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Touqui, Gérard Lambeau, Christiane Deregnacourt

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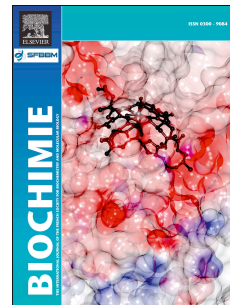
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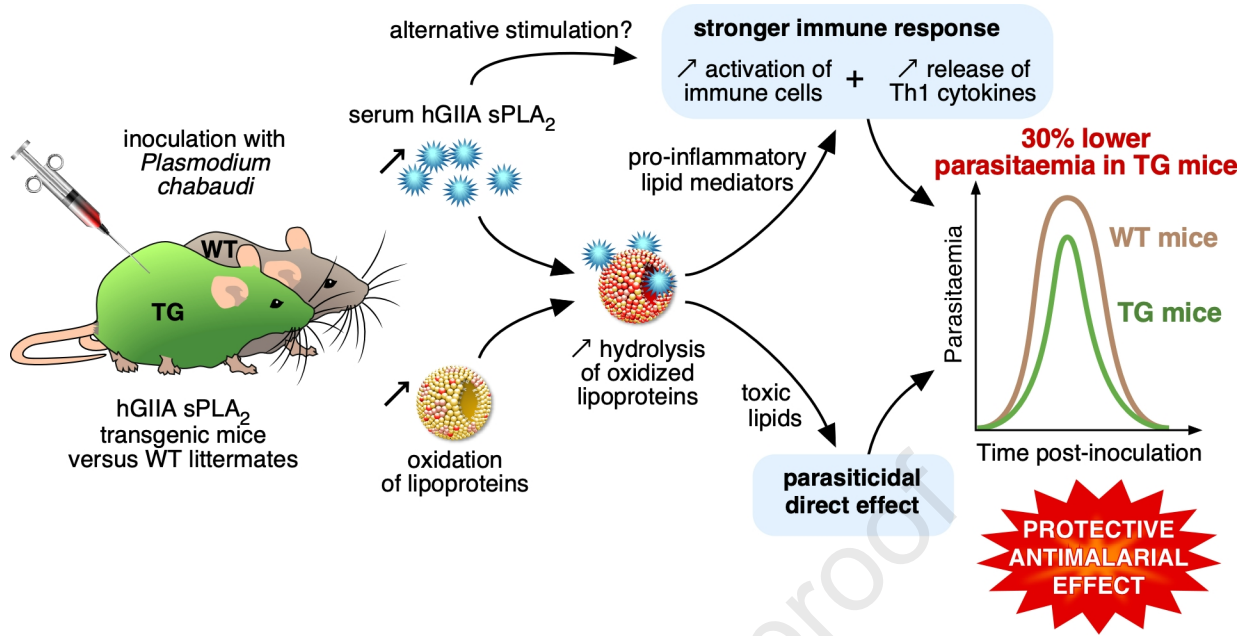
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1 **Role of human group IIA secreted phospholipase A<sub>2</sub> in malaria pathophysiology:**  
 2 **Insights from a transgenic mouse model**

3  
 4 Mélanie Dacheux<sup>1</sup>, Soraya Chaouch<sup>1</sup>, Alonso Joy<sup>1</sup>, Amandine Labat<sup>1</sup>, Christine Payré<sup>2</sup>, Agnès Petit-  
 5 Paitel<sup>2</sup>, Franck Bihl<sup>2</sup>, Isabelle Lagrange<sup>3</sup>, Philippe Grellier<sup>1</sup>, Lhousseine Touqui<sup>4,5\*</sup>, Gérard Lambeau<sup>2\*#</sup>,  
 6 Christiane Deregnacourt<sup>1\*#</sup>

7 <sup>1</sup> UMR 7245 Molécules de Communication et Adaptation des Micro-organismes, Muséum National  
 8 d'Histoire Naturelle, CNRS, CP52, 61 rue Buffon, Paris Cedex 05 75231, France

9 <sup>2</sup> Université Côte d'Azur (UCA), Centre National de la Recherche Scientifique (CNRS), Institut de  
 10 Pharmacologie Moléculaire et Cellulaire (IPMC), UMR7275, Valbonne Sophia Antipolis, France ;

11 <sup>3</sup> Ecole Nationale Vétérinaire d'Alfort, BioPôle, Laboratoire d'hématologie, 94704 Maisons-Alfort,  
 12 France ;

13 <sup>4</sup> Cystic fibrosis and Bronchial diseases team - INSERM U938, Institut Pasteur, 75015 Paris, France

14 <sup>5</sup> Sorbonne Université, INSERM UMRS938, Centre de Recherche Saint-Antoine (CRSA), 75012 Paris,  
 15 France.

16 \* Contributed equally to the development and implementation of the project.

17 Current addresses for M.D.: Mélanie Dacheux, Van Vleet Memorial Cancer Building, Department of  
 18 Physiology, University of Tennessee Health Science Center. 3 North Dunlap Street, Memphis, TN  
 19 38163.

20 # Address correspondence to Christiane Deregnacourt, [christiane.deregnacourt@mnhn.fr](mailto:christiane.deregnacourt@mnhn.fr) or  
 21 Gérard Lambeau, [lambeau@ipmc.cnrs.fr](mailto:lambeau@ipmc.cnrs.fr)

22 **Running title:** *In vivo* antimalarial activity of hGIIA sPLA<sub>2</sub>

23 **Keywords:** Malaria, secreted phospholipase A<sub>2</sub>, transgenic mice, *Plasmodium chabaudi*

24 **Abbreviations:** hGIIA sPLA<sub>2</sub>: human group IIA secreted phospholipase A<sub>2</sub>. A comprehensive  
 25 abbreviation system is used for the various mammalian sPLA<sub>2</sub>s: each sPLA<sub>2</sub> is abbreviated with a  
 26 lowercase letter indicating the sPLA<sub>2</sub> species (m and h for mouse and human, respectively), followed  
 27 by uppercase letters identifying the sPLA<sub>2</sub> group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GX and  
 28 GXII). IFN-γ: Interferon-gamma. KC/GRO: keratinocyte chemoattractant/growth regulated oncogene.  
 29 MDA: malondialdehyde. PUFAs: polyunsaturated fatty acids. RBCs: red blood cells. RT-qPCR: real-  
 30 time quantitative polymerase chain reaction. TBARS: thiobarbituric acid-reactive-substances. WBCs:  
 31 white blood cells. TNF-α: tumor-necrosis factor alpha. TR-FIA: time-resolved fluoroimmunoassay.

32 **ABSTRACT**

33

34 We previously showed that injection of recombinant human group IIA secreted phospholipase A<sub>2</sub>  
35 (hGIIA sPLA<sub>2</sub>) to *Plasmodium chabaudi*-infected mice lowers parasitaemia by 20%. Here, we show  
36 that transgenic (TG) mice overexpressing hGIIA sPLA<sub>2</sub> have a peak of parasitaemia about 30% lower  
37 than WT littermates. During infection, levels of circulating sPLA<sub>2</sub>, enzymatic activity and plasma lipid  
38 peroxidation were maximal at day-14, the peak of parasitaemia. Levels of hGIIA mRNA increased in  
39 liver but not in spleen and blood cells, suggesting that liver may contribute as a source of circulating  
40 hGIIA sPLA<sub>2</sub>. Before infection, baseline levels of leukocytes and pro-inflammatory cytokines were  
41 higher in TG mice than WT littermates. Upon infection, the number of neutrophils, lymphocytes and  
42 monocytes increased and were maximal at the peak of parasitaemia in both WT and TG mice, but  
43 were higher in TG mice. Similarly, levels of the Th1 cytokines IFN- $\gamma$  and IL-2 increased in WT and TG  
44 mice, but were 7.7- and 1.7-fold higher in TG mice. The characteristic shift towards Th2 cytokines was  
45 observed during infection in both WT and TG mice, with increased levels of IL-10 and IL-4 at day-14.  
46 The current data are in accordance with our previous *in vitro* findings showing that hGIIA kills  
47 parasites by releasing toxic lipids from oxidized lipoproteins. They further show that hGIIA sPLA<sub>2</sub> is  
48 induced during mouse experimental malaria and has a protective *in vivo* role, lowering parasitaemia  
49 by likely releasing toxic lipids from oxidized lipoproteins but also indirectly by promoting a more  
50 sustained innate immune response.

51

## 52 1. Introduction

53

54 Malaria is a tropical and sub-tropical disease caused by a protozoan parasite of the genus  
55 *Plasmodium*, which is transmitted to humans by the bite of *Anopheles* mosquitoes [1]. Five  
56 *Plasmodium* species have been identified in humans (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and  
57 *P. knowlesi*). In 2018, an estimated 228 million cases of malaria occurred worldwide (WHO malaria  
58 report 2019 at [www.who.int](http://www.who.int)) with an estimated 405,000 deaths. Most malaria cases (93%) were in  
59 the World Health Organization (WHO) African Region, where *P. falciparum* is the most prevalent  
60 malaria parasite.

61 *Plasmodium* development in the human host mostly takes place in red blood cells (RBCs). The intra-  
62 erythrocyte cycle is responsible for common malaria symptoms including fever, aches and nausea. *P.*  
63 *falciparum* malaria can lead to severe, life-threatening complications including cerebral malaria and  
64 severe anemia. A better understanding of malaria pathophysiology is key to open new perspectives  
65 to fight against *Plasmodium* infection. Among the various mechanisms of innate immunity and host  
66 response engaged following infection [2-4], endogenous secreted phospholipases A<sub>2</sub> (sPLA<sub>2</sub>s) may  
67 play a specific role.

68 Mammalian phospholipases A<sub>2</sub> (PLA<sub>2</sub>, EC 3.1.1.4) comprise numerous intracellular and secreted  
69 enzymes that catalyze the hydrolysis of phospholipids at the *sn*-2 position to release free fatty acids  
70 and lysophospholipids [5, 6]. PLA<sub>2</sub>s play multiple roles in physiological and pathophysiological  
71 conditions. For example, PLA<sub>2</sub> can release arachidonic acid which is a key precursor for the  
72 biosynthesis of eicosanoids such as prostaglandins and leukotrienes, these latter being important in  
73 the host inflammatory response [7]. Among the diversity of PLA<sub>2</sub>s, secreted PLA<sub>2</sub>s (sPLA<sub>2</sub>s) comprise  
74 up to 12 members in mice and humans, called IB, IIA, IIC (pseudogene in humans), IID, IIE, IIF, III, V, X,  
75 XIIA, XIIB and otoconin-90 [8]. It is now clear that each sPLA<sub>2</sub> has distinct cell and tissue distributions  
76 and exerts specific and diverse biological functions [8-11]. Mechanistically, most sPLA<sub>2</sub>s contribute to  
77 multiple pathophysiological functions by hydrolysis of various phospholipid substrates from cellular  
78 membranes or extracellular lipid components, such as lipoproteins, microparticles, lung surfactant,  
79 mitochondria, lipid diet and microbes [10, 12-19].

80 Human group IIA (hGIIA) sPLA<sub>2</sub> is one of the most studied sPLA<sub>2</sub>, especially because it is induced  
81 during inflammation and host defense where it likely plays key but controversial roles [20]. As early  
82 as 1979, this sPLA<sub>2</sub> has been recognized as a potent bactericidal enzyme secreted from  
83 polymorphonuclear leukocytes [21]. Since then, multiple studies have shown that the enzyme is  
84 secreted by inflammatory, immune and stromal cells after stimulation by various pro-inflammatory  
85 molecules including pathogen-associated molecular patterns such as lipopolysaccharides [15, 18, 22,  
86 23]. Accordingly, the enzyme has been detected at high levels in body fluids such as plasma,

87 gastrointestinal tract lumen, tears, bronchial secretions or inflammatory exudates during sepsis,  
88 infection and systemic inflammation [24-30]. hGIIA sPLA<sub>2</sub> and several other sPLA<sub>2</sub>s have been  
89 involved in host defense with antimicrobial activity against bacteria, viruses and parasites [11, 18, 22,  
90 23, 31-40]. sPLA<sub>2</sub>s are also present in various plant and animal genomes, and are particularly  
91 abundant in snake and bee venoms, where they also display antimicrobial activities, suggesting an  
92 evolutionary conserved function [41-46].

93 However, the possible role of hGIIA and other sPLA<sub>2</sub>s in host defense against *Plasmodium* in malaria  
94 has remained unknown until recently. In the early 90's, Vadas and colleagues reported elevated  
95 levels of hGIIA sPLA<sub>2</sub> in the serum of patients with malaria [47, 48] but its role was not investigated.  
96 In the early 2000's, we reported that snake and bee venom sPLA<sub>2</sub>s exert potent antimalarial activity  
97 against *P. falciparum* in an *in vitro* model of human RBCs infected by *P. falciparum* [49-52]. More  
98 recently, we showed that several but not all human sPLA<sub>2</sub>s (hGIIA, hGIIF, hGIII, hGV and hGX) have  
99 the same effect as venom sPLA<sub>2</sub>s, inhibiting the growth of *P. falciparum* with IC<sub>50</sub> values from nM to  
100 μM range [53, 54]. In this *in vitro* model, we identified an indirect mechanism of action by which  
101 sPLA<sub>2</sub>s hydrolyze lipoproteins from the human plasma that supplements parasite culture medium  
102 and release polyunsaturated fatty acids (PUFAs) that are toxic to the parasite [53]. Interestingly,  
103 hGIIA sPLA<sub>2</sub> was inactive against *P. falciparum* in the presence of normal human plasma but became  
104 active in the presence of plasma from malaria patients containing oxidized lipoproteins [54]. We also  
105 found increased levels of hGIIA sPLA<sub>2</sub> associated with the presence of oxidized lipoproteins in the  
106 plasma of patients with malaria [54, 55]. Last, we showed that injection of recombinant hGIIA sPLA<sub>2</sub>  
107 to *Plasmodium*-infected mice significantly reduced parasitaemia, supporting an *in vivo* role [54].  
108 These findings suggest a possible antimalarial role of hGIIA when present at high levels and  
109 concomitantly with oxidized lipoproteins. Concerning other human sPLA<sub>2</sub>s, hGIIF, hGIII, hGV and hGX  
110 sPLA<sub>2</sub>s were active in the presence of both native and oxidized lipoproteins sPLA<sub>2</sub>s, but they were not  
111 detected in the plasma of malaria patients, leaving unknown their possible *in vivo* roles [53, 54].

112 To investigate the pathophysiological role of hGIIA sPLA<sub>2</sub> in an *in vivo* situation close to human  
113 malaria where the sPLA<sub>2</sub> is present at high levels during infection [47, 48, 54], we used transgenic  
114 (TG) mice overexpressing hGIIA sPLA<sub>2</sub> [56] and explored its impact on the pathophysiology of malaria  
115 infection by the self-healing murine parasite *Plasmodium chabaudi chabaudi* 864VD (*P. chabaudi*). In  
116 line with our approach, hGIIA sPLA<sub>2</sub> TG mice have been extensively used by us and others to reveal  
117 the role of hGIIA in host defense against various bacterial pathogens [18, 22, 23, 57-59]. Of note,  
118 studies with the transgenic hGIIA mouse strain were performed in comparison with the parental  
119 inbred C57BL/6 mouse strain that is naturally-deficient for endogenous mouse group IIA sPLA<sub>2</sub>  
120 (*Pla2g2a* gene) [60]. Using this transgenic mouse model, we show that i) the circulating level of hGIIA  
121 sPLA<sub>2</sub> increases during the course of malaria infection, ii) the sPLA<sub>2</sub> inhibits parasite development at

122 the peak of parasitaemia, when the levels of both circulating enzyme and oxidized proteins are  
123 maximally increased, and iii) the expression of the hGIIA sPLA<sub>2</sub> promotes a stronger interferon- $\gamma$  (IFN-  
124  $\gamma$ ) immune response, which is key to fight malaria infection [61].

125

## 126 **2. Materials and methods**

127

### 128 *2.1. Materials*

129 Diff-Quick staining reagents were from Medion Diagnostics AG (Düdingen, Switzerland). Heparin  
130 Choay 5,000 IU/mL was from Sanofi-Aventis. KovaSlides<sup>®</sup> were from Kova International. The U-PLEX  
131 Th1/Th2 Combo Mouse assay kit from Meso Scale Discovery (Meso Scale Diagnostics, Rockville,  
132 Maryland) was used for quantitative determination of IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, KC/GRO, IL-10, IL-  
133 12 p70, IL-13 and TNF- $\alpha$  in mouse plasma. RNA Later<sup>®</sup> and TissueLyser II were from Qiagen. TRIzol<sup>®</sup>  
134 was from Ambion<sup>®</sup>. The Turbo DNase kit was from Invitrogen. RevertAid H Minus Reverse  
135 Transcriptase and Oligo(dT)18 Primer were from Thermo Scientific. SensiFAST SYBR No-ROX Kit  
136 Master Mix 2X was from Bioline Meridian Bioscience.

137

### 138 *2.2. Ethical statement*

139 All experimental procedures were carried out in accordance with the EC Directive 2010/63/UE and  
140 the relevant national legislation, namely the French “Décret n° 2013–118, 1<sup>er</sup> février 2013, Ministère  
141 de l’Agriculture, de l’Agroalimentaire et de la Forêt”. Protocols were approved by the ethical  
142 committee “Comité Cuvier” and registered under the APAFIS file #13845. Mice were housed in the  
143 animal facilities of the MNHN and Institut Pasteur accredited by the French Ministry of Agriculture  
144 for performing experiments in live rodents.

145

### 146 *2.3. Parasite*

147 The rodent malaria parasite *Plasmodium chabaudi chabaudi* 864VD used in this work was originally  
148 described by Landau in 1965 [62]. The 864VD strain of *P. chabaudi chabaudi* was from the MCAM  
149 Research Unit’s *Plasmodium* collection as part of the National Museum of Natural History parasite  
150 collection. The 864VD strain is generally non-lethal, invades both normocytes and reticulocytes,  
151 adheres on vascular endothelium (sequestration) and induces a chronic infection. The asexual cycle  
152 in RBCs is completed within 24 h and is highly synchronous.

153



## 154 2.4. Mice

155 Wild-type C57BL/6J (C57BL/6J JAX<sup>TM</sup> mice strain) mice were from Charles River Laboratories  
156 (Miserey, 27930 France). C57BL/6 mice have a natural frameshift mutation in exon 3 of the *Pla2g2a*  
157 gene encoding for group IIA sPLA<sub>2</sub> (5 exons and 4 introns), making these mice deficient for active  
158 mouse group IIA enzyme [60]. hGIIA sPLA<sub>2</sub> transgenic mice (transgenic model #1005, C57BL/6J-  
159 [Tg]PLA<sub>2</sub>) referred to as TG mice in this study, were from Taconic Biosciences (Taconic EZcohort®  
160 Models). Of note, the hGIIA sPLA<sub>2</sub> transgene is a 6.2-kbp genomic fragment of the *PLA2G2A* gene  
161 containing the full open reading frame of hGIIA sPLA<sub>2</sub> flanked by 1.6 kbp of 5' and 0.35 kbp of 3' non-  
162 coding regions harboring several promoter elements [56]. Previous *in situ* hybridization experiments  
163 have shown that hGIIA sPLA<sub>2</sub> mRNA is present at constitutively high levels in skin, at low levels in  
164 liver, kidney, bladder, small intestine and lung, and is absent in spleen [63]. The 1.6 kbp upstream  
165 region contains transcriptional response element consensus sequences for IL-6, IFN, hepatocyte NF-  
166 3, AP1, AP2, C/EBP, and CRE, and the transcription promoter sequences (CCAAT box and TATA box)  
167 [56, 64, 65]. TG and WT littermates used for experiments were all obtained by breeding TG males  
168 with WT C57BL/6J females. Since TG mice are smaller and present alopecia compared to their WT  
169 counterparts, mice from each group were easily differentiated phenotypically, without the need for  
170 DNA genotyping. Mice were housed with water and food *ad libitum* on a 12 h light-dark cycle. Eight  
171 to 15 weeks-old male mice were used for all experiments.

172

## 173 2.5. Infection and blood cell analysis

174 WT littermates and TG mice were inoculated by the intraperitoneal route (IP) with  $1 \times 10^6$  *P. c.*  
175 *chabaudi* 864VD-infected mouse RBCs in Alsever's solution. Control uninfected mice were injected  
176 with  $1 \times 10^6$  non-infected RBCs in Alsever's solution. Tail blood was collected every 2-3 days within a  
177 maximum of 30 days following inoculation. Parasitaemia was established by optical examination of  
178 Diff-Quik-stained blood smears and counting of 2,000 RBCs (parasitaemia corresponds to the  
179 percentage of infected RBCs over total RBCs). The limit of detection for patent parasitaemia was  
180  $\approx 0.05\%$  infected RBCs.

181 Blood cell distribution in TG mice was analyzed before parasite inoculation and during the course of  
182 infection, and compared to WT littermate mice. Longitudinal study and cross-sectional study were  
183 performed. Longitudinal follow-up of the infection was performed by collecting small volumes (20-  
184 30  $\mu$ L) of tail blood onto heparin every 2-3 days p.i. and manual counting of blood cells. RBC number  
185 per  $\mu$ L of blood was determined by using KovaSlide® chambers after blood dilution into saline  
186 solution (NaCl 9 g/L). WBCs were counted on KovaSlides® after 1:20 dilution into 3% (v/v) acetic acid  
187 solution containing methylene blue. Distribution of leukocyte subpopulations was established by  
188 morphological identification of cells on Diff-Quik-stained blood smears.

189 Automated determination of blood parameters (hematogram) was carried out using the Sysmex XT-  
190 2000iV apparatus configured for mouse blood. TG and WT littermate mice were inoculated by IP with  
191  $1 \times 10^6$  infected RBCs. Blood was collected from facial vein onto EDTA-coated tubes before  
192 inoculation of the parasite (day-0), at the onset of the patent phase ( $\approx$  day-7 p.i.), then at the time of  
193 high parasitaemia ( $\approx$  day-13 p.i.) and early after crisis ( $\approx$  day-16 p.i.), to cover the peak of  
194 parasitaemia. Hundred fifty  $\mu$ L of blood were analyzed within 2 hours with the Sysmex methodology.  
195 Mice were sacrificed upon blood collection. Manual and automated analyses were completed by  
196 optical examination of Giemsa-stained blood smears. Morphological determination of leukocyte  
197 activation was performed by optical examination of Giemsa-stained blood smears.

198

### 199 *2.6. Organ and blood collection during infection*

200 Groups ( $n \geq 5$ ) of uninfected and infected WT and TG mice were sacrificed at day-0 (before  
201 inoculation), day-8 p.i. (onset of the patent phase), day-14 p.i. (parasitaemia peak) and day-21 p.i.  
202 (parasite clearance). Mice were weighed prior to sacrifice. They were anesthetized by IP injection of  
203 ketamine (100 mg/kg)/ xylazine (10 mg/kg) then euthanized by retro-orbital exsanguination. The  
204 blood was collected in tubes containing 5  $\mu$ L heparin (15-30 IU/mL final) and centrifuged at  $1,500 \times g$   
205 for 5 min at room temperature. Plasma was stored frozen at  $-80^\circ\text{C}$  before analysis (cytokine  
206 measurement, TBARS assay, etc). Pelleted blood cells were resuspended in TRIzol<sup>®</sup>, vortexed and  
207 stored frozen at  $-80^\circ\text{C}$  until RT-qPCR analysis. Liver, spleen and kidney from infected and non-  
208 infected mice were removed and weighed. Spleen and liver were sliced into small pieces, collected  
209 into 1 mL of RNA Later<sup>®</sup>, and stored at  $-80^\circ\text{C}$  before RNA extraction for RT-qPCR analysis.

210

### 211 *2.7. Measurement of sPLA<sub>2</sub> activity and time-resolved fluoroimmunoassay for hGIIA sPLA<sub>2</sub>*

212 Mouse tail blood was collected onto heparin (15-30 IU/mL final concentration) and centrifuged at  
213  $1,500 \times g$  for 5 minutes at room temperature. Plasma was frozen at  $-80^\circ\text{C}$  before analysis. sPLA<sub>2</sub>  
214 enzymatic activity was measured by using the <sup>3</sup>H-oleic acid-labeled *E. coli* membrane hydrolysis assay  
215 as described [54]. Time-resolved fluoroimmunoassay (TR-FIA) to detect hGIIA sPLA<sub>2</sub> was performed  
216 as described [28].

217

### 218 *2.8. Real time-quantitative PCR (RT-qPCR)*

219 Organ pieces frozen in RNA Later were thawed on ice, and 50-100 mg of tissues were disrupted in 1  
220 mL of TRIzol<sup>®</sup> using TissueLyser II with RNase-free tungsten carbide beads. Lysates were centrifuged  
221 for 10 min at  $12,000 \times g$  at  $4^\circ\text{C}$ . Blood cell samples in TRIzol<sup>®</sup> were thawed on ice and immediately  
222 centrifuged at  $12,000 \times g$ . Total RNA from supernatants was prepared with TRIzol<sup>®</sup> and quantified  
223 using NanoDrop 2000 spectrophotometer (Thermo Scientific). Samples with  $A_{260/280} > 1.8$  and  $A_{260/230}$

224 > 1.6 were DNase-treated. RNA integrity was assessed by electrophoresis on 1% agarose gels. For  
225 each sample, 500 ng of RNA were converted to first-strand cDNA using RevertAid H Minus Reverse  
226 Transcriptase and Oligo(dT)18 Primer according to the manufacturer's protocol. To assess genomic  
227 DNA contamination, non-reverse transcription controls were performed in parallel.

228 The design of gene-specific primers for sPLA<sub>2s</sub> and housekeeping reference genes were based on  
229 previously published data [66, 67] or designed using the Primer-BLAST software, and synthesized by  
230 Eurofins Genomics (Ebersberg, Germany). Genes ID, primer sequences, and amplicon lengths are  
231 listed in Table S1. Real-time PCR was performed on a LightCycler 480 (Roche Diagnostics France).  
232 Each reaction was performed in duplicate (in triplicate for blood samples) and contained 5 µl of  
233 SensiFAST SYBR No-ROX Kit Master Mix 2X, 500 nM of each primer, and 4 µl of 1:20 diluted cDNA  
234 sample, in a final volume of 10 µl. Cycling conditions were as follows: 1 cycle at 95°C for 2 min, and  
235 then 45 three-phase cycles at 95°C for 5 s, 60°C for 10 s, 72°C for 12 s. At the end of amplification,  
236 the melting temperature of the products was determined using a melting curve program, and for  
237 each primer pair, amplicon length was checked on 2.5% agarose gels. Cq value was automatically  
238 calculated using the "Second Derivative Maximum Method". Reaction efficiency was determined for  
239 each pair of primers using a serial dilution of cDNA as template: a standard curve was obtained by  
240 plotting Cq values against logarithmic dilutions. Reaction efficiency (E) was calculated as  $E = 10^{(-1/\text{slope})}$ ,  
241 from the slope value of a standard curve with  $r^2 > 0.99$ . Efficiencies were comprised between  
242 93% and 108%. To identify suitable reference genes (RGs) for normalization, five candidates were  
243 tested, namely *Actb*, *Hprt*, *Gusb*, *Tbp*, and *Pgk1*. For these genes, Cq values obtained from samples  
244 were analyzed using the mathematical algorithm geNorm [68], which classified RG's stability across  
245 samples, and gave the optimum number of RGs required for an accurate normalization by the  
246 pairwise variation ( $V_n/n+1$ ) calculation [68]. Blood, spleen and liver samples were analyzed  
247 independently. The most stably expressed genes were found to be *Gusb*, *Hprt1* and *Tbp* for spleen,  
248 *Hprt1*, *Tbp* and *Pgk1* for liver, and *Gusb*, *Hprt1*, *Tbp* and *Actb* for blood. To determine the relative  
249 mRNA expression levels of hGIIA, mGIIF, mGIII, mGV and mGX sPLA<sub>2s</sub>, expression levels (Q) were first  
250 expressed as fold changes of each sample relative to day-0 sample from the same organ, using the  
251 formula  $Q = E^{(Cq^{DO} - Cq^{\text{sample}})}$ , where E is the efficacy of the PCR reaction), and then normalized using the  
252 geometric mean of the expression levels of the validated reference genes [69]. Genomic DNA  
253 detection in the non-reverse transcription controls was assessed with GAPDH primer pair, as it was  
254 designed within a single exon. No significant genomic DNA contamination was found in the non-  
255 reverse transcription controls (Cq>34.5 or no target melting peak).

256

### 257 2.9. Lipid peroxidation measurement

258 The level of TBARS (thiobarbituric acid reactive substances) was determined as a marker of plasma  
259 lipid peroxidation. TBARS assay was performed as reported [54].

260

### 261 2.10. Th1/Th2 cytokine quantification during infection

262 The multiplex technology from Meso Scale Discovery was applied to measure the concentration of  
263 cytokines in plasma of non-infected and *P. chabaudi*-infected mice. Plasma samples from day-0 (5 WT  
264 and 5 TG), day-8 p.i. (6 WT and 6 TG), day-14 p.i. (6 WT and 6 TG) and day-21 p.i. (6 WT and 5 TG)  
265 mice were analyzed. Twenty  $\mu\text{L}$  of each plasma were loaded into the MSD 96-well multi-array U-PLEX  
266 TH1/TH2 Combo Mouse assay kit plate from Meso Scale Discovery (Meso Scale Diagnostics, Rockville,  
267 Maryland) and incubated overnight at 4°C. Analysis was performed according to manufacturer's  
268 recommendations using the MESO QuickPlex SQ 120 reader (Meso Scale Diagnostics, Rockville,  
269 Maryland).

270

### 271 2.11. Statistical analyses

272 Data were analyzed using the GraphPad InStat 3 software (San Diego, CA). The choice of statistical  
273 tests was based on sample size and normality examined prior to further analysis. Data from  
274 independent experiments were pooled when possible. Results were analyzed using t-test, one-way  
275 ANOVA test (in order to determine the effect of one factor, *i.e.* the group (genotype) of mice)  
276 followed by a Dunnett's post-test, or two-way ANOVA (in order to determine the effects of two  
277 factors, *i.e.* i) the group (genotype) of mice and ii) the time after infection) and their interaction,  
278 followed by a Bonferroni's post-test. Normality of samples with n number superior to 6 was tested  
279 using the Shapiro-Wilk test. When sample distribution was normal, the parametric unpaired or  
280 paired t-test with a two-tailed P value was applied. When sample distribution was not normal and/or  
281 the n number was too small ( $\leq 6$ ), non-parametric Mann-Whitney U test for independent samples and  
282 Wilcoxon matched-paired test for dependent samples were respectively used. One-way and two-way  
283 ANOVA were applied after normalization of data when required. P values <0.05 (# or \*), <0.01 (## or  
284 \*\*) and <0.001 (### or \*\*\*) were considered as statistically significant.

285

## 286 3. Results

287

### 288 3.1. Parasitaemia is reduced in TG mice infected with *P. chabaudi*

289 We first compared the time course of parasitaemia in TG *versus* WT mice infected with *P. chabaudi*.  
290 Parasite-containing RBCs were detected on blood smears at day-8 post-inoculation (p.i.) in both  
291 groups of mice (Figure 1A). Similar parasitaemia was measured at day-11 p.i., suggesting that hGIIA

292 sPLA<sub>2</sub> has no major effect on parasite multiplication rate during early time points in TG mice.  
293 Parasitaemia then increased at similar rates in both groups of mice, to reach a maximum at day-14  
294 p.i. (parasitaemia peak or crisis). Interestingly, the peak of parasitaemia in TG mice was about one  
295 third lower than in WT mice (Figure 1A and 1B). The mean  $\pm$  SEM peak value of parasitaemia was  
296  $38.6 \pm 2.1$  % in WT mice (n=19) *versus*  $27.9 \pm 8.0$  % in TG mice (n=23), corresponding to a mean  
297 reduction of parasitemia of 27.6% (p value = 0.0002) (Figure 1B). Parasitaemia then decreased  
298 similarly after crisis and blood was almost cleared from all parasites at day-20 p.i. (parasite clearance  
299 or resolution phase) in both groups of mice. Thus, the difference between parasitized TG and WT  
300 mice only occurred at the peak of parasitaemia with no difference during the pre-patent and  
301 resolution phases. These results are in agreement with our previous data showing that recombinant  
302 hGIIA sPLA<sub>2</sub> was effective at lowering parasitaemia in infected C57BL/6 WT mice when injected right  
303 before the peak of parasitaemia but not when injected at earlier time points [54].

304

### 305 3.2. Macroscopic examination of TG and WT mice before and during infection

306 Along with thrombocytopenia and anaemia, weight loss, hepatosplenomegaly and kidney damage  
307 are among the signs of mild malaria in mice and humans, and can occur early or late during the  
308 course of the disease. We thus examined whether differences in these signs occur between TG and  
309 WT mice during *P. chabaudi* infection. It is known from original studies that TG mice overexpressing  
310 hGIIA are roughly 25% smaller than WT littermates [56]. Accordingly, the mean body weights  $\pm$  SEM  
311 of WT and TG mice at baseline were  $25.9 \pm 0.7$  g (n=11) and  $22.1 \pm 0.8$  g (n=7), respectively (9 weeks-  
312 old male mice, p value = 0.0060, Figure 2A). The body weight variation during the course of infection  
313 was similar in both groups of mice, with a significant weight loss at maximal parasitaemia. The mean  
314 weights  $\pm$  SEM at the peak of parasitaemia were  $23.8 \pm 0.8$  g (n=11) for WT mice (weight loss  $\approx$ 10%, p  
315 value <0.0001) and  $20.7 \pm 0.7$  (n=7) for TG mice (weight loss  $\approx$ 7%, p value = 0.0003) (Figure 2A). The  
316 weight of liver, spleen and kidneys from WT and TG mice were also measured before and after  
317 infection.

318 Weight of liver was similar between TG and WT mice at baseline (mean weight  $\pm$  SEM =  $0.97 \pm 0.02$  g  
319 (TG) *versus*  $0.88 \pm 0.12$  g (WT)). During infection, liver from both groups increased in size and turned  
320 brown due to hemozoin accumulation (not shown). At days -14 and -21 p.i., liver from TG mice ( $1.47$   
321  $\pm 0.09$  g at day-14 p.i. and  $1.62 \pm 0.04$  g at day-21 p.i.) was heavier than that of WT mice ( $1.21 \pm 0.07$   
322 g at day-14 p.i.;  $1.35 \pm 0.13$  g at day-21 p.i.), indicating more hepatomegaly in TG mice. Two-way  
323 ANOVA analysis confirmed the global increase of weight of liver during the course of infection (p  
324 value <0.0001) in both groups of mice and indicated that the weight of liver differed according to the  
325 genotype (p value = 0.0079, Figure 2B). Spleen from TG mice was slightly heavier than from WT mice  
326 at baseline, with mean  $\pm$  SEM values of  $0.13 \pm 0.009$  g *versus*  $0.09 \pm 0.007$  g, respectively (Mann

327 Whitney test,  $p$  value = 0.0142). Spleen browning and enlargement (not shown) due to infection  
328 occurred at similar time points in both groups of mice, with a dramatic increase of weight occurring  
329 between day-8 p.i. and day-14 p.i. in WT (5.4-fold) and TG (6.1-fold) mice ( $p$  value <0.0001, Figure  
330 2C). Kidneys from TG and WT mice exhibited similar weights at baseline ( $0.42 \pm 0.02$  g for WT *versus*  
331  $0.41 \pm 0.03$  g for TG mice). No variation of weight was observed in either group of mice during  
332 infection (Figure 2D).

333

### 334 3.3. Both enzymatic activity and circulating levels of hGIIA sPLA<sub>2</sub> are increased in TG mice during 335 parasitaemia

336 Enzymatic activity and circulating levels of hGIIA sPLA<sub>2</sub> were quantified in the plasma of TG and WT  
337 littermate mice before and during infection. In accordance with previous data on the same mouse  
338 line [56], non-infected TG mice exhibit at baseline high levels of sPLA<sub>2</sub> activity (mean  $\pm$  SEM =  $3,106 \pm$   
339  $224.8$  cpm/min  $\times$   $\mu$ L,  $n = 12$ , Figure 3A) and hGIIA sPLA<sub>2</sub> concentration (mean  $\pm$  SEM =  $52.1 \pm 20.6$  nM,  
340  $n = 5$ , Figure 3B). Upon infection, a significant increase of circulating sPLA<sub>2</sub> activity occurred with  
341 more than 2-fold higher enzymatic activity at crisis (mean value =  $7,350 \pm 1821$  cpm/min  $\times$   $\mu$ L,  $n = 12$ )  
342 (Figure 3A). Accordingly, the concentration of circulating hGIIA sPLA<sub>2</sub> increased by almost 3-fold at  
343 day-14 p.i. (mean value =  $144.7 \pm 21.6$  nM,  $n = 6$ ) (Figure 3B). In contrast, no or very marginal sPLA<sub>2</sub>  
344 activity was measured in the plasma of WT mice before infection (mean value =  $55.7 \pm 15.8$   
345 cpm/min  $\times$   $\mu$ L,  $n = 18$ , not shown). This was expected since C57BL/6 mice have a natural frameshift  
346 mutation in the *Pla2g2a* gene and are deficient for a functional mGIIA sPLA<sub>2</sub> enzyme [60, 66]. No  
347 increase of enzymatic activity was measured in the plasma of WT mice infected by *P. chabaudi* (not  
348 shown), suggesting that no other mouse sPLA<sub>2</sub> was stimulated by *Plasmodium* infection at a systemic  
349 level. Thus, infection by *Plasmodium* stimulated the baseline level of hGIIA sPLA<sub>2</sub> in the plasma of TG  
350 mice, with a peak of enzymatic activity and concentration coincident with parasitaemia reduction,  
351 suggesting a causal relationship.

352

### 353 3.4. Expression of hGIIA and mouse sPLA<sub>2</sub>s in tissues from TG and WT mice infected by *P. chabaudi*

354 To analyze whether the expression of the hGIIA sPLA<sub>2</sub> transgene might be modulated by *Plasmodium*  
355 infection in a tissue-specific manner, we performed RT-qPCR on liver, spleen and blood cells collected  
356 from TG mice at different times of infection. By RT-qPCR, we could detect baseline levels of hGIIA  
357 sPLA<sub>2</sub> mRNA in liver, blood cells and spleen of TG mice (Figure 4A). Upon infection, mRNA levels of  
358 hGIIA sPLA<sub>2</sub> increased in liver by about 2-fold between day-8 and day-14 p.i., and then returned to  
359 baseline (Figure 4A). Parasitaemia and mRNA levels were positively correlated (Spearman  $r$  0.6468,  $p$   
360 value = 0.068,  $n=16$ , not shown). By contrast, hGIIA sPLA<sub>2</sub> mRNA decreased over time in spleen  
361 (Figure 4A) and the expression level was inversely correlated to parasitaemia (Spearman  $r$  -0.5036,  $p$

362 value = 0.0393, n=17, not shown). In blood cells, no mRNA variation was observed during infection  
363 (Figure 4A), with no significant correlation between parasitaemia and mRNA levels (Spearman  $r$  -  
364 0.2716,  $p$  value = 0.3931, n=12, not shown). Altogether, these results suggest a tissue-specific  
365 regulation of the hGIIA sPLA<sub>2</sub> transgene during infection, where liver appears as a possible source of  
366 circulating hGIIA sPLA<sub>2</sub>.

367 To determine whether endogenous mouse sPLA<sub>2</sub>s might be regulated by *P. chabaudi* infection, we  
368 performed RT-qPCR for mouse GIIF (mGIIF, *Pla2g2f*), GIII (mGIII, *Pla2g3*), GV (mGV, *Pla2g5*) and GX  
369 (mGX, *Pla2g10*) sPLA<sub>2</sub>s in liver, spleen and blood cells of TG mice and WT littermates before and after  
370 *P. chabaudi* inoculation. In all tissue extracts, mGIII and mGX mRNAs were under the detection limit  
371 in our conditions of RT-qPCR. In contrast, mGIIF and mGV mRNAs were detected before infection in  
372 spleen of both TG and WT mice, but not in liver or blood cells. Upon infection, a marked and early  
373 drop of mGIIF and mGV mRNA was observed in spleen for both groups of mice (Figure 4B). This was  
374 supported by a strong negative correlation between parasitaemia and the relative levels of mRNA for  
375 both groups of mice (for mGIIF: Spearman's  $r$  -0.8956,  $p$  value <0.0001, n=18 (TG mice); Spearman's  $r$   
376 -0.8019,  $p$  value = 0.0030, n=11 (WT mice), and for mGV: Spearman's  $r$  -0.9345,  $p$  value <0.0001,  
377 n=19 (TG mice); Spearman's  $r$  -0.7973,  $p$  value = 0.0033, n=11 (WT mice), not shown). It is interesting  
378 to note that both WT and TG mice exhibit the same pattern of mRNA expression and regulation, that  
379 is detectable levels of mGIIF and mGV but not mGIII and mGX sPLA<sub>2</sub> mRNAs at baseline in spleen and  
380 significant down-regulation of mGIIF and mGV mRNAs after *Plasmodium* infection. This suggests no  
381 compensation effect associated with overexpression of hGIIA sPLA<sub>2</sub>.

382

### 383 3.5. Plasma lipid peroxidation increases in TG mice during infection

384 We previously reported that the *in vitro* anti-*Plasmodium* activity of hGIIA sPLA<sub>2</sub> is mediated by  
385 hydrolysis of oxidized lipoproteins and release of toxic PUFAs [54]. We also showed that injection of  
386 recombinant hGIIA sPLA<sub>2</sub> to infected WT mice leads to a reduction of parasitaemia at the time of  
387 elevation of lipid peroxidation in plasma. To assess whether a similar mechanism operates in TG  
388 mice, we measured the peroxidation level of plasma lipids during infection. In agreement with  
389 previous observation in WT mice [54], malondialdehyde (MDA) concentration in infected TG mice  
390 was elevated at day-14 p.i., but not before (day-0 and day-8 p.i.) or after (day-21 p.i.) (Figure 5). This  
391 was in concordance with the highest concentration of circulating hGIIA sPLA<sub>2</sub> observed at day-14 p.i.,  
392 bringing together the appropriate conditions for hydrolysis of oxidized lipoproteins by hGIIA sPLA<sub>2</sub>,  
393 which may explain, at least in part, the observed reduction of parasitaemia.

394

### 395 3.6. Hematological analysis of TG and WT mice infected by *P. chabaudi*

396 Because hGIIA sPLA<sub>2</sub> has been considered as an extracellular mediator of host immune response to  
397 pathogens since a long time [8, 9, 70, 71], we examined whether the reduced parasitaemia observed  
398 in TG mice might be due to differences in the immune response. For instance, it has been reported  
399 that TG mice exhibit elevated numbers of neutrophils and lymphocytes [72], a phenomenon  
400 attributed to the capacity of hGIIA sPLA<sub>2</sub> to release potent inflammatory lipid mediators. We inferred  
401 that overexpression of hGIIA sPLA<sub>2</sub> may be capable to not only modify the mouse immune system at  
402 baseline but also to promote a stronger antimalarial response to *Plasmodium* infection in TG versus  
403 WT mice. We thus analyzed the variation of red blood cells (RBCs) and white blood cells (WBCs), as  
404 well as cytokines before and after infection by *P. chabaudi* in both mouse genotypes.

405 Blood cell numbers in TG mice and WT littermates were compared before infection by automatic and  
406 manual counting from facial and caudal blood samples, respectively (Table 1). Blood from TG mice  
407 contained significantly less RBCs than WT mice ( $\approx 10\%$  reduction in facial blood and  $\approx 30\%$  in caudal  
408 blood; Table 1 and Figure 6). However, the proportion of reticulocytes among RBCs was comparable  
409 in both groups of mice, with similar distribution between young and mature reticulocytes. In  
410 contrast, in agreement with previous studies [72], TG mice had more WBCs than WT mice at baseline  
411 (Table 1 and Figure 7A). Overall, the number of cells in each population of WBCs was higher. Finally,  
412 TG and WT mice exhibited the same number of platelets (Table 1).

413 Both hemoglobin and RBCs are markers of anemia, a well-known phenomenon observed during  
414 malaria and attributed to lysis of parasitized erythrocytes and dysregulation of erythropoiesis [73].  
415 During infection, hemoglobin concentration and the number of RBCs fell down, concomitantly to the  
416 increase of parasitaemia in both genotypes (Table 2 and Figure 6). Longitudinal overview of RBC  
417 counts from day-0 to day-22 p.i. (Figure 6) showed that recovery from anemia after day-16 p.i. is  
418 slower in TG mice, suggesting a possible alteration of erythropoiesis with a negative impact on RBC  
419 renewal in TG animals.

420 In line with higher numbers at baseline, WBC numbers increased with parasitaemia at higher level in  
421 TG mice than in WT littermates (Figures 7A and S1A). Numbers of lymphocytes and neutrophils were  
422 maximal at day-16 p.i. in both groups of mice (Figures 7B and 7C). Remarkably, the number of  
423 neutrophils in TG mice was higher than in WT mice before and after infection (Table 1 and Figure 7C),  
424 and exhibited a much higher increase at day-16 p.i.. The number of neutrophils was 2.4-fold higher  
425 than in WT mice at day-16 p.i., as measured by manual counting ( $p$  value  $< 0.001$ , Figure 7C) and 14.3-  
426 fold higher, as measured by automated counting ( $p$  value  $< 0.001$ , Figure S1C), indicating a stronger  
427 proliferation of neutrophils in TG mice. Of note, neutrophils constituted about 50% of the total  
428 leukocyte population at day-16 p.i. in infected TG mice. By comparison, no such difference was  
429 observed for lymphocytes (Figure 7B), although automated counting showed a 2.2-fold increase in



430 TG lymphocytes at day-16 p.i., as compared to WT (p value <0.001, Figure S1B). TG mice also had a  
431 higher number of monocytes than WT mice at baseline (Table 1) and increased levels during infection  
432 (Figures 7D and S1D). Finally, TG mice had a higher number of eosinophils than WT mice at baseline,  
433 with a marked variation during infection and the largest increase at day-16 p.i. (Table 1, Figures 7E  
434 and S1E).

435 Immune activation of lymphocytes and monocytes was traced by optical examination of changes in  
436 cell morphology on facial blood smears. At baseline, less than 0.1% of lymphocytes were activated in  
437 both genotypes (Table 3). After infection, the percentage of activated lymphocytes was dramatically  
438 increased at day-7 p.i. ( $\approx 11.4\%$ , 125-fold) and day-13 p.i. ( $\approx 22.8\%$ , 250-fold) in TG mice, while the  
439 increase was lower and occurred later in WT littermates (no activation at day-7 p.i. and  $\approx 1.2\%$ , 12-  
440 fold, at day-13 p.i., Table 3). Activated monocytes were observed on smears from TG mice at day-13  
441 and -16 p.i., but were not observed on smears from WT mice.

442 Collectively, these results indicate that TG mice exhibited a more robust immune cell response to  
443 *Plasmodium* infection than WT mice, especially for neutrophils, lymphocytes and monocytes. Thus,  
444 the lower parasitaemia observed in TG mice may also be explained by a more sustained stimulation  
445 of the immune response due to overexpression of hGIIA sPLA<sub>2</sub>.

446

### 447 3.7. Cytokine expression in TG and WT littermate mice before and during *Plasmodium* infection

448 In view of the above findings on WBCs and to investigate a possible specific cytokine response in  
449 parasitized TG mice, plasma samples from TG and WT mice were analyzed for cytokine content  
450 before and after parasite inoculation. Various interleukins (IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-  
451 13), interferon-gamma (IFN- $\gamma$ ), tumor-necrosis factor alpha (TNF- $\alpha$ ) and keratinocyte  
452 chemoattractant/growth regulated oncogene (KC/GRO) were measured in plasma collected at day-0  
453 (before inoculation), day-8 p.i. (onset of the patent phase), day-14 p.i. (parasitaemia peak) and day-  
454 21 p.i. (parasite clearance).

455 Before infection, baseline levels of the Th1 pro-inflammatory cytokines IL-1 $\beta$  and TNF $\alpha$  were 9.7-fold  
456 and 1.7-fold higher in TG mice than WT littermates, with respective mean values  $\pm$  SEM of  $3.4 \pm 0.8$   
457 versus  $0.3 \pm 0.1$  pg/mL (p value = 0.0043) for IL-1 $\beta$  and  $3.5 \pm 0.1$  versus  $2.0 \pm 0.1$  pg/mL (p value =  
458 0.0022) for TNF $\alpha$  (Figure 8A). The level of the Th2 anti-inflammatory cytokine IL-4 was 2.7-fold higher  
459 in the plasma of TG mice ( $0.08 \pm 0.02$  pg/mL in TG versus  $0.03 \pm 0.01$  pg/mL in WT mice, p value =  
460 0.0364) (Figure 8A). Similar concentrations were measured in both groups of mice for other Th1 (IFN-  
461  $\gamma$ , IL-2, IL-12p70) and Th2 (IL-5, IL-10, IL-13) cytokines, as well as for the chemokine KC/GRO  
462 (Figure 8A).

463 Upon infection, in accordance with the central role of the Th1 cytokine IFN- $\gamma$  in mouse models of  
464 malaria infection [61], there was a marked increase in IFN- $\gamma$  at day-8 p.i. in both TG and WT mice  
465 (Figure 8B). Interestingly, IFN- $\gamma$  level in TG mice was 7.7-fold higher than in WT mice (mean value  $\pm$   
466 SEM =  $56.6 \pm 20.5$  pg/mL *versus*  $7.3 \pm 4.0$  pg/mL, p value  $<0.001$ ), indicating a stronger Th1 response.  
467 Accordingly, IL-12p70, a known inducer of IFN- $\gamma$  production, was continuously produced at higher  
468 concentration during infection in TG mice (p value = 0.0024) (Figure 8B). At day-14 p.i., the  
469 concentration of IL-1 $\beta$  was reduced whereas those of TNF- $\alpha$  and IL-2 were increased in both groups  
470 (Figure 8B). The mean IL-2 concentration in TG mice was about 1.7-fold higher than in WT mice (p  
471 value  $<0.05$ ), whereas that of TNF- $\alpha$  was similar in both groups (Figure 8B). In accordance with the  
472 normal course of *P. chabaudi* infection and the shift from a Th1 to a Th2 response at crisis [74],  
473 plasma levels of the Th2 cytokines IL-4 and IL-10 were increased at day-14 p.i. (Figure 8B). However,  
474 the concentration of IL-4 in TG mice was higher at day-14 p.i. than in WT mice (p value  $<0.01$ ),  
475 whereas that of IL-10 was similar in both groups (Figure 8B). Levels of the other Th2 cytokines IL-5  
476 and IL-13 did not vary during infection in both groups of mice (Figure 8B). Interestingly, TG mice  
477 tended to have higher concentrations of the potent neutrophilic and chemotactic cytokine KC/GRO,  
478 which might explain the elevated numbers of neutrophils in TG mice. The KC/GRO concentration was  
479 then lowered at day-14 p.i. in both groups (Figure 8B).

480 Overall, these results suggest that overexpression of hGIIA sPLA<sub>2</sub> does not modify the pattern of  
481 expression of cytokines during *P. chabaudi* infection, but promotes more sustained levels for several  
482 of them at both baseline and during the course of infection, especially for pro-inflammatory  
483 cytokines including the key player IFN- $\gamma$ .

484

#### 485 **4. Discussion and conclusion**

486

487 Figure 9 summarizes the key findings of this study in the context of our previous data, and support a  
488 beneficial role of hGIIA sPLA<sub>2</sub> in malaria. The level of hGIIA sPLA<sub>2</sub> was found to be increased in the  
489 plasma of patients with malaria [47, 48, 54, 75] but its role was unclear. Because high enzymatic  
490 activity was associated with high parasitaemia and neurological complications, early studies  
491 proposed that hGIIA sPLA<sub>2</sub> might be pro-inflammatory and associated with worsening of the disease  
492 [47, 48, 75]. However, our previous and current data suggest an innate protective role of hGIIA in  
493 malaria, which may be in line with its antibacterial role [15, 18, 22, 23].

494 Specifically, we previously found that hGIIA sPLA<sub>2</sub> inhibits the development of *Plasmodium* both *in*  
495 *vitro* when added to cultures of infected human RBCs in the presence of oxidized lipoproteins, and *in*

496 *in vivo* when injected to *Plasmodium*-infected mice at the time lipoproteins are readily oxidized [54]  
497 (Figure 9).

498 To provide more *in vivo* evidence for a protective role of hGIIA sPLA<sub>2</sub> in host defense against  
499 *Plasmodium* infection, we used TG mice overexpressing hGIIA sPLA<sub>2</sub> which, upon infection with  
500 *Plasmodium*, showed increased blood concentrations similar to those observed in patients with  
501 malaria [47, 48, 54, 75] (Figure 9). When these TG mice were infected by the murine parasite  
502 *Plasmodium chabaudi*, we observed an almost 30% reduction of parasitaemia at crisis, further  
503 supporting the notion that hGIIA sPLA<sub>2</sub> has a protective role in malaria. In addition, thanks to the "old  
504 design" of these TG mice where the transgene contains a significant part of the *PLA2G2A* gene with  
505 key promoter elements [56, 64, 65, 76], we observed that the level of circulating hGIIA sPLA<sub>2</sub> is  
506 increased during infection, and identified liver as a possible source of the circulating enzyme.  
507 However, we do not exclude the possibility that circulating hGIIA sPLA<sub>2</sub> may originate from other  
508 sources not analyzed in this study, including immune or stromal cells activated during *Plasmodium*  
509 infection. Furthermore, the factors inducing hGIIA sPLA<sub>2</sub> expression from liver or other tissues were  
510 not investigated in this study, and cytokines such as IL-6 [77] or IFN- $\gamma$  [78, 79] may play a key role.

511 Mechanistically, our data are consistent with a dual mechanism of action of hGIIA sPLA<sub>2</sub>, exerting its  
512 antimalarial protective effect by directly inhibiting parasite multiplication via hydrolysis of oxidized  
513 lipoproteins and production of toxic lipids, and by stimulating a more robust innate and inflammatory  
514 immune response in both chronic and acute ways (Figure 9). We previously showed that the catalytic  
515 activity of both venom (OS<sub>2</sub>) and human (hGX) sPLA<sub>2</sub>s is required for their *in vitro* antimalarial activity  
516 [52, 53], making likely that the enzymatic activity of hGIIA sPLA<sub>2</sub> is also required for its *in vivo*  
517 antimalarial effect. However, this remains to be ascertained *in vivo* using catalytically-inactive  
518 mutants such as H48Q hGIIA sPLA<sub>2</sub> [80].

519 Interestingly, despite high basal level of circulating hGIIA sPLA<sub>2</sub> in TG mice, no difference in  
520 parasitaemia was observed between WT and TG mice before day-11 of infection. This was in line with  
521 our previous data obtained by acute injection of the recombinant enzyme to infected WT mice,  
522 which induced a reduction of parasitaemia only when the enzyme was injected right before the peak  
523 of parasitaemia [54]. Since neither the circulating level of hGIIA sPLA<sub>2</sub> activity nor the genetically  
524 modified immune background of the recipient TG mice seemed to impact on *Plasmodium*  
525 development at the early time of infection, we inferred that secondary events, presumably an  
526 increase of the enzyme concentration to some threshold and/or modifications in the enzyme  
527 environment including access to new substrates, had to occur before hGIIA sPLA<sub>2</sub> became effective  
528 against *Plasmodium*.

529 Interestingly, the increase of enzymatic activity and concentration of hGIIA sPLA<sub>2</sub> at the peak of  
530 parasitaemia coincides with the increase of lipid peroxidation, suggesting that the anti-*Plasmodium*

531 effect of hGIIA sPLA<sub>2</sub> may result from the concerted presence of high levels of the enzyme and  
532 oxidized lipoproteins acting as a preferred substrate of the enzyme and leading to the production of  
533 toxic lipids to *Plasmodium* [54]. We previously showed that hGIIA sPLA<sub>2</sub> is inhibitory to *Plasmodium*  
534 (*P. falciparum*) in *in vitro* culture conditions when the medium contains oxidized but not native  
535 lipoproteins [54]. This is consistent with the higher enzymatic activity of hGIIA observed on oxidized  
536 versus native lipoproteins [81-83]. We further showed that hydrolysis of phospholipids from  
537 lipoproteins by sPLA<sub>2</sub>s releases free PUFAs which are toxic to *Plasmodium* [53, 54]. In the case of  
538 infected WT mice, we previously showed that lipid peroxidation also occurred at the peak of  
539 parasitaemia [54], but these mice do not express the endogenous mouse group IIA sPLA<sub>2</sub> [60] and no  
540 circulating enzymatic activity was detected during infection, supporting the notion that both high  
541 levels of hGIIA (from TG mice or after injection of recombinant hGIIA) and lipid peroxidation are  
542 required to lower parasitaemia (Figure 9). Together, this strongly suggests that the shift from native  
543 to oxidized state of lipoproteins during infection might be a key event to hGIIA sPLA<sub>2</sub> activation  
544 against *Plasmodium*, resulting from phospholipid hydrolysis and release of lipid products that could  
545 act as mediators or effectors of the parasite inhibition process. Lipidomics experiments measuring  
546 the levels of PUFAs and lysophospholipids present in the plasma of infected TG and WT mice should  
547 be performed to identify which lipids are increased in TG mice and may be toxic to *Plasmodium*.  
548 From a translational perspective, it has been shown that plasma from malaria patients contain high  
549 levels of hGIIA sPLA<sub>2</sub> and of lipid peroxidation but no careful follow-up was performed [54, 55, 84-  
550 86]. It thus remains to determine whether the two factors are increased concomitantly in patients  
551 with malaria.

552 Considering further the mechanisms of the anti-*Plasmodium* effect of hGIIA sPLA<sub>2</sub>, alternative  
553 substrates to lipoproteins may be envisaged. Indeed, it is known that hGIIA sPLA<sub>2</sub> exhibits very low  
554 enzymatic activity on zwitterionic substrates like the phosphatidylcholine-rich outer leaflet of the  
555 plasma membrane of resting cells or the surface of native lipoproteins [87, 88]. However, the enzyme  
556 becomes active in the presence of lipid membranes enriched in negatively charged phospholipid  
557 substrates such as activated or apoptotic cells, oxidized lipoproteins, extracellular vesicles (EVs),  
558 bacteria or even platelet-extruded mitochondria [12, 16, 18, 89, 90]. Such peculiar substrates are  
559 more abundant in pathological situations like infection and inflammation and sPLA<sub>2</sub>s can even be  
560 loaded into exosomes [91-93]. As for malaria, several studies have reported increased levels of EVs  
561 from host and parasite origin during active *Plasmodium* infection, which return to normal levels after  
562 resolution of infection. Accumulating evidence suggests that EVs contribute to malaria-associated  
563 clinical symptoms [94-96]. *P. falciparum*-derived EVs were also shown to mediate cell-cell  
564 communication between parasites, propagating drug resistance among the parasite population or  
565 inducing gametocyte differentiation [97, 98]. As far as we know, interaction of hGIIA sPLA<sub>2</sub> with EVs

566 has not been investigated in the context of malaria. It thus remains to determine whether hGIIA  
567 sPLA<sub>2</sub> might regulate parasite development through hydrolysis of EVs, either by controlling the rate  
568 of EV production/degradation, thereby modulating the role of EVs in malaria, and/or by generating  
569 toxic lipids from EVs, as from oxidized lipoproteins. Enzymatic activity of hGIIA sPLA<sub>2</sub> on the above  
570 substrates might contribute to the reduction of parasitaemia through the generation of toxic PUFAs,  
571 but also through the production of lipid mediators modulating the immune response during infection  
572 [11, 99] (Figure 9).

573 Another important aspect that may explain the anti-*Plasmodium* effect of hGIIA sPLA<sub>2</sub> in TG mice is  
574 the chronically-modified immunological background of these mice induced by the constitutive  
575 overexpression of the enzyme and the clearly higher acute innate immune response to *Plasmodium*  
576 infection. At baseline, hematological analysis revealed a specific blood profile of TG mice. Blood from  
577 TG mice was enriched in neutrophils and lymphocytes whereas the number of platelets was normal,  
578 which confirmed previous data in the same mouse line [72]. Additionally, the number of monocytes  
579 and eosinophils was higher and that of RBCs lower, suggesting marked hematopoiesis disorder in TG  
580 mice. Of note, although the level of anemia occurring at high parasitaemia was similar in infected TG  
581 and WT mice, recovery from anemia seemed to be delayed in TG mice. This erythropoiesis  
582 impairment suggests that hGIIA sPLA<sub>2</sub> might be an as yet unsuspected actor in the implementation of  
583 severe anemia, which is observed in complicated cases of malaria [73]. During infection, the number  
584 of leukocytes remained high in TG mice, and the increase was maximal at crisis. Neutrophils mostly  
585 accounted for this increase. In addition, infected TG mice had higher levels of activated lymphocytes  
586 and monocytes than WT mice, indicating a more robust activation of the immune response. These  
587 observations are in accordance with the well-known ability of sPLA<sub>2</sub>s or their lipid products to induce  
588 leukocyte recruitment and activation during inflammation and infection [11, 14, 36, 100-106]. The  
589 enhanced and/or specific activation of immune cells might contribute to the improved capacity of TG  
590 mice to control parasite development, for example by increasing the phagocytic power of immune  
591 cells, as reported for monocytes from monkeys injected with sPLA<sub>2</sub> [107] or for macrophages  
592 activated by mGV sPLA<sub>2</sub> during infection with *Candida albicans* [105, 108].

593 The baseline cytokine profile of TG mice was different from that of WT mice. Levels of the pro-  
594 inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  were clearly higher and those of IL-12p70 and KC/GRO  
595 chemokine showed a higher trend (also observed during infection), whereas IL-4 was increased  
596 among the anti-inflammatory cytokines. Thus, despite the fact that no significant inflammatory cell  
597 infiltrates were observed in the skin or other tissues of TG mice [56], which suggests that the immune  
598 background of TG mice is not inflammatory, the modified cytokine profile observed in TG mice  
599 suggests a priming of this latter by hGIIA sPLA<sub>2</sub>, leading to low-grade systemic inflammation. In this  
600 regard, it is interesting to note that IL-1 $\beta$  and TNF- $\alpha$ , whose levels are increased in TG mice, are well-

601 known regulators of hematopoiesis [109-111] and might contribute, along with hGIIA sPLA<sub>2</sub> and lipid  
602 mediators, to set up the abnormally elevated leukocyte concentration observed in TG mice.

603 Comparative analysis of the cytokine profile during infection in TG and WT mice showed quantitative  
604 but not qualitative differences. The hallmark of malaria parasite infection in the early stages is a  
605 strong protective cell-mediated Th1 immune response characterized by increased IFN- $\gamma$ . The Th1  
606 response eventually wanes with disease progression with a switch to a Th2 immune response. IL-10 is  
607 believed to play a critical role in the regulation of host immune response following *Plasmodium*  
608 infection and potentially orchestrates the switch of Th1/Th2 immune dominance during disease  
609 progression [112]. As expected from previous data showing a central role of IFN- $\gamma$  in the  
610 development of protective immunity against blood-stage infection by *P. chabaudi* [61], levels of IFN- $\gamma$   
611 were very high at day-8 p.i. in both types of mice, yet much higher in TG mice. In this respect, it is  
612 interesting to note that IFN- $\gamma$  is an inducer of hGIIA sPLA<sub>2</sub> production [78, 79]. The non-coding  
613 upstream region of 1.6 kbp present in the *PLA2G2A* transgene contains the transcriptional response  
614 element consensus sequence for IFN- $\gamma$  [56], suggesting that the increase in sPLA<sub>2</sub> activity observed  
615 during infection might result from IFN- $\gamma$ -induced production of hGIIA sPLA<sub>2</sub>. It is also interesting to  
616 note that, when using the same mouse model of infection by *P. chabaudi*, injection of monoclonal  
617 antibodies blocking IFN- $\gamma$  increased the peak of parasitaemia but did not impact on the course of  
618 infection [113], mirroring the effect of hGIIA sPLA<sub>2</sub>. This in turn suggests that IFN- $\gamma$  and hGIIA sPLA<sub>2</sub>  
619 may act on the same pathway, one possibly activating the other in an autocrine/paracrine loop, and  
620 with the possibility that inhibition of IFN- $\gamma$  would prevent the increase of hGIIA sPLA<sub>2</sub> observed at the  
621 peak of parasitaemia. At day-14 p.i., pro-inflammatory TNF- $\alpha$  and IL-2 were high and IFN- $\gamma$  was low,  
622 whereas anti-inflammatory IL-4 and IL-10 were increased. Thus, the well-described Th1/Th2 switch  
623 observed in *P. chabaudi* infection [114, 115] was also seen in TG mice, indicating that hGIIA sPLA<sub>2</sub>  
624 does not influence the time course of cytokine production during infection, but rather enhance the  
625 production of several cytokines, especially those involved in the pro-inflammatory Th1 pathway, in  
626 particular IFN- $\gamma$ .

627 Finally, in an attempt to reveal the possible involvement of other endogenous sPLA<sub>2</sub>s during infection  
628 by *P. chabaudi*, we measured the mRNA levels of sPLA<sub>2</sub>s known to have an *in vitro* effect on  
629 *Plasmodium*, namely mGIIF, mGIII, mGV and mGX sPLA<sub>2</sub>s [53, 54]. In our RT-qPCR conditions, mRNAs  
630 for these four sPLA<sub>2</sub>s were undetectable in liver and blood cells from both groups of mice, before and  
631 after infection. In contrast, mGIIF and mGV but not mGIII and mGX mRNAs were detectable before  
632 infection in spleen from TG and WT mice. The amounts of mGIIF and mGV mRNAs were decreased  
633 during the course of infection, in line with their possible roles in inflammatory response and host  
634 defense [11, 36, 116]. These results indicate that infection by *P. chabaudi* does not mobilize any of

635 these four sPLA<sub>2</sub>s in the liver, spleen or blood cells, and probably not at the systemic level, since no  
636 circulating enzymatic activity was detected in infected and non-infected WT mice. Our data also  
637 suggest that the overexpression of hGIIA sPLA<sub>2</sub> does not modify the expression of these sPLA<sub>2</sub>s.  
638 In conclusion, we have shown that TG mice overexpressing hGIIA sPLA<sub>2</sub> better control infection by *P.*  
639 *chabaudi* than their WT littermates (Figure 9). In this murine model, the decrease of parasitaemia at  
640 the peak of infection is concomitant with the simultaneous increase of hGIIA sPLA<sub>2</sub> enzymatic activity  
641 and in lipid peroxidation. This suggests that hGIIA sPLA<sub>2</sub> exerts most of its antimalarial activity in an  
642 acute way by a specific and concerted mechanism of action where i) lipoproteins are transiently  
643 oxidized because of infection and activation of the inflammatory and immune response ii) the  
644 enzyme is induced and hydrolyzes oxidized lipoproteins to release active lipid metabolites in the  
645 vicinity of infected RBCs, leading to either direct killing of the parasite and/or stimulation of a more  
646 robust immune response. Collectively, our data support a protective role of hGIIA sPLA<sub>2</sub> in human  
647 malaria, but this remains to be further investigated, especially because the major parasite at play in  
648 humans, *Plasmodium falciparum*, is different. This parasite can also lead to severe malaria where  
649 hGIIA sPLA<sub>2</sub> may play a role in the brain compartment [75], but this could not be investigated in this  
650 animal model of malaria. Notable differences are also known between rodent and human  
651 *Plasmodium* parasites in terms of lipid metabolism [117]. Furthermore, human but not mouse  
652 platelets are an abundant source of group IIA sPLA<sub>2</sub> [12, 16, 118], while platelets are an important  
653 player in *Plasmodium* infection [119]. This brings attention to the possible role of platelets in the  
654 antimalarial effect of hGIIA in humans, as both a source of sPLA<sub>2</sub> enzyme and substrates including EVs  
655 and extruded mitochondria. It will be worth to also investigate on one hand the role of endogenous  
656 mouse group IIA (Pla2g2a) and of other sPLA<sub>2</sub>s using appropriate inbred mouse strains [29] or gene  
657 knockout mice [9], and on the other hand the effect of the broadly specific sPLA<sub>2</sub> inhibitor  
658 varespladib, which has been tested in several clinical trials as a drug candidate for sepsis and other  
659 inflammatory diseases but has led to mitigated therapeutic effects or even adverse effects [120-122].  
660 Interestingly, LY311727, an analog of varespladib, was shown to induce earlier mortality in a mouse  
661 model of toxoplasmosis [123]. From a therapeutical point of view and based on our present studies,  
662 it is thus tempting to speculate that sPLA<sub>2</sub>s should not be inhibited in human malaria but rather  
663 stimulated, at least for non-severe cases of malaria. Last, genome-wide association studies have  
664 shown that single nucleotide polymorphisms present in the *PLA2G2A* gene and in other sPLA<sub>2</sub> genes  
665 may be associated to malaria, inflammatory diseases and cardiovascular diseases [124, 125],  
666 warranting further investigation of hGIIA sPLA<sub>2</sub> and other sPLA<sub>2</sub>s for genetic susceptibility to malaria.  
667 These are as some of the directions for future investigation of the role of hGIIA sPLA<sub>2</sub> and other  
668 sPLA<sub>2</sub>s in human malaria.  
669

670 **Declaration of competing interest**

671

672 The authors have no conflict of interest to declare.

673

674 **Author Contributions**

675 M.D. designed, performed and analyzed the experiments.

676 A.J. was in charge of mouse breeding and participated in animal studies.

677 M.D., S.C. and A.L. designed, performed and analyzed RT-qPCR experiments.

678 A.P.P. and F.B. performed and analyzed multiplex cytokine assays.

679 I.L. performed automated and manual analysis of blood components.

680 C.P. produced recombinant sPLA<sub>2</sub>s and performed enzymatic and TR-FIA assays.

681 P.G. discussed the data at different steps of the study and reviewed the manuscript.

682 M.D. C.D., G.L. and L.T. designed the project and wrote the manuscript.

683

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690

691 **Appendix A. Supplementary data**

692

693 Supplementary data to this article can be found online at xxxx

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695 **Legend to Figures**

696

697 **Figure 1.** Time-course of parasitaemia in TG mice and WT littermates infected with *P. chabaudi*.

698 Results from four independent infection experiments totalizing 23 TG and 19 WT mice have been

699 pooled. Panel A: Parasitaemia profiles from TG (■) and WT (□) mice inoculated at day-0 with  $1 \times 10^6$

700 *P. chabaudi*-infected RBCs by IP injection. Values are mean  $\pm$  SEM. Two-way ANOVA statistical

701 analysis with Bonferroni's post-test (★) was applied after data normalization. A significant difference

702 (\*\*\*p-value < 0.0001) between TG and WT profiles was observed at day-14 p.i. Panel B: Comparative

703 distribution of blood parasitaemia values at day-14 p.i. between infected TG and WT mice. A



704 significant difference of 27.6% (unpaired t test, \*\*\*p-value = 0.0002) was observed between the  
 705 mean peak of parasitaemia for TG versus WT mice. Horizontal lines are mean with SEM.

706 **Figure 2.** Variation of weight in animal body and organs in TG mice and WT littermates during *P.*  
 707 *chabaudi* infection. Panel A: body weight. Results from two independent infection experiments  
 708 totalizing eleven WT and seven TG mice have been pooled. Mice (9-weeks old) were weighed at day-  
 709 0 before inoculation and then every 2-3 days. Box whiskers: body weight values at day-0 (P0) and at  
 710 maximal parasitaemia (Pmax). Statistical analysis: unpaired t-test was used to compare body weight  
 711 of non-infected WT and TG mice; paired t-test was used to compare body weight before parasite  
 712 inoculation and at maximal parasitaemia. Panel B: liver weight from seven WT and seven TG mice at  
 713 each time point. Statistical analysis using two-way ANOVA (#) test with Bonferroni's post-test  
 714 indicates that liver weight increases significantly during infection (###p-value < 0.0001), and with  
 715 differences in increased rates between genotypes (##p-value = 0.0079) at day-14 (\*\*p<0.01) and day-  
 716 21 p.i. (\*\*p<0.01)). Panel C: spleen weight from nine WT and nine TG mice at each time point. Spleen  
 717 weight increases significantly during infection (###p-value < 0.0001) without difference between  
 718 genotypes. Panel D: kidney weight from nine WT and nine TG mice at each time point. No statistical  
 719 difference was observed during infection or between genotypes. Values are mean  $\pm$  SEM.

720 **Figure 3.** Plasma level of sPLA<sub>2</sub> enzymatic activity (A) and hGIIA sPLA<sub>2</sub> concentration (B) during  
 721 infection of TG mice. A: Plasma samples from tail blood were collected before (Day-0) and at  
 722 different days after inoculation of TG mice with  $1 \times 10^6$  *P. chabaudi*-infected RBCs. Samples were  
 723 analyzed for sPLA<sub>2</sub> enzymatic activity using [<sup>3</sup>H]-oleic acid radiolabeled *E. coli* membranes as  
 724 substrate. Results are from two independent infection experiments with groups of six mice (12 mice  
 725 per day until day-20, then six mice per day); mean  $\pm$  SEM values are presented. For statistical  
 726 analysis, one-way ANOVA (#) with Dunnett's post-test (\*) was applied. Enzymatic activity varied  
 727 significantly during infection (#p-value = 0.0032) with a net increase at day-11 p.i. and day-14 p.i.  
 728 (day-11 p.i. versus day-0: \*\*p-value <0.01; day-14 p.i. versus day-0: \*p-value <0.05). B: TR-FIA  
 729 analysis was performed using specific antibodies for hGIIA sPLA<sub>2</sub> on plasma samples from mice  
 730 infected with *P. chabaudi*. Blood was collected from groups of five to six mice at day-0 before  
 731 inoculation, and day-8, -14 and -21 post-inoculation. Mean  $\pm$  SEM values are presented. Significant  
 732 difference was found between day-0 and day-14 using one-way Anova with Dunnett's post-test (\*\*p-  
 733 value = 0.0028).

734 **Figure 4.** Gene expression of sPLA<sub>2</sub>s in TG mice and WT littermates during *P. chabaudi* infection. Total  
 735 RNA extracts were prepared from organs at different time points before (Day-0) and after parasite  
 736 inoculation (Day-8 to 21). Expression level of mRNA for hGIIA, mGIIF and mGV sPLA<sub>2</sub>s was normalized

737 using GUSB, HPRT1 and TBP as housekeeping genes for spleen; HPRT1, TBP and PGK1 for liver; and  
 738 GUSB, HPRT1, TBP and Act B for blood cells. Each point value is the mean of duplicate (liver and  
 739 spleen) or triplicate (blood cells) measurements. Error bars are mean  $\pm$  SEM values. Panel A: RT-qPCR  
 740 analysis of hGIIA sPLA<sub>2</sub> mRNA in liver, spleen and blood cells from infected TG mice. Two  
 741 independent infection experiments were performed and data were pooled. In total, hGIIA sPLA<sub>2</sub>  
 742 mRNA was analyzed from spleen from eleven and seven TG mice, liver from thirteen and six TG mice,  
 743 and blood cells from seven and five TG mice, respectively. Panel B: RT-qPCR analysis of mGV and  
 744 mGIIF sPLA<sub>2</sub> mRNAs from spleen of TG mice versus WT littermates during *P. chabaudi* infection. Two  
 745 independent infection experiments were performed and data were pooled. Total RNA extracts were  
 746 prepared at different time points during infection from spleen of eleven and seven TG mice, and  
 747 eight and three WT mice, respectively.

748 **Figure 5.** Plasma lipid peroxidation in infected TG mice. Results from three independent infection  
 749 experiments have been pooled. Mice were inoculated at day-0 with  $1 \times 10^6$  *P. chabaudi*-infected  
 750 RBCs. Blood was collected right before inoculation (day-0, 19 mice), at day-8 (13 mice), day-14 (18  
 751 mice) and day-21 (6 mice) post-inoculation. Lipid peroxidation was assessed by measuring the end-  
 752 product malondialdehyde using the TBARS assay. Lipid peroxidation varied during infection (One-way  
 753 Anova: ###p-value = 0.0004) with a net increase at day-14 p.i. (Dunnett's post-test: day-14 p.i. versus  
 754 day-0, \*\*\*p-value <0.001).

755 **Figure 6.** Variation of RBC numbers in TG mice and WT littermates during *P. chabaudi* infection.  
 756 Results from three independent infection experiments, totalizing 12 TG mice and 13 WT littermates.  
 757 Tail blood was collected at day-0 (before inoculation) and then every 2-4 days during 22 days.  
 758 Erythrocytes were numbered using Kova slides (manual counting). For statistical analysis, two-way  
 759 ANOVA (#) with Bonferroni's (\*) post-test was applied after data normalization. RBC number varied  
 760 during the course of infection (###p-value < 0.0001). Variation was different between genotypes (#p-  
 761 value = 0.0155). Difference was obvious at day-7 p.i. (\*p-value <0.05) and day-22 p.i. (\*\*p-value  
 762 <0.01). A significant interaction was observed between time and genotype (##p-value = 0.0034).

763 **Figure 7.** Levels of blood leukocytes in TG mice and WT littermates during *P. chabaudi* infection  
 764 (manual counting). Three independent infection experiments were pooled, totalizing 12 TG mice and  
 765 13 WT littermates. About 10-20  $\mu$ L of blood was taken from tail tips of infected animals at day-0  
 766 before parasite inoculation and then every 2-4 days after inoculation of  $1 \times 10^6$  *P. chabaudi*-infected  
 767 RBCs. Total WBCs were counted on Kova slides after lysis of erythrocytes. Relative numbers of WBC  
 768 subpopulations were calculated from distribution on Diff-Quik stained blood smears. For statistical  
 769 analysis, two-way ANOVA (#) with Bonferroni's post-test (\*) was applied.

770

771 **Figure 8.** Plasma levels of cytokines in TG mice and WT littermates before (A) and after (B) *P.*  
772 *chabaudi* inoculation. Levels of cytokines were quantified using the U-Plex Th1/Th2 Combo Mouse kit  
773 (Meso Scale Discovery). Plasma was prepared from blood collected from five WT and five TG mice at  
774 day-0 before inoculation, six WT and six TG mice at day-8 p.i., six WT and six TG mice at day-14 p.i.  
775 and six WT and five TG mice at day-21 p.i.. Values are mean  $\pm$  SEM. (A) Cytokine concentrations in  
776 WT and TG mice before infection. Mann-Whitney U test was applied to compare cytokine  
777 concentrations between genotypes. (B) Cytokine concentrations after parasite inoculation. Two-way  
778 ANOVA test (#) with Bonferroni's post-test ( $\star$ ) was applied to analyze variation in cytokine  
779 concentrations during infection. Results from Bonferroni's test indicate differences between WT and  
780 TG mice at specific days post-inoculation. Where appropriate, steady-state differences between  
781 genotypes during infection, as well as time-genotype dependent interaction, are indicated.

782

783 **Figure 9. Summary of the findings at a glance.** In a previous study [54], we reported that WT mice  
784 (grey) infected by *P. chabaudi* and then treated with recombinant hGIIA sPLA<sub>2</sub> have a 20% lower  
785 parasitaemia than untreated mice. Mechanistically, the enzyme may function by hydrolysis of  
786 oxidized lipoproteins (oxidation is due to infection) and release of lipids directly toxic to *Plasmodium*  
787 growth. In this previous study, we did not evaluate the effects of recombinant sPLA<sub>2</sub> on Th1  
788 activation at the cytokine and immune cell levels, which may also result from the release of lipid  
789 mediators from oxidized lipoproteins or other mechanisms. In the current study, we show that  
790 transgenic mice (TG, green) constitutively expressing hGIIA sPLA<sub>2</sub> better control *Plasmodium*  
791 proliferation than WT littermates. Like in human malaria, a concomitant increase in lipoprotein  
792 oxidation and hGIIA circulating activity/concentration is observed in infected TG mice, along with a  
793 stronger activation of immune cells and enhanced release of pro-inflammatory cytokines. It is known  
794 that hGIIA sPLA<sub>2</sub> can better hydrolyze oxidized lipoproteins. By piling up both studies, we hypothesize  
795 that oxidation of lipoproteins during infection promote their hydrolysis by hGIIA sPLA<sub>2</sub>, and thereby  
796 release lipids directly toxic to the parasite and/or lipid mediators triggering the immune response.  
797 Thus, both direct and indirect mechanisms likely contribute to the 20% (hGIIA-treated mice) and 30%  
798 (TG mice) lower parasitaemia observed in infected mice. Altogether, our data suggest a beneficial  
799 role of hGIIA sPLA<sub>2</sub> in malaria.

800

801 **References**

802

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## TABLES

**Table 1. Blood cell population from TG and WT littermate mice at baseline.** Manual counting: three independent experiments respectively comprising 7 WT and 6 TG mice (exp. 1), 10 WT and 11 TG mice (exp. 2), and 19 WT and 7 TG mice (exp. 3) were performed. Cell counting was carried out from blood tail. Percentages of leukocyte subpopulations were established from Diff-Quik-stained blood smears. Automated counting (Sysmex 2000iV technology) was performed from facial blood from WT (n=6) and TG (n=6) mice. Reticulocytes were distributed into three categories according to fluorescence intensity: LFR (low fluorescence reticulocytes, corresponding to mature reticulocytes), MFR (medium fluorescence reticulocytes, i.e. middle-age immature reticulocytes) and HFR (high fluorescence reticulocytes, i.e. young immature reticulocytes). Values are expressed as mean  $\pm$  SEM. ND: not determined. T test (Mann-Whitney) was applied for statistical analysis. Note that discrepancies were observed between manual and automated counting, which can be attributed to the localization (tail vein or facial vein) and method of blood sampling, known to possibly alter blood values [1] and to the methodology used for cell counting (i.e. automated *versus* manual). P values <0.05 (\*), <0.01 (\*\*), and <0.001 (\*\*\*) were considered statistically significant.

Blood cells	Facial blood (Automated counting)			Caudal blood (Manual counting)		
	WT (n = 6)	TG (n = 6)	P value (TG vs WT)	WT (n=36)	TG (n=24)	P value (TG vs WT)
<b>Total WBCs</b> ( $10^3/\mu\text{L}$ )	6.08 $\pm$ 0.73	10.15 $\pm$ 0.63	0.0043**	20.58 $\pm$ 1.89	26.11 $\pm$ 2.50	0.0164*
Lymphocytes ( $10^3/\mu\text{L}$ )	4.50 $\pm$ 0.50	6.50 $\pm$ 0.30	0.0043**	12.57 $\pm$ 0.98	12.08 $\pm$ 1.21	0.7456
Neutrophils ( $10^3/\mu\text{L}$ )	1.24 $\pm$ 0.45	2.57 $\pm$ 0.38	0.1320	4.29 $\pm$ 0.34	11.78 $\pm$ 1.24	<0.0001***
Monocytes ( $10^3/\mu\text{L}$ )	0.26 $\pm$ 0.05	0.81 $\pm$ 0.03	0.0022**	0.53 $\pm$ 0.08	1.13 $\pm$ 0.13	0.0004***
Eosinophils ( $10^3/\mu\text{L}$ )	0.07 $\pm$ 0.01	0.21 $\pm$ 0.02	0.0050**	0.51 $\pm$ 0.08	1.21 $\pm$ 0.16	<0.0001***
Basophils ( $10^3/\mu\text{L}$ )	0.005 $\pm$ 0.002	0.013 $\pm$ 0.003	0.0906	ND	ND	
<b>Total RBCs</b> ( $10^6/\mu\text{L}$ )	10.67 $\pm$ 0.24	9.72 $\pm$ 0.22	0.0152*	5.94 $\pm$ 0.57	4.16 $\pm$ 0.41	0.0292*
Reticulocytes (%)	5.0 $\pm$ 0.4	4.8 $\pm$ 0.3	0.6991			
LFR (%)	47.9 $\pm$ 0.9	45.6 $\pm$ 1.2	0.3095			
MFR (%)	20.1 $\pm$ 0.7	18.5 $\pm$ 0.7	0.1255			
HFR (%)	32.0 $\pm$ 1.4	35.8 $\pm$ 1.1	0.0931			
<b>Platelets</b> ( $10^3/\mu\text{L}$ )	1494 $\pm$ 76.1	1694 $\pm$ 95.5	0.1320	ND	ND	

**Table 2. Red blood cell number and anemia during infection in TG and WT littermate mice.** Automated analysis of facial blood of mice was performed using the Sysmex 2000iV technology. Values are expressed as mean  $\pm$  SEM (and median under parenthesis).

Day post-inoculation	WT				TG			
	0	7	13	16	0	7	13	16
Mice (n number)	6	5	5	5	6	3	4	3
Red blood cells ( $10^6/\mu\text{L}$ )	10.67 $\pm$ 0.24 (10.51)	10.39 $\pm$ 0.26 (10.71)	4.76 $\pm$ 0.22 (4.5)	2.64 $\pm$ 0.45 (2.59)	9.72 $\pm$ 0.22 (9.72)	9.57 $\pm$ 0.27 (9.49)	6.35 $\pm$ 0.26 (6.25)	2.36 $\pm$ 0.55 (2.25)
hematocrit (%)	47.27 $\pm$ 0.95 (47.4)	45.5 $\pm$ 0.81 (46.3)	17.40 $\pm$ 0.95 (17.7)	25.30 $\pm$ 3.55 (26.6)	45.15 $\pm$ 0.76 (45.4)	44.07 $\pm$ 1.11 (43.8)	22.63 $\pm$ 1.40 (22.6)	17.67 $\pm$ 1.77 (17.0)
hemoglobin (g/dL)	15.33 $\pm$ 0.28 (15.55)	14.96 $\pm$ 0.27 (15.1)	4.76 $\pm$ 0.23 (4.45)	6.10 $\pm$ 0.64 (5.90)	14.68 $\pm$ 0.35 (14.85)	14.50 $\pm$ 0.37 (14.4)	6.35 $\pm$ 0.38 (6.25)	4.23 $\pm$ 0.48 (3.80)

**Table 3. Morphology-based detection of leukocyte activation during infection.** Leukocytes were identified by optical examination of blood smears from TG and WT littermate mice. Activated lymphocytes are presented as the percentage of activated lymphocytes over the total number of lymphocytes. Activated monocytes are presented as the number of smears exhibiting activated monocytes versus the total number of smears. Values are mean  $\pm$  SEM.

Mice		Days after infection			
		Day-0	Day-7	Day-13	Day-16
<b>TG</b>	Mice (n number)	5	5	4	3
	Activated Lymphocytes (%)	0.09 $\pm$ 0.01	11.42 $\pm$ 1.77	22.75 $\pm$ 4.20	3.43 $\pm$ 1.89
	Activated Monocytes	0/5	0/5	4/4	3/3
<b>WT</b>	Mice (n number)	6	5	5	5
	Activated Lymphocytes (%)	0.09 $\pm$ 0.01	0.04 $\pm$ 0.01	1.20 $\pm$ 0.34	0.50 $\pm$ 0.02
	Activated Monocytes	0/6	0/5	0/5	0/5

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Figure 1

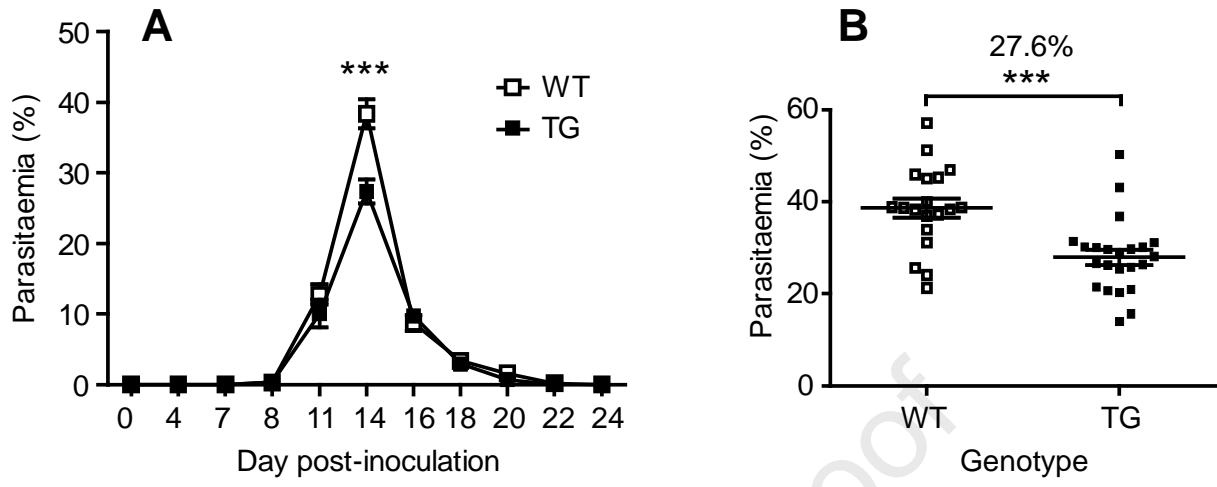




Figure 2

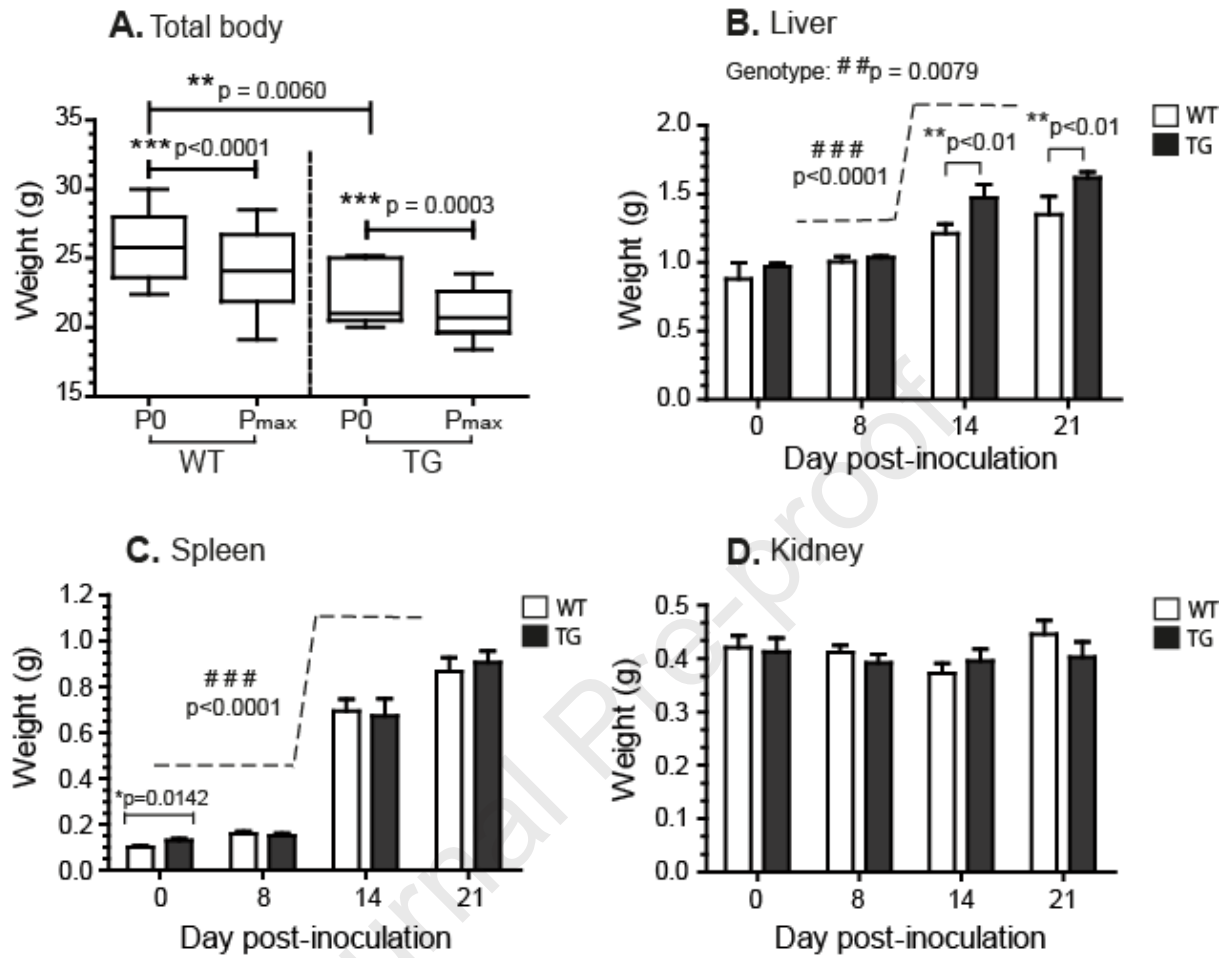


Figure 3

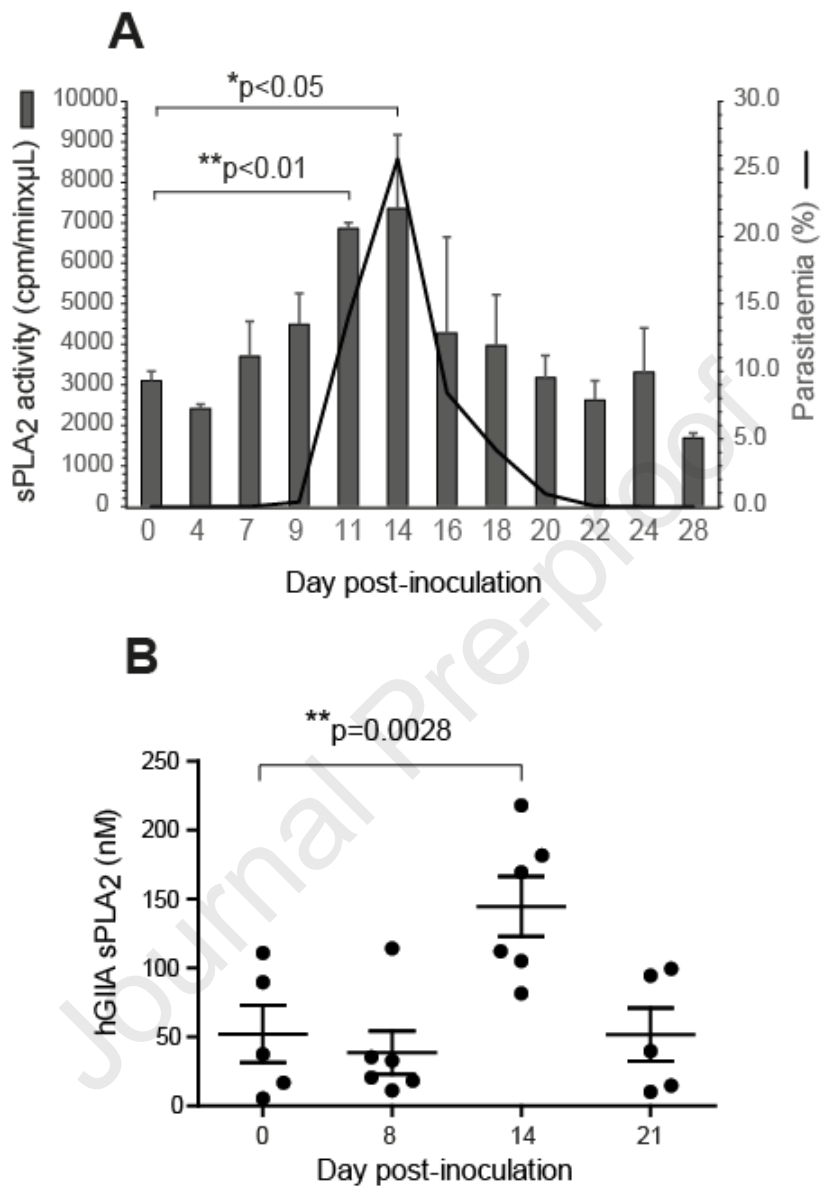
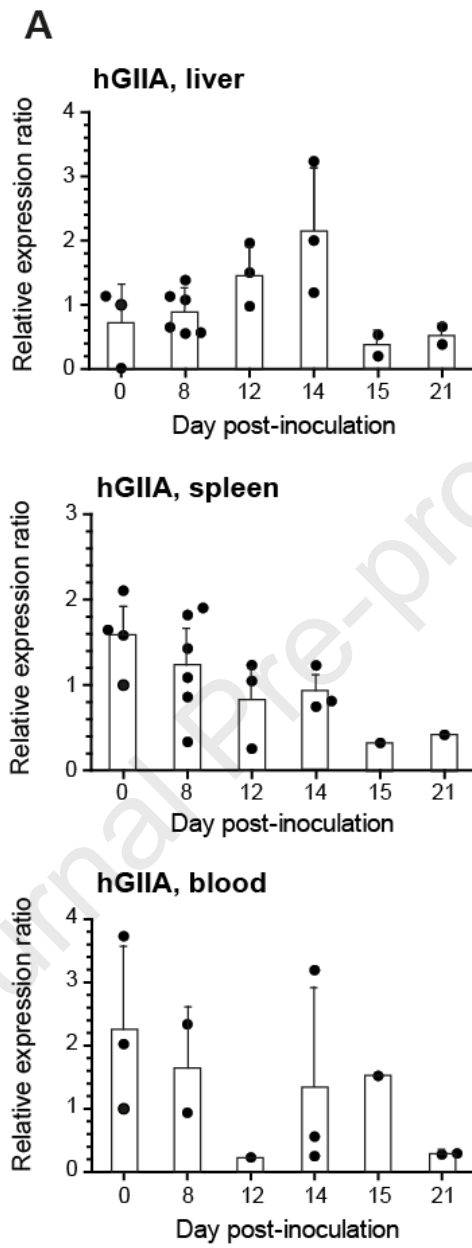


Figure 4



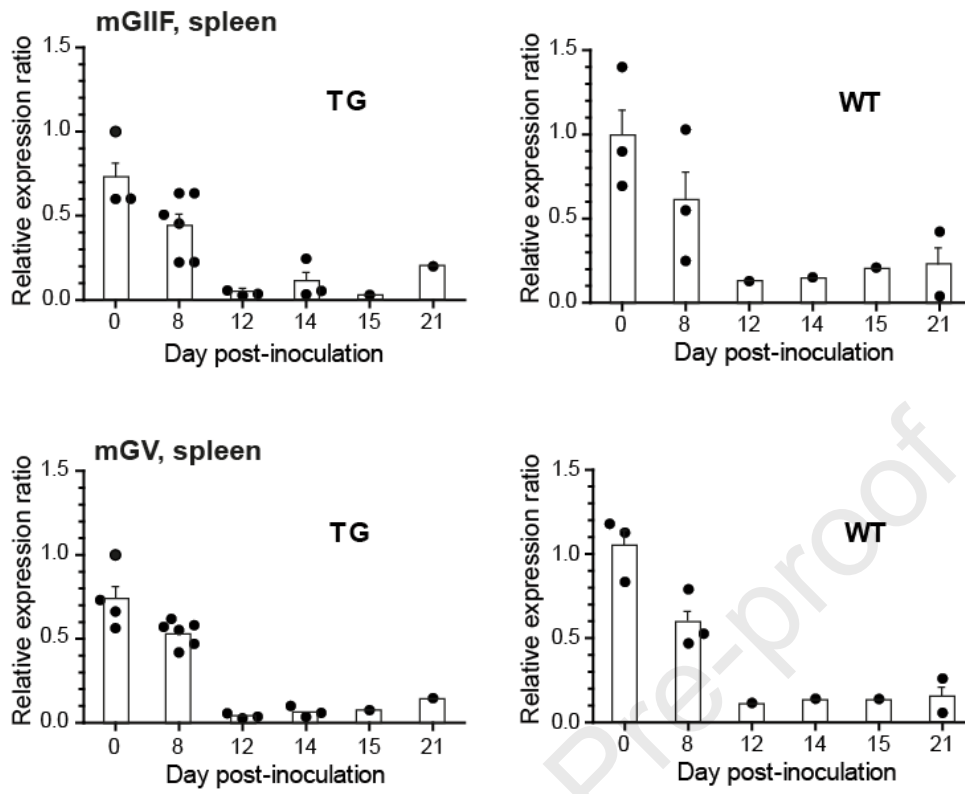
**B**

Figure 5

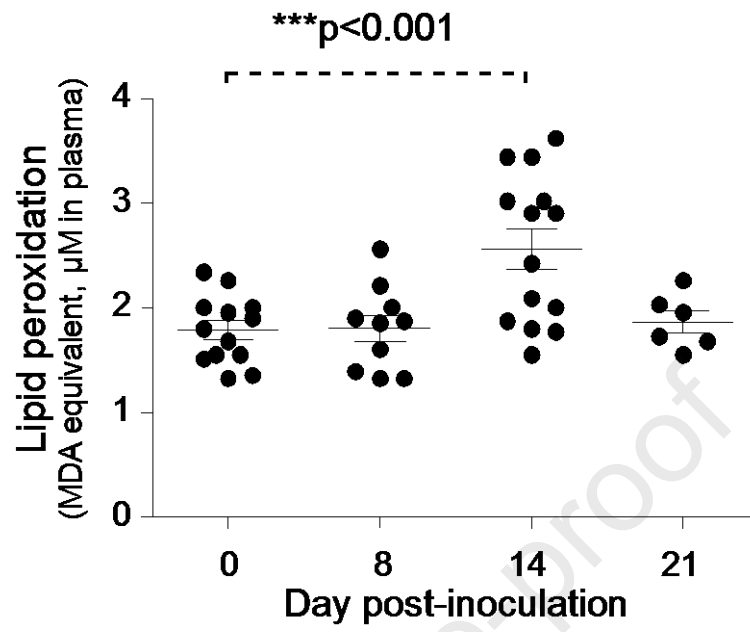


Figure 6

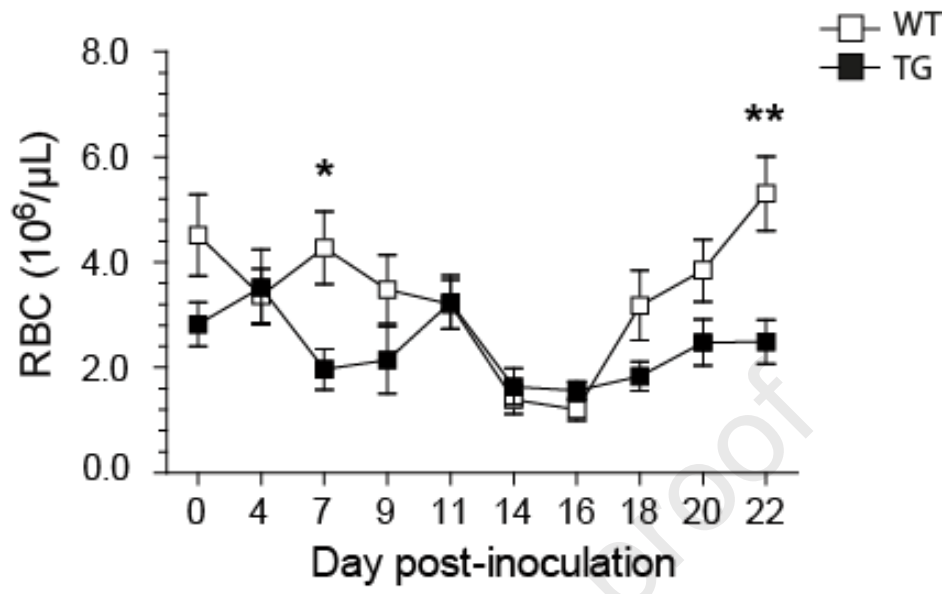


Figure 7

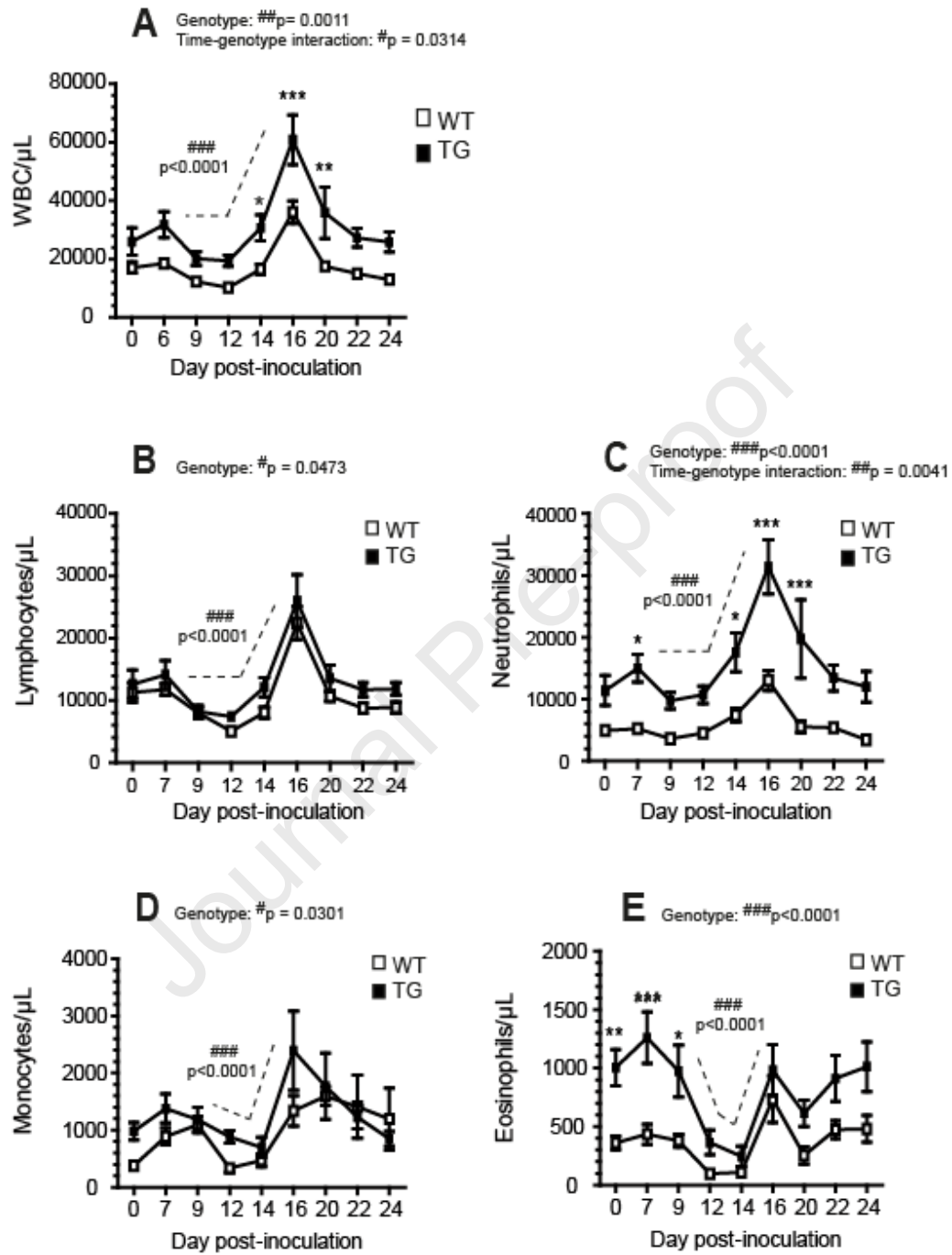
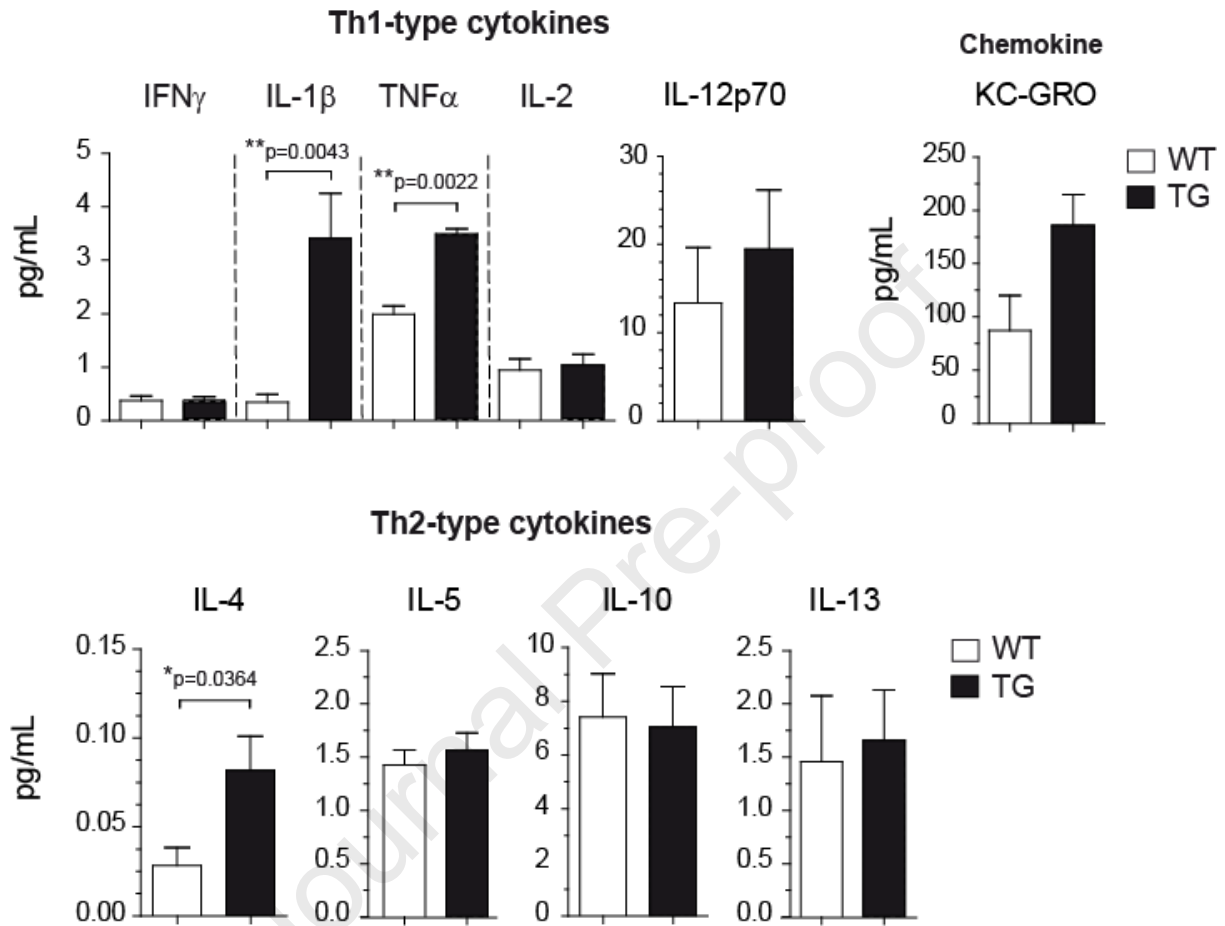


Figure 8

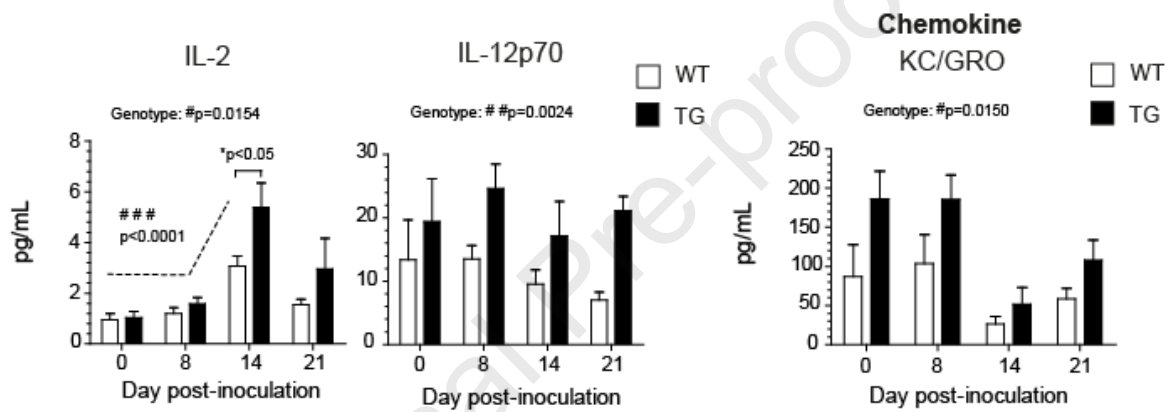
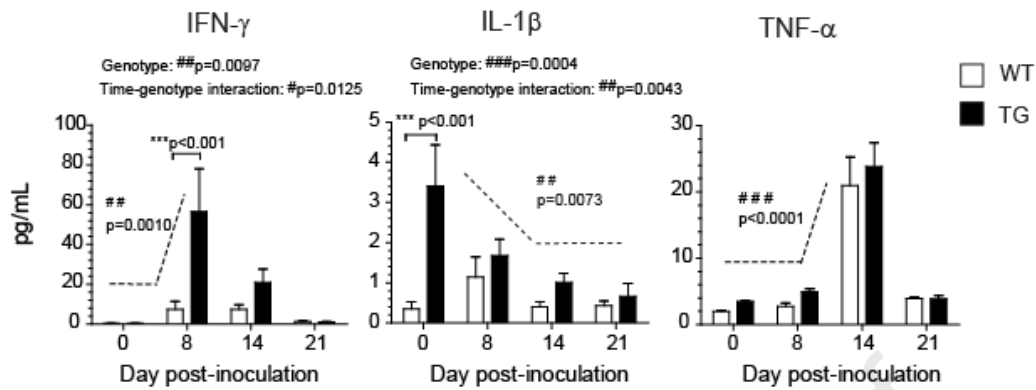
## A. Before parasite inoculation





## B. After parasite inoculation

## Th1-type cytokines



## Th2-type cytokines

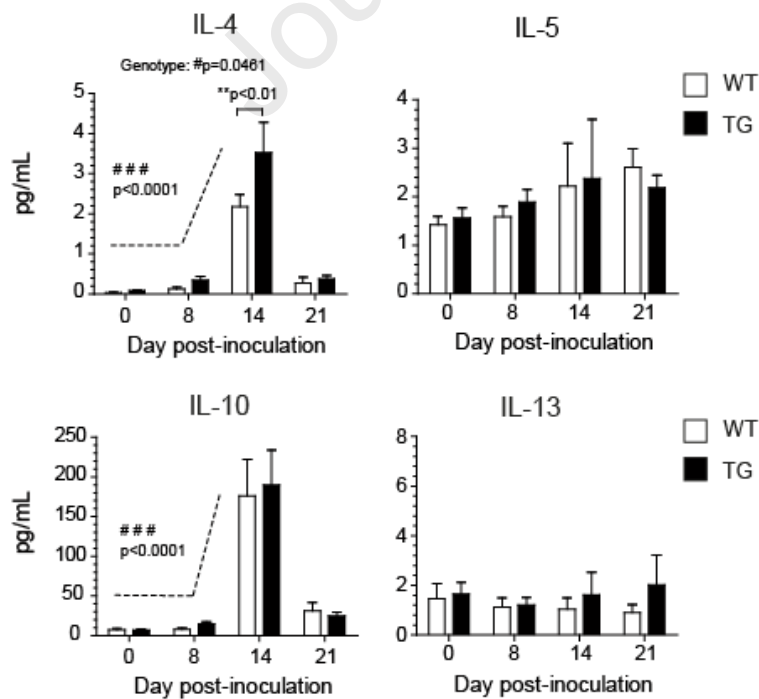
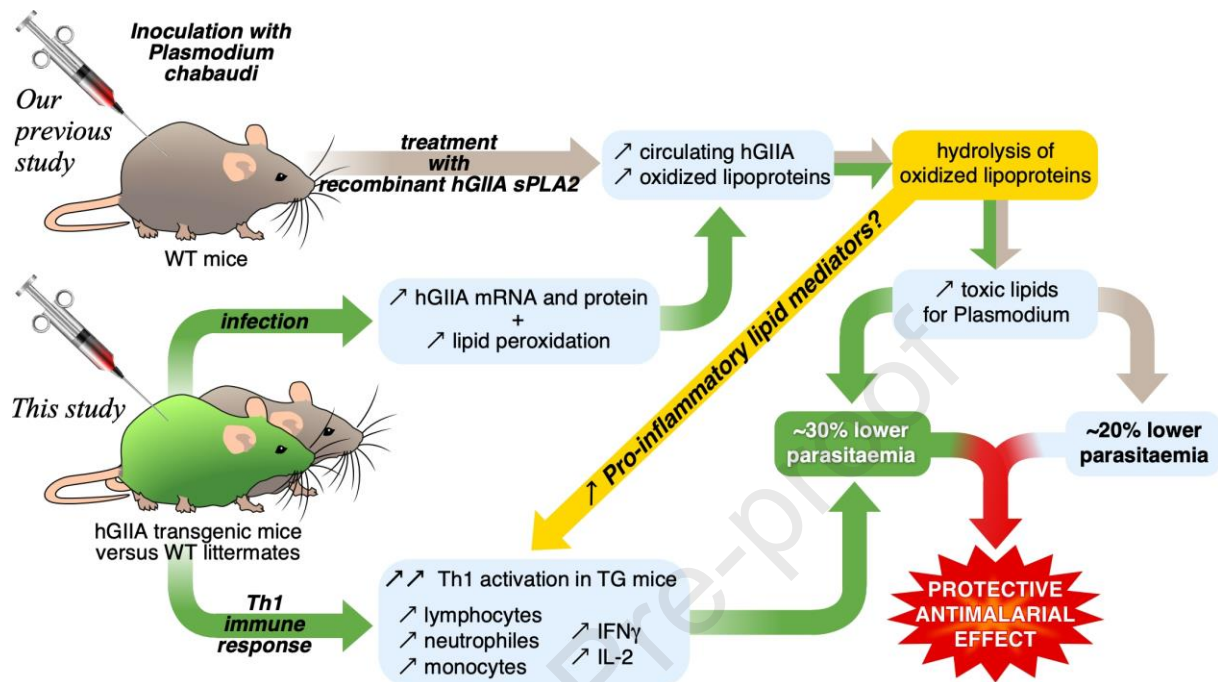


Figure 9



#### HIGHLIGHTS

1. Mice overexpressing hGIIA sPLA<sub>2</sub> (TG) are more resistant to *Plasmodium* infection
2. Infected TG mice have high levels of hGIIA and oxidized lipoproteins
3. Infected TG mice have high levels of host defence pro-inflammatory cytokines
4. Likely mechanism 1: hGIIA releases parasitocidal lipids from oxidized lipoproteins
5. Likely mechanism 2: hGIIA boosts host innate immunity against *Plasmodium*

Journal Pre-proof