSP1<sub>19</sub> formulated with CpG ODN and ISA720 (Figure 3B. and 4D.). This confirms our previous studies<sup>18</sup> that ISA51 is more efficacious than ISA720.

In comparing immunizations either by a single or by four injections with MSP1<sub>19</sub> formulated with CpG ODN and ISA720, the magnitude of total IgG antibody specific for MSP1<sub>19</sub> were not significantly different, but the IgG subclass responses were different. The single-injection immunization induced a significantly lower IgG2a, but not a different IgG1 antibody level than the four-injection immunization. This difference correlates with protection against *P. yoelii* challenge infection, in that the four-injection immunization was more protective (Figure 3B. and F.), and suggests that the IgG2a antibody could play a critical role in protection. When comparing immunization between by two and four injections of MSP1<sub>19</sub> formulated with CpG ODN and ISA720, the total IgG antibody response of the four-injection immunization was lower than that of the two-injection immunization, which was due to the lower IgG1, but not a lower IgG2a antibody level. However, in this case, the efficacy of protection against *P. yoelii* infection was similar, again suggesting of the role of IgG2a antibody. The role of the IgG2a antibody in protection against malaria infection has been demonstrated in our previous studies, when mice immunized with MSP1<sub>19</sub> formulated with CpG ODN

and ISA720 gave 15-fold titer of the MSP1<sub>19</sub>-specific IgG2a antibody but unchanged titers of the IgG1 antibody and were completely protected. However, mice immunized with MSP1<sub>19</sub> formulated with ISA720 succumbed to infection and died with high parasitemia post *P. yoelii* infection<sup>18</sup>. Further, Su et al.<sup>20</sup> showed IgG1-depleted immune sera, but not IgG2a depleted immune sera, could protect mice infected with *P. chabaudi*. Therefore, it is likely that the functional activity of IgG2a is superior to that of IgG1 but its mechanism is not clear. IgG2a can bind FcγR1, which then triggers the processes of antibody-dependent cell-mediated cellular cytotoxicity, antibody-dependent cellular inhibition, and phagocytosis, but the IgG2a antibody specific for MSP1<sub>19</sub> cannot use the Fc function for antibody-mediated protection<sup>21-22</sup>. It may act via a process of inhibition of merozoite invasion which is essential for erythrocyte entry<sup>23-24</sup>.

Our recent studies have shown that a four-injection immunization with MSP1<sub>19</sub> formulated with CpG ODN and ISA51 induces a long-lasting protective response, during which the IgG antibody titers do not change for over 12 months after immunization<sup>19</sup>. Even though in this study we did not follow up the antibody response in the long-term, as was the case of immunization with MSP1<sub>19</sub> formulated with CpG ODN and ISA51, we demonstrated that a single, two-injection, or four-injection immunization with MSP1<sub>19</sub> formulated with CpG ODN and ISA720 could also induce an antibody response of at least 20 weeks duration.

In summary, we have demonstrated that single-injection immunization with MSP1<sub>19</sub> formulated with CpG ODN in Montanide ISA, can provide protection against lethal malaria infection. A strong adjuvant like Montanide ISA 51 induces an earlier maximum antibody response. For more complete protection, boosting immunization is essential and this is achieved by effectively enhancing IgG2a, a cytotoxic antibody. This would pave the way for further studies in the development of a malaria vaccine.

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