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Vaccination with a *Plasmodium chabaudi adami* multivalent DNA vaccine cross protects A/J mice against challenge with *P. c. adami* DK and virulent *P. c. chabaudi* AS parasites

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Abstract

A current goal of malaria vaccine research is the development of vaccines that will cross-protect against multiple strains of malaria. In the present study, the breadth of cross-reactivity induced by a 30K multivalent DNA vaccine has been evaluated in susceptible A/J mice (H-2a) against infection with the *Plasmodium chabaudi adami* DK strain and a virulent parasite subspecies, *Plasmodium chabaudi chabaudi* AS. Immunized A/J mice were significantly protected against infection with both *P. c. adami* DK (31-40% reduction in cumulative parasitemia) and *P. c. chabaudi* AS parasites, where a 30-39% reduction in cumulative parasitemia as well as enhanced survival was observed. The 30K vaccine induced specific IFN-γ production by splenocytes in response to native antigens from both *P. c. chabaudi* AS and *P. c. adami* DK. Specific antibodies reacting with surface antigens expressed on *P.c. adami* DS and *P. c. chabaudi* AS infected red blood cells, and with opsonizing properties, were detected. These results suggest that multivalent vaccines encoding conserved antigens can feasibly induce immune cross-reactivity that span *Plasmodium* strains and subspecies and can protect hosts of distinct major histocompatibility complex haplotypes.

Keywords: Malaria; *Plasmodium chabaudi*; DNA vaccines; Multivalency; Cross-protection; Genetic restriction; Cellular immune responses; Opsonizing antibodies

1. Introduction

There is an urgent need for a vaccine against malaria which represents the most serious parasitic disease in Africa. Considerable effort has been invested in the identification of candidate blood-stage vaccine antigens and, although several clinical trials have been assessed, the results so far have been hampered by the antigenic diversity and variation observed in different parasite isolates (reviewed in Bull and Marsh, 2002; Genton et al., 2002; Plebanski et al., 2002; Doolan et al., 2003; Mahanty et al., 2003; Ballou et al., 2004; Moorthy et al., 2004). Numerous studies involving immunization with single candidate malaria blood-stage antigens report protection against challenge with homologous parasites (reviewed in Wipasa et al., 2002a; Mahanty et al., 2003; Moorthy et al., 2004; Smooker et al., 2004) but other studies have demonstrated that the protection elicited by the leading vaccine candidates Apical Membrane Antigen 1 (AMA1) and Merozoite Surface Antigen 1 (MSP1) is strain-specific. Vaccination of mice with the *Plasmodium chabaudi adami* AMA-1 sequence conferred protection against homologous infection with the *P. c. adami* DS strain but failed to protect against heterologous infection with the DK strain (Crewther et al., 1996; Anders et al., 1998). The *Plasmodium falciparum* MSP1 vaccine also shows strain-specific protection (Renia et al., 1997; Rotman et al., 1999). Such observations suggest that existing single vaccine candidate antigens may not be sufficient to protect against *Plasmodium* populations in the field due to allelic heterogeneity among antigens. The major challenge remains the identification of potentially cross-protective antigens and it is possible that novel protective antigens exist in the malaria genome that have not yet been evaluated (Doolan and Hoffman, 2001;

Gardner et al., 2002; Genton et al., 2002; Doolan et al., 2003; Haddad et al., 2004; Elliot et al., 2005; Scorza et al., 2005).

Support for this notion comes from two recent studies using a genomic approach to vaccine discovery that have identified novel candidates for liver or blood-stage vaccines (Haddad et al., 2004; Scorza et al., 2005). The protective efficacy of a *P. c. adami* DS genomic expression library has been demonstrated in challenge experiments with homologous and heterologous *P. c. adami* strains (Smooker et al., 2000; 2004; Rainczuk et al., 2003a; Scorza et al., 2005). We believe that two important properties of this vaccine, the multivalency (the vaccine comprises about 2,300-long open reading frames encoded in 30,000 genomic sequences) and the presence of conserved epitopes, are key elements for the vaccine-induced protection against infection with a heterologous parasite strain (Scorza et al., 2005). Such cross-protection has not been achieved when assessing single antigens such as MSP1 or AMA-1 (Renia et al., 1997; Rotman et al., 1999; Crewther et al., 1996) although Merozoite Surface Antigen 4/5 (MSP4/5) has been validated as a cross-protective vaccine (Goschnick et al., 2004).

Based on DNA sequence analysis of clones within the library, we hypothesized that epitopes within the library may be sufficiently conserved to induce cross-protective immune responses against other malaria subspecies in mouse strains of different major histocompatibility complex (MHC) haplotypes (Scorza et al., 2005). Indeed, the use of polytope vaccines increases the chances of circumventing the restriction imposed by the host genetic background and MHC haplotype. In the present study, we evaluated the cross-protective capacity of the *P. c. adami* genomic library (30K vaccine) against infection with *P. c. adami* DK and the distinct subspecies *Plasmodium chabaudi chabaudi* AS in A/J mice. The A/J mice/ *P. c. chabaudi* host-parasite system is a well

characterized, stringent model for susceptibility to malaria (Taylor-Robinson, 1995; Caroline and Langhorne, 1998; Mota et al., 2001; Stevenson et al, 2001; Langhorne et al., 2002). The cross-reactivity of cellular responses and the breadth of the opsonising antibody responses generated by the vaccine were assessed against antigens from *P. c. adami* DK and *P. c. chabaudi* AS parasites. Our results show that multivalent vaccination promotes a high degree of immune reactivity to these two different parasite sub-species and generates protective immune responses capable of overcoming host genetic restriction.

2. Materials and methods

2.1. Creation of plasmid pools and isolation of plasmid DNA

A genomic expression library from *P. c. adami* DS was produced in the DNA vaccine vector VR1020 as described previously (Smooker et al., 2000; Rainczuk et al., 2003a). The VR1020/30K genomic expression library contains approximately 30,000 cloned *P. c. adami* DS sequences and delivers approximately 2,300 open reading frames > 50 aminoacids in size (Scorza et al., 2005). Preparation of plasmid DNA from the 30K library and removal of endotoxin were performed as described (Scorza et al., 2005). DNA purified under endotoxin-free conditions was used for i.m. immunization.

*2.*2. Vaccination and infection of mice

Procedures for animal experiments and care of animals were approved by the Animal Care committees of the Macdonald Campus McGill University (protocol XXXX) and the University of Quebec in Montreal (protocol 0705-R1-508-0706).

The breadth of the cross-protection elicited by the VR1020/30K vaccine was assessed in a susceptible strain of mice of different MHC haplotype (A/J mice, H-2a MHC haplotype) against heterologous infection with both *P. c. adami* DK and the virulent subspecies *P. c. chabaudi* AS. The latter represents a stringent test of the vaccine since *P. c. chabaudi* AS parasites gives rise to virulent infections in A/J mice, resulting in high parasitemia (50% or more) and 80-100% lethality (Stevenson et al., 2001; Langhorne et al., 2002). An i.m. route of vaccination was used in A/J mice as the IED [define] vaccination route used previously in BALB/c mice (Rainczuk et al., 2003a; Scorza et al., 2005) did not induce significant levels of survival in A/J mice (data not shown). Female A/J mice 4-5 weeks old were purchased from Jackson Laboratories or were bred at the Animal Resources Centre (McGill University). A/J mice were vaccinated with DNA by the i.m. route. The mice received three doses of plasmid DNA into the *tibialis anterior* muscle (50 μl per muscle for a total of 100 μg DNA per dose) in physiological saline. Each dose was separated by a 2 week interval. Two weeks after the last DNA dose, the mice were infected i.p. with 5×10^4 *P. c. adami* DK or *P. c. chabaudi* AS infected red blood cells (IRBCs) obtained from syngeneic infected mice. Parasitemia was assessed from day 3-4 through the period of crisis until the resolution of infection by examination of Giemsa stained blood smears and was expressed as mean parasitemia. Cumulative parasitemia and mean peak parasitemia levels were compared using a non-parametric (Mann Whitney) two tailed *t*-test. Cumulative parasitemia represents the total parasitemia over the period of patent infection (Cravo et al., 2001; Scorza et al., 2005).

2.3. Analysis of survival curves

Survival curves for vaccinated and control mice were compared using the Mantel-Haenszel test. Statistical analysis was performed using Prizm 3.02 software (GraphPad, San Diego, CA, USA).

2.4. In vitro spleen cell culture and ELISA for IFN-γ

In order to evaluate cellular responses to native *Plasmodium* antigens, groups of A/J mice were vaccinated by the IM route with the VR1020 empty vector (four mice) or with the 30K vaccine (five mice). As a source of native malaria antigens, blood was provided from syngeneic mice infected with the homologous *P. c. adami* DS strain or the heterologous parasites *P. c. adami* DK and *P. c. chabaudi* AS. One batch of splenocytes was stimulated on the same day with each source of IRBC. .Mice were sacrificed 2 weeks after the last DNA dose and splenocyte cultures were performed as described by Scorza et al. (2005). Cell viability was greater than 98% as determined by trypan blue exclusion (Invitrogen). Splenocytes were plated at a final concentration of 5 x 10^6cells /ml in 24 well plates and were stimulated with 2 x 10^6 IRBC/ml, or non-infected RBCs, for 72 h. Cell culture supernatants were harvested and tested for the presence of IFN-γ by ELISA as described by Scorza et al. (2005). Statistical differences were estimated using a non-parametric analysis of variance (ANOVA).

2.5. Assessment of antibodies to antigens on IRBCs

To allow the recovery of a high percentage of IRBCs, parasitized blood from *P. c .adami* DS and *P .c. chabaudi* AS-infected A/J mice at a comparable parasitemia (40%)

was used for the analysis of antibody reactivity by flow cytometry. The reactivities of the sera from 30K-vaccinated and control mice were assessed both with live and fixed IRBCs. For live cells, 5µl of blood pellets were incubated individually with 50 µl of sera (1:10 dilution in PBS) from 30K-vaccinated or control mice (six mice per category) at 37ºC for 1 h, after which the cells were washed twice in PBS and treated with a rabbit anti-mouse-total IgG polyclonal antibody (labeled with Fluorescein isothiocyanate (FITC), 1:100 dilution) for 30 min at 37ºC. Live IRBCs were washed twice in PBS and directly analyzed by Fluorescence-activated cell sorting (FACS). IRBCs were fixed with 0.025% glutaraldehyde overnight at 4ºC, after which the cells were washed twice in PBS and incubated with sera as described above. Some cells were then permeabilized by treatment with 0.015% saponin in PBS at room temperature for 15 min. The cells were then washed and resuspended in 1 ml of PBS, and 1µl of propidium iodide (PI, 10µg/ml) was added. The percentages of fixed IRBCs reacting with mouse sera (FITC+) were determined by gating on PI-positive RBCs. Hyperimmune serum from mice that had been infected three times with *P .c. adami* DK was used as a positive control, using IRBCs that were fixed, permeabilized and stained with PI. Acquisition of at least 30,000 events was done using a FACScan (Becton Dickinson, USA).

2.6. Opsonization and phagocytosis assays

Opsonisation assays were performed essentially as described by Mota et al. (1998) and Scorza et al. (2005) using 3 x 10^6 peritoneal exudate cells per well. Adherent cells were incubated for 2 h with *P. c. adami* (DS and DK strains) IRBCs or *P. c. chabaudi* AS IRBCs (parasitemias up to 40% with the DS and AS virulent strains; 15-25% for the avirulent DK strain), previously incubated for 1 h with heat-inactivated

immune sera or sera from DNA vaccinated mice collected before parasite challenge (1:20 serum dilution). As controls, RBCs from naïve mice were used. The percentage of macrophages ingesting IRBCs and non-infected RBCs (300 macrophages per individual sample) was then quantified by light microscopy. Statistical analysis was carried out using a non-parametric (Mann-Whitney) two tailed *t* test.

3. Results

3.1. The 30K vaccine cross-protects A/J mice against heterologous challenge with P. c. adami DK and P. c. chabaudi AS parasites

Parasitemia was significantly lower in A/J mice vaccinated with the 30K vaccine by the i.m. route and challenged with *P. c. adami* DK parasites compared with control mice that received the empty VR1020 vector. In two independent experiments, a 31-40% decrease in mean cumulative parasitemia (total parasite burden during the days of patent infection, Cravo et al., 2001; Scorza et al., 2005) and a significant reduction (33-34%) in peak parasitemia was observed. Fig. 1 shows the results from one experiment in which vaccinated mice displayed a reduction in both cumulative parasitemia (40%, $P < 0.01$, Fig. 1B) as well as peak parasitemia (33%, $P < 0.05$, Fig. 1A, C).

The breadth of the protective efficacy of the 30K vaccine was further evaluated against challenge with virulent *P. c. chabaudi* AS parasites. Vaccination by the i.m. route conferred a reduction (39, 32 and 30%, respectively) in cumulative parasitemia in three independent experiments, of which the results of one experiment are shown (Fig. 2A, B; $P < 0.05$). A significant reduction in peak parasitemia was observed (Fig. 2C, $P < 0.01$). Increased survival of A/J mice challenged with *P. c. chabaudi* AS was observed in three experiments using i.m. vaccinated mice, with 4/5, 4/5 and 5/6 vaccinated mice surviving

infection relative to 1/5, 1/5 and 1/6 control mice, respectively. Fig. 2D shows the result of one experiment with statistically significant survival ($P < 0.05$). Overall, 13/16 vaccinates survived compared to 3/16 control mice. These results show that the multivalent *P. c. adami* DS 30K vaccine induces significant levels of cross-protection which extends to other subspecies of *P. chabaudi*.

3.2. Immune responses induced by i.m. administration of the 30K vaccine in A/J mice

Production of IFN-γ by splenocytes from vaccinated mice was observed upon stimulation with IRBCs from all three strains of parasite (Fig. 3). No IFN-γ was produced when splenocytes from control mice vaccinated with empty vector DNA were stimulated with IRBCs from any parasite strain or when splenocytes from vaccinated or control mice were left unstimulated (Fig. 3) or were stimulated with non-infected RBCs (data not shown). These results agree with those observed previously with splenocytes from BALB/c mice vaccinated intradermally (Scorza et al., 2005), and suggest that the *P. c. adami* DS vaccine-primed cells to respond to epitopes that are conserved among strains and subspecies of *P. chabaudi.*

The reactivity of the sera from 30K vaccinated A/J mice against parasite antigens expressed on the surface of *P.c. adami* DS and *P.c. chabaudi* AS IRBCs was assessed in live IRBCs. A significant percentage of RBCs infected with homologous *P. c. adami* DS (1.09 ± 0.27%) and heterologous *P. c. chabaudi* AS (0.7 ± 0.05%) parasites reacted with the 30K sera (Fig. 4A). Using glutaraldehyde-fixed IRBCs, the percentage of cells reacting with the 30K sera increased with both homologous *P. c. adami* DS (3.1 ± 0.14%) and heterologous *P. c. chabaudi* AS (1.59 ± 0.19%) (Fig. 4B). This increase in reactivity is most likely due to alteration of the cell membrane by fixation, allowing the entry of

antibodies and their reaction with intracellular antigens. The reactivity of the sera from 30K DNA vaccinated mice was also measured in fixed and permeabilized IRBCs, by gating on PI-positive RBCs (Fig. 4C, gate R1). A marker (M1) allowed the determination of the percentages of PI ¯positive RBCs (IRBCs) recognized by the sera from 30K vaccinated mice (Fig. 4D) The 30K vaccine sera reacted significantly both with homologous *P. c. adami* DS (6.63 ± 1.57%) and with heterologous *P. c. chabaudi* AS (4.05 ± 1.72%) IRBCs, relative to control naïve sera (Fig. 4E). Comparatively, sera from *P. c. adami* DK hyperimmune mice were assessed as positive cross-reaction controls, reacting with *P. c. adami* DS (10.5 ± 2.91%) and *P. c. chabaudi* AS (3.94 ± 0.63%) IRBCs, respectively (Fig. 4F).

In order to assess a functional role for these antibodies in vaccine-mediated cross-protection, the opsonizing capacity of the sera from mice vaccinated with the 30K library (n = 4 per group) was assessed using homologous *P. c. adami* DS IRBCs (Fig. 5A), heterologous *P. c. adami* DK IRBCs (Fig. 5B) or heterologous *P. c. chabaudi* AS IRBCs (Fig. 5C). Sera from 30K-vaccinated A/J mice opsonized *P. c. adami* DS and DK IRBCs as well as RBCs infected with *P. c. chabaudi* AS at a level similar to that observed with immune sera from mice that had recovered from a homologous infection (Fig. 5A-C, $P < 0.05$). Sera from control mice vaccinated with empty VR1020 vector DNA did not promote opsonisation (Fig. 5A-C).

4. Discussion

The key observation from this study is that the multivalent 30K DNA vaccine induces cross-protection in susceptible A/J mice against heterologous infections with a different parasite strain (*P. c. adami* DK) as well as a different virulent subspecies (*P. c. chabaudi* AS). Specific cross-reacting cellular and humoral immune responses (IFN-γ and opsonising antibodies) were generated in A/J mice immunized by the i.m. route with the 30K vaccine. These results show that a multivalent vaccine can prime T cells in mice of different MHC haplotypes to respond to epitopes that are conserved in the genomes of *P. chabaudi* subspecies and identification of these sequences could provide novel T cell epitopes for inducing strain-transcending T cell responses. Our observations are consistent with previous results showing enhanced protection in mice of three distinct MHC haplotypes following vaccination with a combination of two pre-erythrocytic antigens in a DNA vaccine formulation (Doolan et al., 1996). Thus, in the long-term, multivalency may address the problems of both inter-strain variation in the parasite and genetic heterogeneity in the human population (Doolan et al., 1996, 2003; Plebanski et al., 2002; Mahanty et al., 2003).

As antibody-dependent cellular inhibition (ADCI) is involved in protection against blood stage malaria in humans, vaccine-induced ADCI represents an important parameter to be considered in a malaria blood stage vaccine (Bouharoun-Tayoun et al., 1995; Shi et al., 1999a, b, 2001; Mahanty et al., 2003). As shown by FACS analysis with live cells, specific antibodies reacting with surface antigens on *P. c. adami* DS and *P. c. chabaudi* AS IRBCs were induced by the 30K vaccine, enhancing the phagocytosis of both homologous (*P. c. adami* DS) and heterologous (*P. c. adami* DK, *P. c. chabaudi* AS) IRBCs by macrophages. Such cross-reactivity is presumptively targeted to conserved

epitopes/antigen domains expressed on the IRBC surface, and has been previously observed within strains of *P. c. chabaudi* (Mota et al., 1998, 2001; Elliot et al., 2005).). These results extend data using BALB/c mice and the *P. c. adami* DS and DK strains (Rainczuk et al., 2003a; Scorza et al., 2005), and suggest the existence of antigenic cross-reactivity on the surface of IRBCs between *P. c. adami* DS and *P. c. chabaudi* AS parasites. Antibodies against intracellular antigens were also induced by the 30K vaccine, as enhanced reactivity was measured in glutaraldehyde-fixed IRBC. Sequences encoding intracellular antigens have been demonstrated in the vaccine (Scorza et al, 2005).

We hypothesize that the multivalent character of the 30K vaccine, as well as the high proportion of conserved orthologues within the 30K pool (Scorza et al., 2005), accounts for these responses. In an earlier study, sequence analysis of 38 plasmids in the 30K library identified seven orthologues with properties of exported proteins. These open reading frames exhibit a predicted signal sequence and transmembrane domain and amino acid sequence conservation (25-72%) with orthologues from *P. falciparum*: erythrocyte membrane-associated antigen, ubiquitin activating enzyme e11, subtilisin-like protease 2 and four hypothetical proteins (Scorza et al, 2005). The highest conservation (72%) was shown to a *P. falciparum* hypothetical protein PF14_0504. It is noteworthy that approximately 57% of the sequences >50 residues in length encoded by the 30K vaccine are conserved in three or more *Plasmodium* species, including *P. falciparum* (Scorza et al., 2005).

Interestingly, the i.m. delivery route elicited protection in A/J mice in contrast to earlier results in BALB/c mice, in which IED vaccination was more effective (Rainczuk et al., 2003a; Scorza et al., 2005), suggesting differences in responsiveness to DNA vaccines inherent to different mouse strains, as reported earlier (Kang et al., 1998; Doolan and Hoffman, 2000,, 2001). Several studies have shown that i.m. and IED delivery of DNA vaccines leads to different types of immune responses in mice, as well as differences in antibody titres (Kang et al., 1998; Rainczuk et al, 2003b).

The specific nature of the immune responses elicited by the 30K vaccine has been corroborated by three lines of evidence. Only splenocytes from mice vaccinated with the 30K vaccine respond to native antigens from IRBCs and opsonization is only measured with sera from the 30K vaccinated mice, as shown previously (Rainczuk et al., 2003a; Scorza et al., 2005). In addition, plasmids that contain malarial DNA inserts but do not encode a reading frame do not induce protection or IFN-γ secretion by splenocytes from primed mice (Scorza, et al., 2005). Finally, the 30K library when delivered in a different vector (CTLA4 vector) does not protect BALB/c mice against homologous infection (Rainczuk et al, 2003a).

Altogether, these observations are important in the context of human malaria where immunity is thought to result from antibody responses against a repertoire of polymorphic parasite antigens on the surface of infected red blood cells and merozoites, in addition to T cell responses to epitopes processed from parasite proteins (Bouharoun-Tayoun et al., 1995; Bull and Marsh, 2002; Doolan et al., 2003; Moorthy et al., 2004; Wipasa et al., 2002a, b). A multivalent vaccine is necessary to induce such responses in

the outbred human population exposed to multiple parasite genotypes (Shi et al., 1999b; Doolan and Hoffman, 2001; Genton et al., 2002; Plebanski et al., 2002; Cortes et al., 2003; Doolan et al., 2003). MSP4/5 has recently been validated as exhibiting a high level of cross-protective immunity in the *Plasmodium yoelii* mouse model (Goschnick et al., 2004). Although strain-transcending immunity against malaria has been demonstrated (Goschnick et al., 2004; Elliot et al., 2005; Scorza et al., 2005) it may be more difficult to apply this approach with *P. falciparum* due to the presence of the variable PfEMP1 sequences expressed on the infected human red blood cell surface (Kraemer and Smith, 2006).

Cross-immunity has been induced in humans and primates following exposure to different strains of *P. falciparum* (Jeffrey, 1966; Fandeur and Chalvet, 1998; Bull and Marsh, 2002; McConkey et al., 2003). It is also known that certain antigens of *P. falciparum* cross-protect mice against murine malaria infections (Saul et al., 1992; Chauhan et al., 1993; Lord et al., 1993; Lougovskoi et al., 1999; Chatterjee et al., 2000; Brahimi et al., 2001; Sauzet et al., 2001; Makobongo et al., 2003; Xu et al., 2007) and antigenic cross-reactivity at the IRBC surface has been observed among malaria species (Sulzer et al., 1988; Tan et al., 1996; Mota et al., 1998, 2001; Elliot et al., 2005). Importantly, functional conservation of domains in malaria proteins during speciation has been reported (O'Donnell et al., 2000; Triglia et al., 2000; Healer et al., 2005). Further definition and evaluation of strain-transcending conserved antigens within a multivalent vaccine may reveal hitherto unrecognised novel epitopes that could be engineered for delivery as a component of a polyepitope vaccine (Hanke et al., 1998; Shi et al., 1999b; Doolan and Hoffman, 2001; Jones et al., 2002; Doolan et al., 2003; McConkey et al.,

2003). These antigens could synergise with allelic heterogeneous lead candidates such as MSP1 and AMA1, providing a broader coverage against diverse malaria strains (Shi et al., 1999b; Jones et al., 2002; Cortes et al., 2003). Such a vaccine could be delivered using bicistronic DNA vaccine vectors (Rainczuk et al., 2004) in a prime-boost approach (Plebanski et al., 2002; Wipasa et al., 2002a; Dunachie and Hill, 2003; McConkey et al., 2003; Smooker et al., 2004).

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Legends to figures

Fig. 1. Efficacy of the *Plasmodium chabaudi adami* DS VR1020 30K vaccine delivered i.m. in A/J mice against *P. c. adami* DK challenge. Results from a representative challenge experiment of A/J mice (n = 6) are shown. A) Kinetics of infection; B) cumulative parasitemia values representing the sum of daily parasitemia throughout the period of patent infection per individual mouse in each group; C) the relative peak parasitemia in control and vaccinated mice. Statistical differences were estimated using a non-parametric (Mann Whitney) two-tailed *t* test. $^{**}P < 0.01$, $^{*}P < 0.05$.

Fig. 2. Efficacy of the *Plasmodium chabaudi adami* DS VR1020 30K vaccine delivered i.m. in A/J mice against virulent *Plasmodium chabaudi chabaudi* AS challenge. Results from a representative challenge experiment with A/J mice (n = 5) are shown. A) Kinetics of infection; B) cumulative parasitemia; C) peak parasitemia; D) survival of mice. Cumulative parasitemia values, calculated only during the infection period preceding the death of any of the mice, represent the sum of daily parasitemia throughout the period of patent infection per individual mouse in each group. Statistical differences (B, C) were estimated using a non-parametric (Mann Whitney) two-tailed *t* test. Statistical significances between survival curves were determined using the Mantel-Haenszel (or Logrank) test. $^{**}P < 0.01$, $^{*}P < 0.05$.

Fig. 3: IFN-γ secretion by splenocytes from A/J mice vaccinated by the i.m. route with the VR1020 *Plasmodium chabaudi.adami* DS 30K vaccine. Control values represent data from splenocyte cultures harvested from mice that received the VR1020 empty vector. As

a further control, splenocytes from control and 30K vaccinated mice were also left unstimulated (Unst.). IFN-γ was measured by ELISA and responses were compared between control splenocytes and splenocytes from 30K-vaccinated mice stimulated with the same source of parasite antigen. Statistical differences were estimated using a non-parametric ANOVA. AS infected red blood cell (AS IRBC), $P = 0.06$; *$P < 0.05$, **$P < 0.01$.

Fig. 4. Reactivity of 30K sera with parasite antigens expressed on *Plasmodium chabaudi adami* DS and *Plasmodium chabaudi chabaudi* AS infected red blood cells (IRBCs). Live (A) and glutaraldehyde-fixed (B) *P. c. adami* DS and *P. c. chabaudi* AS IRBC were incubated with sera from 30K DNA vaccinated mice or from control mice (six mice per group), and the antigen-antibody reactions were developed with a FITC -labeled rabbit anti-mouse IgG-polyclonal antibody. In addition, permeabilisation with saponin allowed gating on fixed propidium iodide $(PI)^+$ IRBC (R1, C) (Y axis: side light scatter, X axis: forward light scatter) and definition of a marker region (M1) to determine the percentage of $FITC^+$ IRBC (D). The histogram (D) shows the cell number (Y-axis) versus fluorescence intensity (X-axis): the dotted and full lines represent the reactivity of control and 30K sera, respectively. The percentages of *P.c. adami* and *P.c. chabaudi* AS IRBC reacting with 30K and control sera (E) or with sera from *P.c. adami* DK hyperimmune (HI) and naïve mice (F) were determined and compared using a one way ANOVA. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$.

Fig. 5. Phagocytosis of *Plasmodium* infected red blood cell (IRBC) pre-incubated with heat-inactivated sera. Immune pool sera were obtained from mice recovered from a

homologous infection; 30K sera were from mice immunized with the *Plasmodium chabaudi adami* DS VR1020/30K vaccine; control sera were from mice that received the empty VR1020 plasmid DNA. A) *P. c. adami* DS; B) *P. c. adami* DK; C) *Plasmodium chabaudi chabaudi* AS. Statistical differences were estimated using a non-parametric (Mann Whitney) two-tailed *t* test. *$P < 0.05$.