1 Synthetic TLR4 agonists enhance functional antibodies and CD4+ T-cell responses against the
2 Plasmodium falciparum GMZ2.6C multi-stage vaccine antigen

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22 **Abstract**

23 A subunit vaccine targeting both transmission and pathogenic asexual blood stages of *Plasmodium*
24 *falciparum*, i.e., a multi-stage vaccine, could be a powerful tool to combat malaria. Here, we report
25 production and characterization of the recombinant protein GMZ2.6C, which contains a fragment
26 of the sexual-stage protein *Pfs48/45-6C* genetically fused to GMZ2, an asexual vaccine antigen in
27 advanced clinical development. To select the most suitable vaccine formulation for downstream
28 clinical studies, GMZ2.6C was tested with various immune modulators in different adjuvant
29 formulations (stable emulsions, liposomes, and alum) in C57BL/6 mice. Some, but not all,
30 formulations containing either the synthetic TLR4 agonist GLA or SLA elicited the highest parasite-
31 specific antibody titers, the greatest IFN- γ responses in CD4⁺ T_H1 cells, and the highest percentage
32 of multifunctional CD4⁺ T cells expressing IFN- γ and TNF in response to GMZ2.6C. Both of these
33 agonists have good safety records in humans.

34

35 *Keywords:* *Plasmodium falciparum*; GMZ2; *Pfs48/45*; Transmission Blocking; CD4 T-helper cells;
36 GLA; SLA.

37 **1. Introduction**

38 Malaria is one of the world's greatest public health problems [1]. Since efforts to eliminate malaria
39 transmission through use of insecticides and / or bednets have only been a limited success [2], an
40 effective malaria vaccine is highly desirable for control and elimination of the disease burden.
41 Multiple stages of the *Plasmodium falciparum* malaria parasite life cycle can be targeted by
42 humoral immunity (antibodies), including the asexual blood-stage and the transmission stages. If
43 successful, a subunit vaccine targeting these stages may concomitantly prevent disease and reduce
44 the spread of the parasite in the population [3]. We have previously generated several multi-stage
45 hybrid proteins composed of the N-terminal portion of the *P. falciparum* asexual blood-stage
46 antigen Glutamate Rich Protein (GLURP) genetically fused to C-terminal fragments of the sexual
47 stage protein *Pfs48/45* [3, 4]. Here, we generated a chimera (GMZ2.6C) between the GMZ2
48 malaria vaccine candidate, which has advanced to phase 2 clinical trials [5-7], and the *Pfs48/45*-6C
49 fragment. The GMZ2 component is itself a hybrid protein [8] consisting of conserved domains of
50 the two *P. falciparum* asexual blood-stage antigens GLURP and Merozoite Surface Protein 3
51 (MSP3) [9, 10]. The rationale for combining these two asexual-stage antigens was based on a series
52 of immune epidemiological studies [10-12] and functional *in vitro* studies [13-15]. Thus,
53 vaccination with GMZ2 aims to emulate the benefits of naturally acquired immunity against
54 malaria through increasing the magnitude and quality of this response.
55 The *Pfs48/45* antigen is expressed during the sexual differentiation of the parasite into
56 gametocytes [16] and plays a key role in parasite fertilization [17]. Once inside the mosquito
57 midgut, the parasite develops into an extra-erythrocytic gamete, exposing *Pfs48/45* on its surface
58 where the molecule may be targeted by specific antibodies [16]. Naturally acquired anti-*Pfs48/45*
59 antibodies are present in endemic populations, and the occurrence of these antibodies is

60 associated with transmission-blocking (TB)-activity when tested in the standard membrane
61 feeding assay (SMFA) [18-21].

62 In the classical approach to subunit vaccination, the protein antigen is combined with one or more
63 immune modulatory molecule(s) selected to induce the desired immune responses. However, this
64 approach is somewhat constrained by the difficulties associated with identifying potent adjuvant
65 formulations suitable for human use [22]. For the GMZ2.6C fusion protein an adjuvant needs to
66 promote and induce sustainable high levels of specific antibodies, which simultaneously inhibit
67 asexual blood-stage multiplication and parasite fertilization in the infected mosquito. In addition
68 to IgG antibodies, cellular immune responses may also play important roles not only for producing
69 a robust antibody response through the help of T_H1 and T_H2 cells, but also through CD4⁺ effector
70 functions [23]. In this model T_H1 effector memory T cells producing IFN- γ and TNF were positively
71 correlated with protection against asexual blood-stage multiplication [23].

72 The objective of this study was to select the most potent adjuvant/GMZ2.6C combination for
73 induction of high levels of functional antibodies and CD4⁺ T helper cell responses in mice in
74 preparation for further downstream development. Adjuvants containing the synthetic TLR4
75 agonist glucopyranosyl lipid adjuvant (GLA) or IDRI's synthetic TLR4 agonist second-generation
76 lipid adjuvant (SLA) elicited the strongest antibody and CD4⁺ T_H1 cell responses. The GLA and SLA
77 agonists have been assessed in human clinical trials as a part of vaccines against several infectious
78 diseases, and GLA specifically has been included in malaria vaccine trials (e.g., NCT01540474).

79

80 **2. Materials and Methods**

81 *2.1 Bacterial strains, plasmids, and purification of recombinant GMZ2.6C*

82 *Escherichia coli* TOP10 (Invitrogen, Denmark) and *L. lactis* strain MG1363 were cultured as
83 previously described [8]. To facilitate the construction of GMZ2.6C, a synthetic DNA fragment
84 (GeneArt Germany) containing the 3D7 msp3₄₆₂₋₇₄₇ fragment was digested with *Bam*HI and *Bg*II
85 and cloned into *Bg*II-digested pSS2 [4] yielding pSS3. The *Pfs*48/45₈₅₉₋₁₂₈₄ fragment (6C) was
86 amplified from pLEA5 using the primers 5'-CCA TGG ATC CGA AAA AAA AGT CAT ACA CGG ATG
87 TAA CTT C-3' and 5'-CCA TAG ATC TTG CTG AAT CTA TAG TAA CTG TCA TAT AAG C-3'. The purified
88 amplicon was digested with *Bam*HI and *Bg*II (underlined) and inserted into *Bg*II-digested pSS3
89 giving rise to pSS4.

90 *L. lactis* MG1363 containing pSS4 was cultured in LAB medium supplemented 5mM cysteamine /
91 0.5mM cystamine as previously described [4]. Culture-filtrates were concentrated 10-fold and
92 buffer-exchanged to phosphate-buffered saline (PBS), pH 7.4 containing 10 mM imidazole on a
93 QuixStand Benchtop system (GE Healthcare, Sweden). The recombinant protein was first purified
94 on a 5-ml HisTrapTMHP column (GE Healthcare, Sweden) followed by a 5-ml HiTrap NHS-activated
95 HP column containing monoclonal antibody (mAb) 45.1 (epitope I) as previously described [3].

96

97 2.2. Immunizations

98 Female C57BL/6 mice, 5-7 weeks of age, were purchased from Charles River Laboratories
99 (Wilmington, MA) and were housed in the Infectious Disease Research Institute (Seattle, WA)
100 animal care facility under specific pathogen-free conditions. All animals were treated in
101 accordance with the regulations and guidelines of the Infectious Disease Research Institute Animal
102 Care and Use Committee. Ten mice per group were immunized three times, 3 weeks apart with 5
103 µg of GMZ2.6C per injection. This dose was chosen because it was suboptimal for induction of
104 humoral immunity in rats [4]. Animals were injected intramuscularly (i.m.), 50 µl per thigh muscle

105 (100 µl total volume), with GMZ2.6C plus several different adjuvants including: stable emulsion
106 (SE) at 2% final oil; GLA in SE (GLA-SE with 5 µg GLA); alum with 5 µg GLA (GLA-alum) prepared and
107 mixed with protein as previously described [24]; GLA-SE formulated with 10 µg of the saponin
108 QS21 (GLA-SEQ); neutral liposomes (LS) consisted of a phosphatidylcholine-based composition
109 manufactured using a process similar to previously reported liposomes [24]; a liposomal
110 formulation containing 10 µg QS21 (LSQ); GLA (5 µg) formulated in liposomes (GLA-LS); GLA (5 µg)
111 formulated in liposomes containing QS21 (GLA-LSQ); SLA (second-generation lipid adjuvant, 5 µg)
112 formulated in liposomes (SLA-LS); SLA (5 µg) in a liposomal QS21 formulation (SLA-LSQ); and alum
113 alone.

114 Rats (Wistar, Hannover) were housed in the University of Copenhagen animal care facility with
115 two rats per cage and were immunized with 20 µg of either GMZ2 or immune-purified GMZ2.6C
116 adjuvanted in alum in a volume of 200 µl administered subcutaneously three times at two-week
117 intervals. The rats were bled on days 0, 14, 28 and 43. IgG was purified as previously described
118 [25].

119

120 2.3. Flow cytometry

121 Splenocytes from 5 mice per group were isolated 1 week after the final immunization for
122 evaluation of effector T-cell responses and 4 weeks after the final immunization for memory T-cell
123 responses. Cells were plated at 2×10^6 cells/well in a 96-well U-bottom plate and re-stimulated
124 with either media (negative control) or "unfolded" RO-MSP3-6C (0.9 µg/ml). After 2 h GolgiPlug
125 (BD Biosciences, San Jose, CA) was added (1 µg/ml) and incubated with cells for 8 h at 37°C. Cells
126 were then surface stained with fluorochrome-conjugated mAbs to CD4 (clone RM4-5), CD8 (clone
127 53-6.7), and CD44 (clone IM7) (BioLegend and eBioscience) in the presence of anti-CD16/32 (clone

128 2.4G2) for fifteen minutes. Cells were fixed and permeabilized for 20 minutes at room
129 temperature using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's
130 instructions and stained intracellularly for 15 minutes at room temperature with fluorochrome-
131 conjugated mAbs to IFN- γ (Clone XMG1.2, Affymetrix, SanDiego, CA) and TNF (clone MP6-XT22, BD
132 Biosciences, San Jose, CA) . Cells were washed twice with BD Perm/Wash buffer 1X (BD
133 Biosciences) and re-suspended in PBS with 1% BSA prior to collection on a four-laser LSR Fortessa
134 flow cytometer (BD Biosciences) and analysis using FlowJo software (Tree Star). Lymphocytes were
135 gated by forward and side scatter, and 20,000 CD4+ events were acquired for each sample.
136 Analysis and presentation of distributions were performed using SPICE version 5.3 downloaded
137 from the National Institutes of Health site (<http://exon.niaid.nih.gov/spice>).

138

139 2.4. ELISpots

140 ELISpots were performed on splenocytes from immunized mice one and four weeks after the third
141 immunization (IL-5 and IFN- γ). Mice (n=5 mice/group) were euthanized at each timepoint, and
142 spleens were harvested. A MultiScreen 96-well filtration plate (Millipore, Bedford, MA) was coated
143 with 10 μ g/ml rat anti-mouse IL-5 or IFN- γ capture antibodies (eBioscience) and incubated
144 overnight at 4°C. Plates were washed with PBS, blocked with RPMI 1640 and 10% FBS for at least 1
145 hour at RT, and washed again. Splenocytes were plated in duplicate at 2×10^5 cells/well and
146 stimulated with media or GMZ2.6C (10 μ g/ml) for 48 hrs at 37°C. The plates were washed with
147 0.1% PBS-Tween 20 and incubated overnight with a biotin-conjugated rat anti-mouse IL-5 or IFN- γ
148 secondary antibody (eBioscience) diluted 1:250 in 0.1% PBS-Tween 20/0.5 % BSA. The filters were
149 developed using the VectaStain ABC avidin-peroxidase conjugate and Vectastain AEC substrate kits
150 (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. The reaction was

151 stopped by washing the plates with deionized water. Plates were dried in the dark, and spots were
152 counted on an automated ELISPOT reader (C.T.L. Seri3A Analyzer; Cellular Technology, Cleveland,
153 OH) and analyzed with Immunospot software (CTL Analyzer).

154

155 2.5. Antibody Assays

156 Enzyme-linked immunosorbent assays (ELISA) were performed with cultured asexual and sexual
157 stage of *P. falciparum* NF54 parasites as previously described [3]. Secondary antibodies were goat
158 anti-mouse IgG1-HRP, goat anti-mouse IgG2c-HRP, and total IgG-HRP (Thermo Scientific Pierce)
159 diluted 1:10,000. Pooled serum from R0.10C (adjuvanted with GLA-SE) immunized mice was used
160 as a positive control. Midpoint (EC₅₀) values were calculated using GraphPad Prism (GraphPad
161 Software, USA).

162 The Standard Membrane Feeding Assay (SMFA) was performed with *P. falciparum* NF54 as
163 previously described [3]. Rat and mouse antisera were not heat inactivated prior to analysis, and
164 no additional complement components were added to the SMFA. Estimates of transmission
165 reducing activity (TRA) were generated using oocyst densities in individual mosquitoes, comparing
166 test feeders with control feeders from the same experiment as described [26]. Generalized linear
167 mixed models (GLMMs), assuming a negative binomial distribution of oocyst data, were used to
168 estimate TRA with 95% confidence intervals. Analyses were performed using R (v. 3.0.2, The R
169 Foundation, Boston, USA).

170 The Antibody-Dependent Cellular Inhibition Assay was performed as described [25, 27] using
171 purified IgG (Day 43) from individual rats immunized with either GMZ2.6C (n=6) or GMZ2 (n=6). In
172 brief, Danish blood donor peripheral blood mononuclear cells (MNs) were isolated by LymphoPrep
173 (Lonza), and approximately 2×10^5 MNs/well were selected by adherence in a flat-bottom 96-well

174 culture plate (Nunc, Denmark). Highly synchronized schizont-stage *P. falciparum* NF54 at 0.5%
175 parasitemia and 2.5% hematocrit were added at 100 μ L/ well followed by test and control IgG at
176 0.5 mg/mL and 1.0 mg/mL final concentrations, respectively, to designated duplicate wells.
177 Volume was adjusted to 200 μ L with parasite growth medium (PGM) (RPMI 1640 [Lonza] + 0.5%
178 Albumax) [25]. An additional 50 μ L PGM was added per well at 48 hours and 72 hours, and the
179 assay was stopped after 96 hours. Final parasitemia was determined as described previously [27],
180 and a specific growth inhibitory index (SGI) was calculated: $SGI = 100 \times (1 - [\% \text{ parasitemia with}$
181 $MN \text{ and test antibodies} / \% \text{ parasitemia test antibodies}] / [\% \text{ parasitemia with MN and PNIG} / \%$
182 $\text{parasitemia PNIG}])$. PNIG is a pool of normal IgG from Danish blood-donors never exposed to
183 malaria.

184

185 2.6. Statistical Analysis

186 Statistical analyses were performed using GraphPad Prism software (La Jolla, CA). Standard one-
187 way ANOVA, followed by a Bonferroni multiple comparison test was used for analysis of antibody
188 titers or as indicated in the Figure Legends. P values ≤ 0.05 were considered significant.

189

190 3. Results

191 3.1. Expression of a GM22.6C fusion protein in *L. lactis*

192 A multi-stage multi-component construct was created from *glurp*₇₉₋₁₅₀₀ and *msp3*₄₆₉₋₇₇₄ fragments
193 genetically fused to the *Pfs48/45*₈₇₁₋₁₂₈₄ region (6C), and the fusion protein (GM22.6C) was
194 expressed in *L. lactis* MG1363 (Fig. 1A). The protein was affinity-purified from the supernatant of
195 the *L. lactis* culture on a HisTrap HP column (Fig. 1B, lanes 1&2) followed by immune-purification
196 on mAb45.1 coupled to agarose (Fig. 1B, lanes 3&4). Immune-purified GM22.6C showed a single

band, which reacted strongly with conformation-dependent mAb45.1 in the absence, but not in the presence of DDT (Fig. 1B), confirming the importance of disulfide bridges for the folding of epitope 1 of *Pfs48/45*. In order to determine whether the GMZ2.6C chimera elicits an adequate immune response against the individual GMZ2 and *Pfs48/45* components, a group of 10 rats were immunized 3 times with 20 µg of GMZ2.6C in alum. For comparison a group of 6 rats received GMZ2/alum. Two weeks after the last injection (Day 43), all rats had relatively high antibody titers against both asexual- and sexual-stage parasites with median titers of 1/657 (range 1/228- 1/912) and 1/1516 (1/363- 1/5781), respectively (Fig. 1C). TB activity of anti-GMZ2.6C antisera was assessed in the SMFA. The pre-bleed (Day 0) of rats did not show any TB activity and was used to calculate the percent inhibition of oocyst counts. Five out of 10 rats inhibited oocyst development >70% (Fig. 1D).

Functional activity against asexual blood-stage parasites was tested in the ADCI assay [25, 27]. Purified IgG (Day 43) from GMZ2.6C-immunized rats showed relatively high levels of growth inhibition as measured by the specific growth index (SGI) (Fig. 1E). The level of SGI was similar to that obtained with IgG from the GMZ2-immunized controls (Fig. 1E). Interestingly, there was a clear relationship between levels of parasite-specific antibodies by IFA and SGI (Fig. 1F).

213

214 3.2. Humoral immune responses

215 A large and durable antibody response to the component antigens of GMZ2.6C is thought to be
216 critical for protection against clinical disease and the reduction of transmission. In order to identify
217 a potent adjuvant formulation suitable for human use, we have screened eleven adjuvants in
218 combination with GMZ2.6C (Supplementary Table 1). The formulations either have previously
219 been in humans or have that potential and consist of stable oil-in-water emulsion (SE), liposomal,

220 and alum platforms to which the immune modulators GLA, QS21, and/or SLA were added. Inbred
221 mice were immunized three times with 5µg of GMZ2.6C combined with one of these adjuvants.
222 We used a suboptimal vaccine dose to enable detection of even subtle adjuvant effects. The
223 humoral immune response to the component antigens was investigated one week after the third
224 immunization using a schizont extract for detection of asexual blood-stage antibodies and a
225 gametocyte extract to detect antibodies to the sexual stage. In general, GLA enhanced anti-
226 asexual-stage antibody responses when added to any of the three formulation platforms (Fig. 2A).
227 For SE-based formulations, this increase was only statistically significant when the platform was
228 further supplemented with QS21 (GLA-SEQ) (Fig. 2A). The impact of SLA was investigated using
229 liposomal (LS) formulations only. The addition of SLA resulted in statistically significant asexual-
230 stage IgG titers and, while sexual-stage IgG titers were enhanced with SLA, they were not
231 significant. The combination of SLA and QS21 (SLA-LSQ), however, increased antibody responses
232 against both components of GMZ2.6C (Fig. 2A & 2B). Notably, the SLA-LSQ formulation gave rise to
233 the highest IgG2c antibody titer of all the formulations tested (Fig. 2C). No significant differences
234 were observed with IgG1 antibody responses to gametocytes in any of the adjuvanted groups
235 compared to controls (Fig. 2D). The durability of the circulating antibody responses was assessed
236 by examining antibody levels four weeks after the third immunization. Not surprisingly, antibodies
237 specific for the asexual and sexual antigens declined between weeks one and four for most of the
238 adjuvants tested; the anti-sexual-stage antibodies produced with either alum formulation were
239 exceptions to this (Supplementary Fig. 1).

240 Anti-GMZ2.6C antisera were also tested for functional activity against sexual-stage parasites in the
241 SMFA. The strongest TB activity one week after the third immunization was seen for the GLA-SE
242 and SLA-LSQ formulations (Fig. 3A). The highest, and thus most durable, responses at four weeks

243 were to GLA- and SLA-LSQ, while the response to GLA-SE declined somewhat at this point. There
244 was a strong correlation between SMFA activity and antibodies against sexual stage parasites as
245 measured either by ELISA using a gametocyte extract (Fig. 3B, Spearman rank $R^2 = 0.7233$) or by a
246 suspension IFA (SIFA; Fig. 3C, Spearman rank $R^2 = 0.6228$). Due to limiting amounts of mouse sera
247 available, we were unable to quantify the biological activity of the antisera in ADCl.

248

249 *3.3. Cellular immune responses to GMZ2.6C are broad and strong*

250 In order to further down-select adjuvants for use in humans, the number of splenocytes secreting
251 IFN- γ or IL-5 in response to stimulation by antigen *ex vivo* was assessed one or four weeks after
252 the 3rd immunization. At both time points, the highest numbers of cells secreting IFN- γ were
253 detected in mice immunized with GMZ2.6C plus either of five adjuvant formulations: GLA-SE, GLA-
254 SEQ, LSQ, GLA-LSQ, or SLA-LSQ (Fig. 4A and 4C). The frequency of cells secreting IFN- γ from the
255 antigen + GLA-Alum group was elevated at one week following the last boost with GLA-Alum, but
256 was greatly diminished at four weeks (Fig. 4A & 4C). SE was the only adjuvant that enhanced the
257 number of IL-5-secreting splenocytes at both one and four weeks (Fig. 4B & 4D).

258 To determine whether CD4⁺ T-cells were responsible for some of this IFN- γ secretion by
259 splenocytes and to assess the phenotype of the T cells, intracellular cytokine staining (ICS) was
260 performed after GMZ2.6C stimulation both one and four weeks following the third immunization
261 (Fig. 5A). The results in Figures 5B & 5D show that the same five adjuvant formulations, when
262 used in combination with GMZ2.6C, had elevated frequencies of antigen-specific IFN- γ -producing
263 cells. ICS analysis showed these same five adjuvanted vaccines also induced TNF-producing CD4⁺ T
264 cells at both time points (Fig. 5B & 5D). GLA-Alum also induced a small increase in antigen-specific
265 IFN- γ and TNF-secreting CD4⁺ T cells after one week (Fig. 5B), but the observed recall response

266 was not maintained at the four-week time point (Fig. 5D). No significant CD8+ T-cell responses
267 were observed (data not shown).

268

269 3.4. GMZ2.6C plus adjuvant combinations elicit multifunctional CD4+ T-cell responses

270 T cells that produce more than one cytokine may provide a more effective immune response than
271 T cells producing a single cytokine [28-30]. We analyzed the expression of IFN- γ and TNF in CD4+ T
272 cells from mice immunized with GMZ2.6C plus one of eleven different adjuvant formulations (Fig.
273 5C, 5E). Mice receiving GMZ2.6C with five of these adjuvants (GLA-SE, GLA-SEQ, LSQ, GLA-LSQ, and
274 SLA-LSQ) had an enhanced percentage of antigen-specific CD44+ (activated) T cells that secreted
275 both IFN- γ and TNF when analyzed one week after the third immunization (Fig. 5C). Memory recall
276 responses were also observed four weeks after the last immunization (Fig. 5E). GLA-Alum had a
277 small, but significant percentage of CD4+ T cells with this double-positive phenotype at one week
278 after the last immunization; however, the frequency of these cells remained quite low when
279 compared with the other five noted formulations (Fig. 5C). These double-positive cytokine-
280 secreting CD4+ T cell responses in the GMZ2.6C + Alum group were not observed at four weeks
281 (Fig. 5E).

282

283 4. Discussion

284 GMZ2 is a recombinant subunit vaccine candidate that targets the asexual blood-stage of *P.*
285 *falciparum* and is currently in advanced clinical development [5-7, 31]. Here we report the design,
286 expression, and testing of the antigen GMZ2.6C containing GMZ2 fused with the 6C portion of
287 *Pfs48/45* containing a major epitope for TB antibodies. When tested in rats, a GMZ2.6C – alum
288 formulation elicited specific antibodies with the capacity to inhibit parasite fertilization in the

289 SMFA and reduce asexual parasite growth in the ADCI assay. Thus, highly immunogenic
290 formulations of GMZ2.6C promoting increased levels of bi-functional antibodies may help to
291 control both clinical disease and transmission of the parasite under natural exposure.

292 Adjuvants are proving to be a vital element of an efficacious subunit vaccine, enhancing vaccine
293 antigen immunogenicity and host immune responses. In the case of a vaccine antigen targeting
294 both disease (asexual stages) and transmission (sexual stages), it is important that the adjuvant
295 boost responses to both types of target antigens and thus thorough adjuvant evaluation is
296 important. Beginning this evaluation process only with those adjuvants having a human clinical
297 development path, including a record of both safety and manufacturability, is critical.

298 Here, we have investigated the effects of eleven different adjuvant formulations with human
299 clinical potential on the humoral and cell-mediated immune responses generated by our multi-
300 stage malaria vaccine candidate GMZ2.6C. With the goal of developing robust responses to the
301 antigen components of GMZ2.6C, several immunomodulatory molecules, all with good safety
302 records in people, were evaluated in these eleven formulations: synthetic TLR4 agonists (GLA and
303 SLA), QS21, squalene, and alum. Liposomes, comprised of the neutral phospholipids used here, are
304 generally considered to be inert vehicles useful for delivery of insoluble TLR agonists and
305 otherwise hemolytic saponins. While squalene emulsions and alum serve as delivery vehicles for
306 an immunomodulatory molecule, they also have their own adjuvant activity. Whereas the
307 receptor and signaling pathways for TLR4 ligands [22] are generally well-established [32], the
308 identities of potential molecular receptors for QS21, squalene, and alum are less clear.

309 Nevertheless, it has been postulated that the mechanisms of action of squalene emulsions,
310 saponins such as QS21, and alum are related to the production of danger-type signals (danger-
311 associated molecular patterns [DAMPs]) [22, 33, 34]. In contrast, TLR agonists belong to the class

312 of immune modulators known as pathogen-associated molecular patterns (PAMPs). Thus,
313 combining a PAMP (such as aTLR4 agonist) with a DAMP (such as QS21, Alum, or squalene
314 emulsion) may provide activation of multiple bioactivity pathways.

315 Indeed, a combination of SLA and QS21 in a liposome formulation (SLA-LSQ) induced the highest
316 titers of antibodies to antigens of both the sexual and asexual stages. When tested in the SMFA,
317 pooled serum from this group of mice produced the highest transmission-reducing activity
318 (TRA $\geq$ 90%). A similar high %TRA was observed with serum from mice immunized with GLA-SE.

319 These same GLA-SE-immunized mice also produced relatively high levels of sexual-stage specific
320 antibodies, thus explaining the observed %TRA. This finding is in agreement with results obtained
321 for antibodies against the *Pfs48/45*-10C domain [3] and suggests that the 6C-domain of *Pfs48/45*
322 containing epitope 1 may suffice for induction of TB antibodies. While most functional antibody
323 titers had declined within four weeks after the 3rd immunization, the %TRA observed in SLA-LSQ
324 immunized mice was durable over this time period. Unexpectedly, functional antibody responses
325 (%TRA) seemed to increase from week-1 to week-4 in alum immunized mice, although these levels
326 failed to achieve the levels observed with GLA- and SLA-LSQ. We have previously observed that
327 functional antibody responses against *Pfs48/45* [4] are dependent on the antigen dose, suggesting
328 than an increased dose of adjuvanted GMZ2.6C may further increase titers of TB antibodies.

329 Whereas it is generally accepted that IgG antibodies are a major mediator of protection against
330 clinical disease, cellular immune responses may also play an important role through CD4⁺ effector
331 functions [23]. A recent study involving controlled human malaria infection (CHMI) has
332 demonstrated that volunteers repeatedly exposed to infective mosquito bites, while being treated
333 with an anti-malaria drug, developed protective immunity against a subsequent sporozoite
334 challenge [23]. These immune individuals had solid cell-mediated immune (CMI) responses

335 consisting of multifunctional effector memory T cells producing IFN- γ , TNF, and IL-2 [23]. Mice
336 immunized with some of the adjuvants described here produced high numbers of IFN- γ -positive
337 cells by ELISpot and a significant frequency of CD4⁺ T cells producing both IFN- γ and TNF in the
338 following order: GLA-SEQ> GLA-SE> SLA-LSQ = GLA-LSQ. LSQ alone also induced significant IFN- γ
339 and TNF responses, but these CD4⁺ specific responses were typically lower in magnitude than the
340 adjuvants that contained the TLR4 agonists. For the most part antigen-specific CD4⁺ effector
341 responses peaked with these candidate vaccines one week after the third immunization, while
342 memory recall responses observed four weeks after the third immunization tapered off when
343 most of the antigen-specific T cells are expected to contract. The precise epitope(s) recognized by
344 these CD4⁺ T cells are unknown; however, past experiments have shown that GMZ2 can elicit
345 strong CMI responses in mice [35], suggesting that T cells producing multiple cytokines are, at
346 least in part, directed against the GMZ2 component of GMZ2.6C. It remains to be investigated
347 whether T cells recognizing the GLURP and MSP3 regions of GMZ2.6C contribute to the control of
348 asexual parasite multiplication in naturally exposed individuals. In addition to the IFN- γ -promoting
349 activity seen with the above mentioned adjuvants, an IL-5-promoting activity was seen with SE and
350 alum in the absence of immune modulator molecules. However, this activity did not coincide with
351 high levels of specific antibodies. As noted above for humoral immune responses, we have
352 observed in separate studies an agonist dose-T-cell response relationship for several of these
353 immunomodulatory molecules. Optimization of the dosages of both antigen and adjuvant
354 molecules for maximal antibody and cellular responses is one important area for further
355 investigation.

356 In conclusion, we show that GMZ2.6C combined with adjuvants containing TLR4-agonists and
357 QS21 elicited both enhanced humoral immunity, including increased titers against sexual

358 (gametocyte) and asexual stage (schizont) antigens of *P. falciparum*, and enhanced antigen-
359 specific CD4+ T_H1 cellular responses (T cells secreting both IFN-γ and TNF in response to the
360 immunizing antigen). The results of these adjuvant studies provide a rationale for further
361 development of GMZ2 fused with this selected region of *Pfs48/45* using an adjuvant formulation
362 containing GLA or SLA.

363

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371

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476 **Figure Legends**

477 **Figure 1.** Expression and characterization of GMZ2.6C. **A)** An overview of Chimeric GMZ2.6C. **B)**
478 Purification of GMZ2.6C. Upper panel; Coomassie blue-stained 4 -12.5% polyacrylamide gel of
479 protein fractions (2 µg per well) from GMZ2.6C purified by IMAC (lanes 1 and 2) and by mAb45.1-
480 bound agarose (lanes 3 and 4). Lower panel; an immune blot analysis of the same samples shown
481 in the upper panel (1 µg protein per well) using mAb 45.1 as primary antibody. Reduction using 5
482 mM DTT and the sizes (kDa) of the molecular mass markers are as indicated. **C)** Recognition of
483 asexual- and sexual-stage antigens of individual mice by ELISA. Functional activity in **D)** the SMFA
484 and **E)** the ADCI assay. **F)** The relationship between ADCI-activity and antibody level for individual
485 rats is shown. The coefficient of correlation and P-value are provided in the panel.

486
487 **Figure 2.** Antibody responses against asexual- and sexual-stage parasites. **A)** Asexual-stage
488 (schizont) and **B)** sexual-stage (gametocyte) antibody titers (EC_{50}) from mice immunized with
489 GMZ2.6C and eleven different adjuvants. **C-D)** isotype response of mice for which results are
490 presented in panel **(B)**. Results for individual mice are shown, and a horizontal bar represents each
491 mean value. All antigen-adjuvant combinations were compared to the respective adjuvant
492 “vehicle” for each adjuvant formulation; i.e. the SE group was compared to all other SE-containing
493 adjuvants; the Liposome group was compared to all Liposome-containing adjuvants; and Alum was
494 compared to GLA-Alum. Represented P-values <0.05 were considered significant.

495
496 **Figure 3.** Functional activity of mouse anti-GMZ2.6C antisera in SMFA assay. **A)** Specific oocyst
497 transmission reducing activity (TRA) compared to control feeds in the SMFA of pooled serum
498 samples taken one week and four weeks after the 3rd immunization. **A)** pooled sera taken 1 and 4

499 weeks after the 3rd immunization from groups of mice (n=10) immunized with GMZ2.6C in the
500 indicated adjuvants were assessed for functional activity in the SMFA. The relationships between
501 functional activity and antibody level determined in **B)** the gametocyte ELISA and **C)** by staining of
502 live gametes by suspension IFA (SIFA). The coefficient of correlation and P-value is provided in the
503 panel.

504

505 **Figure 4.** Enhanced numbers of cells producing IFN- γ with synthetic TLR4 agonists. **A & C)** IFN- γ
506 and **B & D)** IL-5 ELISPots from the splenocytes of C57BL/6 mice immunized i.m. with GMZ2.6C plus
507 each of eleven different adjuvant formulations. Spleens were harvested from mice one (**A & B**) or
508 four (**C, D**) weeks after the 3rd immunization. Symbols denote significance (*P < 0.05 vs.
509 GMZ2.6C/Saline and ^P < 0.05 vs. Saline alone) using one-way ANOVA with Tukey's multiple
510 comparison post-test. Each dot represents the number of IFN- γ or IL-5 spot-forming units per
511 million cells from a single mouse, and the horizontal bar designates the mean value for the group
512 of mice.

513

514 **Figure 5.** Percent frequency of cytokine-expressing CD4+ T cells from GMZ2.6C-stimulated
515 splenocytes. **A)** Gating scheme used. Percent frequency of CD4+ T cells producing IFN- γ or TNF
516 after GMZ2.6C *in vitro* stimulation **B)** one week and **D)** four weeks after the 3rd immunization, or
517 producing both cytokines **C)** one week and **E)** four weeks after the 3rd immunization. Bar graphs
518 represent the average $\pm$ SD. The "+" indicates statistical significance based on Student's *t*-test, and
519 the "#" indicates statistical significance based on the Wilcoxon signed rank test.

520

521 **Supplementary Figure 1.** Duration of antibody responses against asexual- and sexual-stage
522 parasites. Pooled Anti-GMZ2.6C serum tested in the **A)** asexual schizont-stage and **B)** sexual
523 gametocyte-stage ELISA, presented as a percentage of a mouse positive control immunized with
524 R0.10C adjuvanted with Alum/GLA (Roeffen *et al.*, unpublished). Dark bars represent results one
525 week after the 3rd immunization, and light bars represent results four weeks after the 3rd
526 immunization.

Figure 1

Figure 2

Figure 3

A

B

C

Figure 4

A [1 week]

B [1 week]

C [4 week]

D [4 week]

Figure 5