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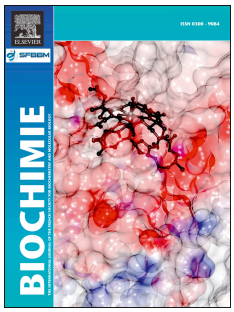

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PII: S0300-9084(21)00155-3

DOI: https://doi.org/10.1016/j.biochi.2021.06.009

Reference: BIOCHI 6151

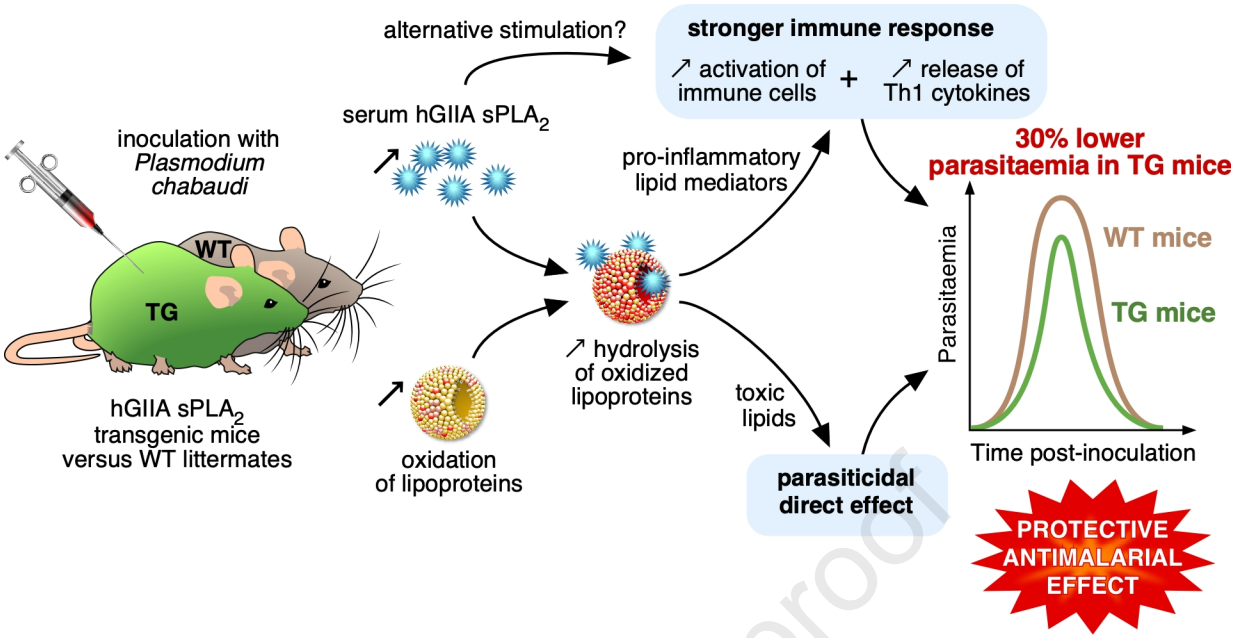
To appear in: *Biochimie*

Received Date: 6 May 2021

Revised Date: 17 June 2021

Accepted Date: 19 June 2021

Please cite this article as: Mé. Dacheux, S. Chaouch, A. Joy, A. Labat, C. Payré, Agnè. Petit-Paitel, F. Bihl, I. Lagrange, P. Grellier, L. Touqui, Gé. Lambeau, C. Deregnaucourt, Role of human group IIA secreted phospholipase A2 in malaria pathophysiology: Insights from a transgenic mouse model, *Biochimie* (2021), doi: https://doi.org/10.1016/j.biochi.2021.06.009.



Role of human group IIA secreted phospholipase A_2 in malaria pathophysiology: Insights from a transgenic mouse model

Mélanie Dacheux[1], Soraya Chaouch[1], Alonso Joy[1], Amandine Labat[1], Christine Payré[2], Agnès Petit-Paitel[2], Franck Bihl[2], Isabelle Lagrange[3], Philippe Grellier[1], Lhousseine Touqui[4,5]*, Gérard Lambeau[2]*#, Christiane Deregnaucourt[1]*#

[1] UMR 7245 Molécules de Communication et Adaptation des Micro-organismes, Muséum National d'Histoire Naturelle, CNRS, CP52, 61 rue Buffon, Paris Cedex 05 75231, France

[2] Université Côte d'Azur (UCA), Centre National de la Recherche Scientifique (CNRS), Institut de Pharmacologie Moléculaire et Cellulaire (IPMC), UMR7275, Valbonne Sophia Antipolis, France ;

[3] Ecole Nationale Vétérinaire d'Alfort, BioPôle, Laboratoire d'hématologie, 94704 Maisons-Alfort, France ;

[4] Cystic fibrosis and Bronchial diseases team - INSERM U938, Institut Pasteur, 75015 Paris, France

[5] Sorbonne Université, INSERM UMRS938, Centre de Recherche Saint-Antoine (CRSA), 75012 Paris, France.

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

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

Address correspondence to Christiane Deregnaucourt, christiane.deregnaucourt@mnhn.fr or Gérard Lambeau, lambeau@ipmc.cnrs.fr

Running title: *In vivo* antimalarial activity of hGIIA $sPLA_2$

Keywords: Malaria, secreted phospholipase A_2, transgenic mice, *Plasmodium chabaudi*

Abbreviations: hGIIA $sPLA_2$: human group IIA secreted phospholipase A_2. A comprehensive abbreviation system is used for the various mammalian $sPLA_2$s: each $sPLA_2$ is abbreviated with a lowercase letter indicating the $sPLA_2$ species (m and h for mouse and human, respectively), followed by uppercase letters identifying the $sPLA_2$ group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GX and GXII). IFN-γ: Interferon-gamma. KC/GRO: keratinocyte chemoattractant/growth regulated oncogene. MDA: malondialdehyde. PUFAs: polyunsaturated fatty acids. RBCs: red blood cells. RT-qPCR: real-time quantitative polymerase chain reaction. TBARS: thiobarbituric acid-reactive-substances. WBCs: white blood cells. TNF-α: tumor-necrosis factor alpha. TR-FIA: time-resolved fluoroimmunoassay.

ABSTRACT

We previously showed that injection of recombinant human group IIA secreted phospholipase A_2 (hGIIA $sPLA_2$) to *Plasmodium chabaudi*-infected mice lowers parasitaemia by 20%. Here, we show that transgenic (TG) mice overexpressing hGIIA $sPLA_2$ have a peak of parasitaemia about 30% lower than WT littermates. During infection, levels of circulating $sPLA_2$, enzymatic activity and plasma lipid peroxidation were maximal at day-14, the peak of parasitaemia. Levels of hGIIA mRNA increased in liver but not in spleen and blood cells, suggesting that liver may contribute as a source of circulating hGIIA $sPLA_2$. Before infection, baseline levels of leukocytes and pro-inflammatory cytokines were higher in TG mice than WT littermates. Upon infection, the number of neutrophils, lymphocytes and monocytes increased and were maximal at the peak of parasitaemia in both WT and TG mice, but were higher in TG mice. Similarly, levels of the Th1 cytokines IFN-γ and IL-2 increased in WT and TG mice, but were 7.7- and 1.7-fold higher in TG mice. The characteristic shift towards Th2 cytokines was observed during infection in both WT and TG mice, with increased levels of IL-10 and IL-4 at day-14. The current data are in accordance with our previous *in vitro* findings showing that hGIIA kills parasites by releasing toxic lipids from oxidized lipoproteins. They further show that hGIIA $sPLA_2$ is induced during mouse experimental malaria and has a protective *in vivo* role, lowering parasitaemia by likely releasing toxic lipids from oxidized lipoproteins but also indirectly by promoting a more sustained innate immune response.

1. Introduction

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

Plasmodium development in the human host mostly takes place in red blood cells (RBCs). The intra-erythrocyte cycle is responsible for common malaria symptoms including fever, aches and nausea. *P. falciparum* malaria can lead to severe, life-threatening complications including cerebral malaria and severe anemia. A better understanding of malaria pathophysiology is key to open new perspectives to fight against *Plasmodium* infection. Among the various mechanisms of innate immunity and host response engaged following infection [2-4], endogenous secreted phospholipases A_2 ($sPLA_2$s) may play a specific role.

Mammalian phospholipases A_2 (PLA_2, EC 3.1.1.4) comprise numerous intracellular and secreted enzymes that catalyze the hydrolysis of phospholipids at the *sn-2* position to release free fatty acids and lysophospholipids [5, 6]. PLA_2s play multiple roles in physiological and pathophysiological conditions. For example, PLA_2 can release arachidonic acid which is a key precursor for the biosynthesis of eicosanoids such as prostaglandins and leukotrienes, these latter being important in the host inflammatory response [7]. Among the diversity of PLA_2s, secreted PLA_2s ($sPLA_2$s) comprise up to 12 members in mice and humans, called IB, IIA, IIC (pseudogene in humans), IID, IIE, IIF, III, V, X, XIIA, XIIB and otoconin-90 [8]. It is now clear that each $sPLA_2$ has distinct cell and tissue distributions and exerts specific and diverse biological functions [8-11]. Mechanistically, most $sPLA_2$s contribute to multiple pathophysiological functions by hydrolysis of various phospholipid substrates from cellular membranes or extracellular lipid components, such as lipoproteins, microparticles, lung surfactant, mitochondria, lipid diet and microbes [10, 12-19].

Human group IIA (hGIIA) $sPLA_2$ is one of the most studied $sPLA_2$, especially because it is induced during inflammation and host defense where it likely plays key but controversial roles [20]. As early as 1979, this $sPLA_2$ has been recognized as a potent bactericidal enzyme secreted from polymorphonuclear leukocytes [21]. Since then, multiple studies have shown that the enzyme is secreted by inflammatory, immune and stromal cells after stimulation by various pro-inflammatory molecules including pathogen-associated molecular patterns such as lipopolysaccharides [15, 18, 22, 23]. Accordingly, the enzyme has been detected at high levels in body fluids such as plasma,

gastrointestinal tract lumen, tears, bronchial secretions or inflammatory exudates during sepsis, infection and systemic inflammation [24-30]. hGIIA $sPLA_2$ and several other $sPLA_2$s have been involved in host defense with antimicrobial activity against bacteria, viruses and parasites [11, 18, 22, 23, 31-40]. $sPLA_2$s are also present in various plant and animal genomes, and are particularly abundant in snake and bee venoms, where they also display antimicrobial activities, suggesting an evolutionary conserved function [41-46].

However, the possible role of hGIIA and other $sPLA_2$s in host defense against *Plasmodium* in malaria has remained unknown until recently. In the early 90's, Vadas and colleagues reported elevated levels of hGIIA $sPLA_2$ in the serum of patients with malaria [47, 48] but its role was not investigated. In the early 2000's, we reported that snake and bee venom $sPLA_2$s exert potent antimalarial activity against *P. falciparum* in an *in vitro* model of human RBCs infected by *P. falciparum* [49-52]. More recently, we showed that several but not all human $sPLA_2$s (hGIIA, hGIIF, hGIII, hGV and hGX) have the same effect as venom $sPLA_2$s, inhibiting the growth of *P. falciparum* with IC_{50} values from nM to µM range [53, 54]. In this *in vitro* model, we identified an indirect mechanism of action by which $sPLA_2$s hydrolyze lipoproteins from the human plasma that supplements parasite culture medium and release polyunsaturated fatty acids (PUFAs) that are toxic to the parasite [53]. Interestingly, hGIIA $sPLA_2$ was inactive against *P. falciparum* in the presence of normal human plasma but became active in the presence of plasma from malaria patients containing oxidized lipoproteins [54]. We also found increased levels of hGIIA $sPLA_2$ associated with the presence of oxidized lipoproteins in the plasma of patients with malaria [54, 55]. Last, we showed that injection of recombinant hGIIA $sPLA_2$ to *Plasmodium*-infected mice significantly reduced parasitaemia, supporting an *in vivo* role [54]. These findings suggest a possible antimalarial role of hGIIA when present at high levels and concomitantly with oxidized lipoproteins. Concerning other human $sPLA_2$s, hGIIF, hGIII, hGV and hGX $sPLA_2$s were active in the presence of both native and oxidized lipoproteins $sPLA_2$s, but they were not detected in the plasma of malaria patients, leaving unknown their possible *in vivo* roles [53, 54].

To investigate the pathophysiological role of hGIIA $sPLA_2$ in an *in vivo* situation close to human malaria where the $sPLA_2$ is present at high levels during infection [47, 48, 54], we used transgenic (TG) mice overexpressing hGIIA $sPLA_2$ [56] and explored its impact on the pathophysiology of malaria infection by the self-healing murine parasite *Plasmodium chabaudi chabaudi* 864VD (*P. chabaudi*). In line with our approach, hGIIA $sPLA_2$ TG mice have been extensively used by us and others to reveal the role of hGIIA in host defense against various bacterial pathogens [18, 22, 23, 57-59]. Of note, studies with the transgenic hGIIA mouse strain were performed in comparison with the parental inbred C57BL/6 mouse strain that is naturally-deficient for endogenous mouse group IIA $sPLA_2$ (*Pla2g2a* gene) [60]. Using this transgenic mouse model, we show that i) the circulating level of hGIIA $sPLA_2$ increases during the course of malaria infection, ii) the $sPLA_2$ inhibits parasite development at

the peak of parasitaemia, when the levels of both circulating enzyme and oxidized proteins are maximally increased, and iii) the expression of the hGIIA $sPLA_2$ promotes a stronger interferon-γ (IFN-γ) immune response, which is key to fight malaria infection [61].

2. Materials and methods

2.1. Materials

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

2.2. Ethical statement

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

2.3. Parasite

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

154 *2.4. Mice*

155 Wild-type C57BL/6J (C57BL/6J JAX™ 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 $sPLA_2$ (5 exons and 4 introns), making these mice deficient for active
158 mouse group IIA enzyme [60]. hGIIA $sPLA_2$ transgenic mice (transgenic model #1005, C57BL/6J-
159 [Tg]PLA_2) referred to as TG mice in this study, were from Taconic Biosciences (Taconic EZcohort®
160 Models). Of note, the hGIIA $sPLA_2$ transgene is a 6.2-kbp genomic fragment of the *PLA2G2A* gene
161 containing the full open reading frame of hGIIA $sPLA_2$ 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 $sPLA_2$ 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 1×10^6 *P. c.*
175 *chabaudi* 864VD-infected mouse RBCs in Alsever's solution. Control uninfected mice were injected
176 with 1×10^6 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 ≈ 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 µL) of tail blood onto heparin every 2-3 days p.i. and manual counting of blood cells. RBC number
185 per µL 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.

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

2.6. Organ and blood collection during infection

Groups (n≥5) of uninfected and infected WT and TG mice were sacrificed at day-0 (before inoculation), day-8 p.i. (onset of the patent phase), day-14 p.i. (parasitaemia peak) and day-21 p.i. (parasite clearance). Mice were weighed prior to sacrifice. They were anesthetized by IP injection of ketamine (100 mg/kg)/ xylazine (10 mg/kg) then euthanized by retro-orbital exsanguination. The blood was collected in tubes containing 5 µL heparin (15-30 IU/mL final) and centrifuged at 1,500×g for 5 min at room temperature. Plasma was stored frozen at -80°C before analysis (cytokine measurement, TBARS assay, etc). Pelleted blood cells were resuspended in TRIzol®, vortexed and stored frozen at -80°C until RT-qPCR analysis. Liver, spleen and kidney from infected and non-infected mice were removed and weighed. Spleen and liver were sliced into small pieces, collected into 1 mL of RNA Later©, and stored at -80°C before RNA extraction for RT-qPCR analysis.

2.7. Measurement of $sPLA_2$ activity and time-resolved fluoroimmunoassay for hGIIA $sPLA_2$

Mouse tail blood was collected onto heparin (15-30 IU/mL final concentration) and centrifuged at 1,500×g for 5 minutes at room temperature. Plasma was frozen at -80°C before analysis. $sPLA_2$ enzymatic activity was measured by using the ^{3}H-oleic acid-labeled *E. coli* membrane hydrolysis assay as described [54]. Time-resolved fluoroimmunoassay (TR-FIA) to detect hGIIA $sPLA_2$ was performed as described [28].

2.8. Real time-quantitative PCR (RT-qPCR)

Organ pieces frozen in RNA Later were thawed on ice, and 50-100 mg of tissues were disrupted in 1 mL of TRIzol® using TissueLyser II with RNAse-free tungsten carbide beads. Lysates were centrifuged for 10 min at 12,000×g at 4°C. Blood cell samples in TRIzol® were thawed on ice and immediately centrifuged at 12,000×g. Total RNA from supernatants was prepared with TRIzol® and quantified 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 $sPLA_2$s and housekeeping reference genes were based on
229 previously published data [66, 67] or designed using the Primer-BLAST software, and synthetized 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(-
241 1/slope), 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 (Vn/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 $sPLA_2$s, 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 $Q=E(Cq^{D0}-Cq^{sample}$, 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

2.9. Lipid peroxidation measurement

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

2.10. Th1/Th2 cytokine quantification during infection

The multiplex technology from Meso Scale Discovery was applied to measure the concentration of cytokines in plasma of non-infected and *P. chabaudi*-infected mice. Plasma samples from day-0 (5 WT 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) mice were analyzed. Twenty µL of each plasma were loaded into the MSD 96-well multi-array U-PLEX TH1/TH2 Combo Mouse assay kit plate from Meso Scale Discovery (Meso Scale Diagnostics, Rockville, Maryland) and incubated overnight at 4°C. Analysis was performed according to manufacturer's recommendations using the MESO QuickPlex SQ 120 reader (Meso Scale Diagnostics, Rockville, Maryland).

2.11. Statistical analyses

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

3. Results

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

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

292 $sPLA_2$ 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 ± SEM peak value of parasitaemia was
296 38.6 ± 2.1 % in WT mice (n=19) *versus* 27.9 ± 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 $sPLA_2$ 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 ± SEM
311 of WT and TG mice at baseline were 25.9 ± 0.7 g (n=11) and 22.1 ± 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 ± SEM at the peak of parasitaemia were 23.8 ± 0.8 g (n=11) for WT mice (weight loss ≈10%, p
315 value <0.0001) and 20.7 ± 0.7 (n=7) for TG mice (weight loss ≈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 ± SEM = 0.97 ± 0.02 g
319 (TG) *versus* 0.88 ± 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 ± 0.09 g at day-14 p.i. and 1.62 ± 0.04 g at day-21 p.i.) was heavier than that of WT mice (1.21 ± 0.07
322 g at day-14 p.i.; 1.35 ± 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 ± SEM values of 0.13 ± 0.009 g *versus* 0.09 ± 0.007 g, respectively (Mann

Whitney test, p value = 0.0142). Spleen browning and enlargement (not shown) due to infection occurred at similar time points in both groups of mice, with a dramatic increase of weight occurring 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 2C). Kidneys from TG and WT mice exhibited similar weights at baseline (0.42 ± 0.02 g for WT *versus* 0.41 ± 0.03 g for TG mice). No variation of weight was observed in either group of mice during infection (Figure 2D).

3.3. Both enzymatic activity and circulating levels of hGIIA sPLA$_2$ are increased in TG mice during parasitaemia

Enzymatic activity and circulating levels of hGIIA sPLA$_2$ were quantified in the plasma of TG and WT littermate mice before and during infection. In accordance with previous data on the same mouse line [56], non-infected TG mice exhibit at baseline high levels of sPLA$_2$ activity (mean ± SEM = 3,106 ± 224.8 cpm/min×µL, n = 12, Figure 3A) and hGIIA sPLA$_2$ concentration (mean ± SEM = 52.1 ± 20.6 nM, n = 5, Figure 3B). Upon infection, a significant increase of circulating sPLA$_2$ activity occurred with more than 2-fold higher enzymatic activity at crisis (mean value = 7,350 ± 1821 cpm/min×µL, n = 12) (Figure 3A). Accordingly, the concentration of circulating hGIIA sPLA$_2$ increased by almost 3-fold at day-14 p.i. (mean value = 144.7 ± 21.6 nM, n = 6) (Figure 3B). In contrast, no or very marginal sPLA$_2$ activity was measured in the plasma of WT mice before infection (mean value = 55.7 ± 15.8 cpm/min×µL, n = 18, not shown). This was expected since C57BL/6 mice have a natural frameshift mutation in the *Pla2g2a* gene and are deficient for a functional mGIIA sPLA$_2$ enzyme [60, 66]. No increase of enzymatic activity was measured in the plasma of WT mice infected by *P. chabaudi* (not shown), suggesting that no other mouse sPLA$_2$ was stimulated by *Plasmodium* infection at a systemic level. Thus, infection by *Plasmodium* stimulated the baseline level of hGIIA sPLA$_2$ in the plasma of TG mice, with a peak of enzymatic activity and concentration coincident with parasitaemia reduction, suggesting a causal relationship.

3.4. Expression of hGIIA and mouse sPLA$_2$s in tissues from TG and WT mice infected by P. chabaudi

To analyze whether the expression of the hGIIA sPLA$_2$ transgene might be modulated by *Plasmodium* infection in a tissue-specific manner, we performed RT-qPCR on liver, spleen and blood cells collected from TG mice at different times of infection. By RT-qPCR, we could detect baseline levels of hGIIA sPLA$_2$ mRNA in liver, blood cells and spleen of TG mice (Figure 4A). Upon infection, mRNA levels of hGIIA sPLA$_2$ increased in liver by about 2-fold between day-8 and day-14 p.i., and then returned to baseline (Figure 4A). Parasitaemia and mRNA levels were positively correlated (Spearman r 0.6468, p value = 0.068, n=16, not shown). By contrast, hGIIA sPLA$_2$ mRNA decreased over time in spleen (Figure 4A) and the expression level was inversely correlated to parasitaemia (Spearman r -0.5036, p

value = 0.0393, n=17, not shown). In blood cells, no mRNA variation was observed during infection (Figure 4A), with no significant correlation between parasitaemia and mRNA levels (Spearman r -0.2716, p value = 0.3931, n=12, not shown). Altogether, these results suggest a tissue-specific regulation of the hGIIA $sPLA_2$ transgene during infection, where liver appears as a possible source of circulating hGIIA $sPLA_2$.

To determine whether endogenous mouse $sPLA_2$s might be regulated by *P. chabaudi* infection, we performed RT-qPCR for mouse GIIF (mGIIF, *Pla2g2f*), GIII (mGIII, *Pla2g3*), GV (mGV, *Pla2g5*) and GX (mGX, *Pla2g10*) $sPLA_2$s in liver, spleen and blood cells of TG mice and WT littermates before and after *P. chabaudi* inoculation. In all tissue extracts, mGIII and mGX mRNAs were under the detection limit in our conditions of RT-qPCR. In contrast, mGIIF and mGV mRNAs were detected before infection in spleen of both TG and WT mice, but not in liver or blood cells. Upon infection, a marked and early drop of mGIIF and mGV mRNA was observed in spleen for both groups of mice (Figure 4B). This was supported by a strong negative correlation between parasitaemia and the relative levels of mRNA for both groups of mice (for mGIIF: Spearman's r -0.8956, p value <0.0001, n=18 (TG mice); Spearman's r -0.8019, p value = 0.0030, n=11 (WT mice), and for mGV: Spearman's r -0.9345, p value <0.0001, n=19 (TG mice); Spearman's r -0.7973, p value = 0.0033, n=11 (WT mice), not shown). It is interesting to note that both WT and TG mice exhibit the same pattern of mRNA expression and regulation, that is detectable levels of mGIIF and mGV but not mGIII and mGX $sPLA_2$ mRNAs at baseline in spleen and significant down-regulation of mGIIF and mGV mRNAs after *Plasmodium* infection. This suggests no compensation effect associated with overexpression of hGIIA $sPLA_2$.

3.5. Plasma lipid peroxidation increases in TG mice during infection

We previously reported that the *in vitro* anti-*Plasmodium* activity of hGIIA $sPLA_2$ is mediated by hydrolysis of oxidized lipoproteins and release of toxic PUFAs [54]. We also showed that injection of recombinant hGIIA $sPLA_2$ to infected WT mice leads to a reduction of parasitaemia at the time of elevation of lipid peroxidation in plasma. To assess whether a similar mechanism operates in TG mice, we measured the peroxidation level of plasma lipids during infection. In agreement with previous observation in WT mice [54], malondialdehyde (MDA) concentration in infected TG mice 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 was in concordance with the highest concentration of circulating hGIIA $sPLA_2$ observed at day-14 p.i., bringing together the appropriate conditions for hydrolysis of oxidized lipoproteins by hGIIA $sPLA_2$, which may explain, at least in part, the observed reduction of parasitaemia.

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

Because hGIIA $sPLA_2$ has been considered as an extracellular mediator of host immune response to pathogens since a long time [8, 9, 70, 71], we examined whether the reduced parasitaemia observed in TG mice might be due to differences in the immune response. For instance, it has been reported that TG mice exhibit elevated numbers of neutrophils and lymphocytes [72], a phenomenon attributed to the capacity of hGIIA $sPLA_2$ to release potent inflammatory lipid mediators. We inferred that overexpression of hGIIA $sPLA_2$ may be capable to not only modify the mouse immune system at baseline but also to promote a stronger antimalarial response to *Plasmodium* infection in TG *versus* WT mice. We thus analyzed the variation of red blood cells (RBCs) and white blood cells (WBCs), as well as cytokines before and after infection by *P. chabaudi* in both mouse genotypes.

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

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

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

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

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

Collectively, these results indicate that TG mice exhibited a more robust immune cell response to *Plasmodium* infection than WT mice, especially for neutrophils, lymphocytes and monocytes. Thus, the lower parasitaemia observed in TG mice may also be explained by a more sustained stimulation of the immune response due to overexpression of hGIIA $sPLA_2$.

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

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

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

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

Overall, these results suggest that overexpression of hGIIA $sPLA_2$ does not modify the pattern of expression of cytokines during *P. chabaudi* infection, but promotes more sustained levels for several of them at both baseline and during the course of infection, especially for pro-inflammatory cytokines including the key player IFN-γ.

4. Discussion and conclusion

Figure 9 summarizes the key findings of this study in the context of our previous data, and support a beneficial role of hGIIA $sPLA_2$ in malaria. The level of hGIIA $sPLA_2$ was found to be increased in the plasma of patients with malaria [47, 48, 54, 75] but its role was unclear. Because high enzymatic activity was associated with high parasitaemia and neurological complications, early studies proposed that hGIIA $sPLA_2$ might be pro-inflammatory and associated with worsening of the disease [47, 48, 75]. However, our previous and current data suggest an innate protective role of hGIIA in malaria, which may be in line with its antibacterial role [15, 18, 22, 23].

Specifically, we previously found that hGIIA $sPLA_2$ inhibits the development of *Plasmodium* both *in vitro* when added to cultures of infected human RBCs in the presence of oxidized lipoproteins, and *in*

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

To provide more *in vivo* evidence for a protective role of hGIIA $sPLA_2$ in host defense against *Plasmodium* infection, we used TG mice overexpressing hGIIA $sPLA_2$ which, upon infection with *Plasmodium*, showed increased blood concentrations similar to those observed in patients with malaria [47, 48, 54, 75] (Figure 9). When these TG mice were infected by the murine parasite *Plasmodium chabaudi*, we observed an almost 30% reduction of parasitaemia at crisis, further supporting the notion that hGIIA $sPLA_2$ has a protective role in malaria. In addition, thanks to the "old design" of these TG mice where the transgene contains a significant part of the *PLA2G2A* gene with key promoter elements [56, 64, 65, 76], we observed that the level of circulating hGIIA $sPLA_2$ is increased during infection, and identified liver as a possible source of the circulating enzyme. However, we do not exclude the possibility that circulating hGIIA $sPLA_2$ may originate from other sources not analyzed in this study, including immune or stromal cells activated during *Plasmodium* infection. Furthermore, the factors inducing hGIIA $sPLA_2$ expression from liver or other tissues were not investigated in this study, and cytokines such as IL-6 [77] or IFN-γ [78, 79] may play a key role.

Mechanistically, our data are consistent with a dual mechanism of action of hGIIA $sPLA_2$, exerting its antimalarial protective effect by directly inhibiting parasite multiplication via hydrolysis of oxidized lipoproteins and production of toxic lipids, and by stimulating a more robust innate and inflammatory immune response in both chronic and acute ways (Figure 9). We previously showed that the catalytic activity of both venom (OS_2) and human (hGX) $sPLA_2$s is required for their *in vitro* antimalarial activity [52, 53], making likely that the enzymatic activity of hGIIA $sPLA_2$ is also required for its *in vivo* antimalarial effect. However, this remains to be ascertained *in vivo* using catalytically-inactive mutants such as H48Q hGIIA $sPLA_2$ [80].

Interestingly, despite high basal level of circulating hGIIA $sPLA_2$ in TG mice, no difference in parasitaemia was observed between WT and TG mice before day-11 of infection. This was in line with our previous data obtained by acute injection of the recombinant enzyme to infected WT mice, which induced a reduction of parasitaemia only when the enzyme was injected right before the peak of parasitaemia [54]. Since neither the circulating level of hGIIA $sPLA_2$ activity nor the genetically modified immune background of the recipient TG mice seemed to impact on *Plasmodium* development at the early time of infection, we inferred that secondary events, presumably an increase of the enzyme concentration to some threshold and/or modifications in the enzyme environment including access to new substrates, had to occur before hGIIA $sPLA_2$ became effective against *Plasmodium.*

Interestingly, the increase of enzymatic activity and concentration of hGIIA $sPLA_2$ at the peak of parasitaemia coincides with the increase of lipid peroxidation, suggesting that the anti-*Plasmodium*

effect of hGIIA $sPLA_2$ may result from the concerted presence of high levels of the enzyme and oxidized lipoproteins acting as a preferred substrate of the enzyme and leading to the production of toxic lipids to *Plasmodium* [54]. We previously showed that hGIIA $sPLA_2$ is inhibitory to *Plasmodium* (*P. falciparum*) in *in vitro* culture conditions when the medium contains oxidized but not native lipoproteins [54]. This is consistent with the higher enzymatic activity of hGIIA observed on oxidized *versus* native lipoproteins [81-83]. We further showed that hydrolysis of phospholipids from lipoproteins by $sPLA_2$s releases free PUFAs which are toxic to *Plasmodium* [53, 54]. In the case of infected WT mice, we previously showed that lipid peroxidation also occurred at the peak of parasitaemia [54], but these mice do not express the endogenous mouse group IIA $sPLA_2$ [60] and no circulating enzymatic activity was detected during infection, supporting the notion that both high levels of hGIIA (from TG mice or after injection of recombinant hGIIA) and lipid peroxidation are required to lower parasitaemia (Figure 9). Together, this strongly suggests that the shift from native to oxidized state of lipoproteins during infection might be a key event to hGIIA $sPLA_2$ activation against *Plasmodium*, resulting from phospholipid hydrolysis and release of lipid products that could act as mediators or effectors of the parasite inhibition process. Lipidomics experiments measuring the levels of PUFAs and lysophospholipids present in the plasma of infected TG and WT mice should be performed to identify which lipids are increased in TG mice and may be toxic to *Plasmodium*. From a translational perspective, it has been shown that plasma from malaria patients contain high levels of hGIIA $sPLA_2$ and of lipid peroxidation but no careful follow-up was performed [54, 55, 84-86]. It thus remains to determine whether the two factors are increased concomitantly in patients with malaria.

Considering further the mechanisms of the anti-*Plasmodium* effect of hGIIA $sPLA_2$, alternative substrates to lipoproteins may be envisaged. Indeed, it is known that hGIIA $sPLA_2$ exhibits very low enzymatic activity on zwitterionic substrates like the phosphatidylcholine-rich outer leaflet of the plasma membrane of resting cells or the surface of native lipoproteins [87, 88]. However, the enzyme becomes active in the presence of lipid membranes enriched in negatively charged phospholipid substrates such as activated or apoptotic cells, oxidized lipoproteins, extracellular vesicles (EVs), bacteria or even platelet-extruded mitochondria [12, 16, 18, 89, 90]. Such peculiar substrates are more abundant in pathological situations like infection and inflammation and $sPLA_2$s can even be loaded into exosomes [91-93]. As for malaria, several studies have reported increased levels of EVs from host and parasite origin during active *Plasmodium* infection, which return to normal levels after resolution of infection. Accumulating evidence suggests that EVs contribute to malaria-associated clinical symptoms [94-96]. *P. falciparum*-derived EVs were also shown to mediate cell-cell communication between parasites, propagating drug resistance among the parasite population or inducing gametocyte differentiation [97, 98]. As far as we know, interaction of hGIIA $sPLA_2$ with EVs

has not been investigated in the context of malaria. It thus remains to determine whether hGIIA $sPLA_2$ might regulate parasite development through hydrolysis of EVs, either by controlling the rate of EV production/degradation, thereby modulating the role of EVs in malaria, and/or by generating toxic lipids from EVs, as from oxidized lipoproteins. Enzymatic activity of hGIIA $sPLA_2$ on the above substrates might contribute to the reduction of parasitaemia through the generation of toxic PUFAs, but also through the production of lipid mediators modulating the immune response during infection [11, 99] (Figure 9).

Another important aspect that may explain the anti-*Plasmodium* effect of hGIIA $sPLA_2$ in TG mice is the chronically-modified immunological background of these mice induced by the constitutive overexpression of the enzyme and the clearly higher acute innate immune response to *Plasmodium* infection. At baseline, hematological analysis revealed a specific blood profile of TG mice. Blood from TG mice was enriched in neutrophils and lymphocytes whereas the number of platelets was normal, which confirmed previous data in the same mouse line [72]. Additionally, the number of monocytes and eosinophils was higher and that of RBCs lower, suggesting marked hematopoiesis disorder in TG mice. Of note, although the level of anemia occurring at high parasitaemia was similar in infected TG and WT mice, recovery from anemia seemed to be delayed in TG mice. This erythropoiesis impairment suggests that hGIIA $sPLA_2$ might be an as yet unsuspected actor in the implementation of severe anemia, which is observed in complicated cases of malaria [73]. During infection, the number of leukocytes remained high in TG mice, and the increase was maximal at crisis. Neutrophils mostly accounted for this increase. In addition, infected TG mice had higher levels of activated lymphocytes and monocytes than WT mice, indicating a more robust activation of the immune response. These observations are in accordance with the well-known ability of $sPLA_2$s or their lipid products to induce leukocyte recruitment and activation during inflammation and infection [11, 14, 36, 100-106]. The enhanced and/or specific activation of immune cells might contribute to the improved capacity of TG mice to control parasite development, for example by increasing the phagocytic power of immune cells, as reported for monocytes from monkeys injected with $sPLA_2$ [107] or for macrophages activated by mGV $sPLA_2$ during infection with *Candida albicans* [105, 108].

The baseline cytokine profile of TG mice was different from that of WT mice. Levels of the pro-inflammatory cytokines IL-1β and TNF-α were clearly higher and those of IL-12p70 and KC/GRO chemokine showed a higher trend (also observed during infection), whereas IL-4 was increased among the anti-inflammatory cytokines. Thus, despite the fact that no significant inflammatory cell infiltrates were observed in the skin or other tissues of TG mice [56], which suggests that the immune background of TG mice is not inflammatory, the modified cytokine profile observed in TG mice suggests a priming of this latter by hGIIA $sPLA_2$, leading to low-grade systemic inflammation. In this regard, it is interesting to note that IL-1β and TNF-α, whose levels are increased in TG mice, are well-

known regulators of hematopoiesis [109-111] and might contribute, along with hGIIA $sPLA_2$ and lipid mediators, to set up the abnormally elevated leukocyte concentration observed in TG mice.

Comparative analysis of the cytokine profile during infection in TG and WT mice showed quantitative but not qualitative differences. The hallmark of malaria parasite infection in the early stages is a strong protective cell-mediated Th1 immune response characterized by increased IFN-γ. The Th1 response eventually wanes with disease progression with a switch to a Th2 immune response. IL-10 is believed to play a critical role in the regulation of host immune response following *Plasmodium* infection and potentially orchestrates the switch of Th1/Th2 immune dominance during disease progression [112]. As expected from previous data showing a central role of IFN-γ in the development of protective immunity against blood-stage infection by *P. chabaudi* [61], levels of IFN-γ were very high at day-8 p.i. in both types of mice, yet much higher in TG mice. In this respect, it is interesting to note that IFN-γ is an inducer of hGIIA $sPLA_2$ production [78, 79]. The non-coding upstream region of 1.6 kbp present in the *PLA2G2A* transgene contains the transcriptional response element consensus sequence for IFN-γ [56], suggesting that the increase in $sPLA_2$ activity observed during infection might result from IFN-γ-induced production of hGIIA $sPLA_2$. It is also interesting to note that, when using the same mouse model of infection by *P. chabaudi*, injection of monoclonal antibodies blocking IFN-γ increased the peak of parasitaemia but did not impact on the course of infection [113], mirroring the effect of hGIIA $sPLA_2$. This in turn suggests that IFN-γ and hGIIA $sPLA_2$ may act on the same pathway, one possibly activating the other in an autocrine/paracrine loop, and with the possibility that inhibition of IFN-γ would prevent the increase of hGIIA $sPLA_2$ observed at the peak of parasitaemia. At day-14 p.i., pro-inflammatory TNF-α and IL-2 were high and IFN-γ was low, whereas anti-inflammatory IL-4 and IL-10 were increased. Thus, the well-described Th1/Th2 switch observed in *P. chabaudi* infection [114, 115] was also seen in TG mice, indicating that hGIIA $sPLA_2$ does not influence the time course of cytokine production during infection, but rather enhance the production of several cytokines, especially those involved in the pro-inflammatory Th1 pathway, in particular IFN-γ .

Finally, in an attempt to reveal the possible involvement of other endogenous $sPLA_2$s during infection by *P. chabaudi*, we measured the mRNA levels of $sPLA_2$s known to have an *in vitro* effect on *Plasmodium*, namely mGIIF, mGIII, mGV and mGX $sPLA_2$s [53, 54]. In our RT-qPCR conditions, mRNAs for these four $sPLA_2$s were undetectable in liver and blood cells from both groups of mice, before and after infection. In contrast, mGIIF and mGV but not mGIII and mGX mRNAs were detectable before infection in spleen from TG and WT mice. The amounts of mGIIF and mGV mRNAs were decreased during the course of infection, in line with their possible roles in inflammatory response and host defense [11, 36, 116]. These results indicate that infection by *P. chabaudi* does not mobilize any of

these four $sPLA_2$s in the liver, spleen or blood cells, and probably not at the systemic level, since no circulating enzymatic activity was detected in infected and non-infected WT mice. Our data also suggest that the overexpression of hGIIA $sPLA_2$ does not modify the expression of these $sPLA_2$s.

In conclusion, we have shown that TG mice overexpressing hGIIA $sPLA_2$ better control infection by *P. chabaudi* than their WT littermates (Figure 9). In this murine model, the decrease of parasitaemia at the peak of infection is concomitant with the simultaneous increase of hGIIA $sPLA_2$ enzymatic activity and in lipid peroxidation. This suggests that hGIIA $sPLA_2$ exerts most of its antimalarial activity in an acute way by a specific and concerted mechanism of action where i) lipoproteins are transiently oxidized because of infection and activation of the inflammatory and immune response ii) the enzyme is induced and hydrolyzes oxidized lipoproteins to release active lipid metabolites in the vicinity of infected RBCs, leading to either direct killing of the parasite and/or stimulation of a more robust immune response. Collectively, our data support a protective role of hGIIA $sPLA_2$ in human malaria, but this remains to be further investigated, especially because the major parasite at play in humans, *Plasmodium falciparum*, is different. This parasite can also lead to severe malaria where hGIIA $sPLA_2$ may play a role in the brain compartment [75], but this could not be investigated in this animal model of malaria. Notable differences are also known between rodent and human *Plasmodium* parasites in terms of lipid metabolism [117]. Furthermore, human but not mouse platelets are an abundant source of group IIA $sPLA_2$ [12, 16, 118], while platelets are an important player in *Plasmodium* infection [119]. This brings attention to the possible role of platelets in the antimalarial effect of hGIIA in humans, as both a source of $sPLA_2$ enzyme and substrates including EVs and extruded mitochondria. It will be worth to also investigate on one hand the role of endogenous mouse group IIA (Pla2g2a) and of other $sPLA_2$s using appropriate inbred mouse strains [29] or gene knockout mice [9], and on the other hand the effect of the broadly specific $sPLA_2$ inhibitor varespladib, which has been tested in several clinical trials as a drug candidate for sepsis and other inflammatory diseases but has led to mitigated therapeutic effects or even adverse effects [120-122]. Interestingly, LY311727, an analog of varespladib, was shown to induce earlier mortality in a mouse model of toxoplasmosis [123]. From a therapeutical point of view and based on our present studies, it is thus tempting to speculate that $sPLA_2$s should not be inhibited in human malaria but rather stimulated, at least for non-severe cases of malaria. Last, genome-wide association studies have shown that single nucleotide polymorphisms present in the *PLA2G2A* gene and in other $sPLA_2$ genes may be associated to malaria, inflammatory diseases and cardiovascular diseases [124, 125], warranting further investigation of hGIIA $sPLA_2$ and other $sPLA_2$s for genetic susceptibility to malaria. These are as some of the directions for future investigation of the role of hGIIA $sPLA_2$ and other $sPLA_2$s in human malaria.

Declaration of competing interest

The authors have no conflict of interest to declare.

Author Contributions

M.D. designed, performed and analyzed the experiments.
A.J. was in charge of mouse breeding and participated in animal studies.
M.D., S.C. and A.L. designed, performed and analyzed RT-qPCR experiments.
A.P.P. and F.B. performed and analyzed multiplex cytokine assays.
I.L. performed automated and manual analysis of blood components.
C.P. produced recombinant $sPLA_2$s and performed enzymatic and TR-FIA assays.
P.G. discussed the data at different steps of the study and reviewed the manuscript.
M.D. C.D., G.L. and L.T. designed the project and wrote the manuscript.

Acknowledgements

The authors sincerely thank Franck Aguila for expertise in graphic art and help in designing the figures. This work was supported by the Museum National d'Histoire Naturelle (ATM blanche 2016-2017-2018 to C.D.) and by grants from Centre National de la Recherche Scientifique (CNRS), the Fondation du Rein (Award FdR 2018/FRM_G. Lambeau) and the Fondation Jean Valade/Fondation de France (Award FJV_FDF-00112090) to G.L.

Appendix A. Supplementary data

Supplementary data to this article can be found online at xxxx

Legend to Figures

Figure 1. Time-course of parasitaemia in TG mice and WT littermates infected with *P. chabaudi.* Results from four independent infection experiments totalizing 23 TG and 19 WT mice have been pooled. Panel A: Parasitaemia profiles from TG (■) and WT (□) mice inoculated at day-0 with 1×10^6 *P. chabaudi*-infected RBCs by IP injection. Values are mean ± SEM. Two-way ANOVA statistical analysis with Bonferroni's post-test (✱) was applied after data normalization. A significant difference (***p-value < 0.0001) between TG and WT profiles was observed at day-14 p.i. Panel B: Comparative distribution of blood parasitaemia values at day-14 p.i. between infected TG and WT mice. A

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

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

Figure 3. Plasma level of $sPLA_2$ enzymatic activity (A) and hGIIA $sPLA_2$ concentration (B) during infection of TG mice. A: Plasma samples from tail blood were collected before (Day-0) and at different days after inoculation of TG mice with 1×10^6 *P. chabaudi*-infected RBCs. Samples were analyzed for $sPLA_2$ enzymatic activity using [^{3}H]-oleic acid radiolabeled *E. coli* membranes as substrate. Results are from two independent infection experiments with groups of six mice (12 mice per day until day-20, then six mice per day); mean ± SEM values are presented. For statistical analysis, one-way ANOVA (#) with Dunnett's post-test (*) was applied. Enzymatic activity varied significantly during infection (#p-value = 0.0032) with a net increase at day-11 p.i. and day-14 p.i. (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 analysis was performed using specific antibodies for hGIIA $sPLA_2$ on plasma samples from mice infected with *P. chabaudi*. Blood was collected from groups of five to six mice at day-0 before inoculation, and day-8, -14 and -21 post-inoculation. Mean ± SEM values are presented. Significant difference was found between day-0 and day-14 using one-way Anova with Dunnett's post-test (**p-value = 0.0028).

Figure 4. Gene expression of $sPLA_2$s in TG mice and WT littermates during *P. chabaudi* infection. Total RNA extracts were prepared from organs at different time points before (Day-0) and after parasite inoculation (Day-8 to 21). Expression level of mRNA for hGIIA, mGIIF and mGV $sPLA_2$s was normalized

using GUSB, HPRT1 and TBP as housekeeping genes for spleen; HPRT1, TBP and PGK1 for liver; and GUSB, HPRT1, TBP and Act B for blood cells. Each point value is the mean of duplicate (liver and spleen) or triplicate (blood cells) measurements. Error bars are mean ± SEM values. Panel A: RT-qPCR analysis of hGIIA $sPLA_2$ mRNA in liver, spleen and blood cells from infected TG mice. Two independent infection experiments were performed and data were pooled. In total, hGIIA $sPLA_2$ mRNA was analyzed from spleen from eleven and seven TG mice, liver from thirteen and six TG mice, and blood cells from seven and five TG mice, respectively. Panel B: RT-qPCR analysis of mGV and mGIIF $sPLA_2$ mRNAs from spleen of TG mice versus WT littermates during *P. chabaudi* infection. Two independent infection experiments were performed and data were pooled. Total RNA extracts were prepared at different time points during infection from spleen of eleven and seven TG mice, and eight and three WT mice, respectively.

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

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

Figure 7. Levels of blood leukocytes in TG mice and WT littermates during *P. chabaudi* infection (manual counting). Three independent infection experiments were pooled, totalizing 12 TG mice and 13 WT littermates. About 10-20 µL of blood was taken from tail tips of infected animals at day-0 before parasite inoculation and then every 2-4 days after inoculation of $1x10^6$ *P. chabaudi*-infected RBCs. Total WBCs were counted on Kova slides after lysis of erythrocytes. Relative numbers of WBC subpopulations were calculated from distribution on Diff-Quik stained blood smears. For statistical analysis, two-way ANOVA (#) with Bonferroni's post-test (★) was applied.

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

Figure 9. Summary of the findings at a glance. In a previous study [54], we reported that WT mice (grey) infected by *P. chabaudi* and then treated with recombinant hGIIA $sPLA_2$ have a 20% lower parasitaemia than untreated mice. Mechanistically, the enzyme may function by hydrolysis of oxidized lipoproteins (oxidation is due to infection) and release of lipids directly toxic to *Plasmodium* growth. In this previous study, we did not evaluate the effects of recombinant $sPLA_2$ on Th1 activation at the cytokine and immune cell levels, which may also result from the release of lipid mediators from oxidized lipoproteins or other mechanisms. In the current study, we show that transgenic mice (TG, green) constitutively expressing hGIIA $sPLA_2$ better control *Plasmodium* proliferation than WT littermates. Like in human malaria, a concomitant increase in lipoprotein oxidation and hGIIA circulating activity/concentration is observed in infected TG mice, along with a stronger activation of immune cells and enhanced release of pro-inflammatory cytokines. It is known that hGIIA $sPLA_2$ can better hydrolyze oxidized lipoproteins. By piling up both studies, we hypothesize that oxidation of lipoproteins during infection promote their hydrolysis by hGIIA $sPLA_2$, and thereby release lipids directly toxic to the parasite and/or lipid mediators triggering the immune response. Thus, both direct and indirect mechanisms likely contribute to the 20% (hGIIA-treated mice) and 30% (TG mice) lower parasitaemia observed in infected mice. Altogether, our data suggest a beneficial role of hGIIA $sPLA_2$ in malaria.

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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 ± 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)
Total WBCs (10^3/µL)	6.08 ± 0.73	10.15 ± 0.63	0.0043**	20.58 ± 1.89	26.11 ± 2.50	0.0164*
Lymphocytes (10^3/µL)	4.50 ± 0.50	6.50 ± 0.30	0.0043**	12.57 ± 0.98	12.08 ± 1.21	0.7456
Neutrophils (10^3/µL)	1.24 ± 0.45	2.57 ± 0.38	0.1320	4.29 ± 0.34	11.78 ± 1.24	<0.0001***
Monocytes (10^3/µL)	0.26 ± 0.05	0.81 ± 0.03	0.0022**	0.53 ± 0.08	1.13 ± 0.13	0.0004***
Eosinophils (10^3/µL)	0.07 ± 0.01	0.21 ± 0.02	0.0050**	0.51 ± 0.08	1.21 ± 0.16	<0.0001***
Basophils (10^3/µL)	0.005 ± 0.002	0.013 ± 0.003	0.0906	ND	ND	
Total RBCs (10^6/µL)	10.67 ± 0.24	9.72 ± 0.22	0.0152*	5.94 ± 0.57	4.16 ± 0.41	0.0292*
Reticulocytes (%)	5.0 ± 0.4	4.8 ± 0.3	0.6991			
LFR (%)	47.9 ± 0.9	45.6 ± 1.2	0.3095			
MFR (%)	20.1 ± 0.7	18.5 ± 0.7	0.1255			
HFR (%)	32.0 ± 1.4	35.8 ± 1.1	0.0931			
Platelets (10^3/µL)	1494 ± 76.1	1694 ± 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 ± SEM (and median under parenthesis).

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

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

1. Hoggatt J, Hoggatt AF, Tate TA, Fortman J, Pelus LM. 2016. Bleeding the laboratory mouse: Not all methods are equal. Exp Hematol 44:132-137 e1.

Figure 1

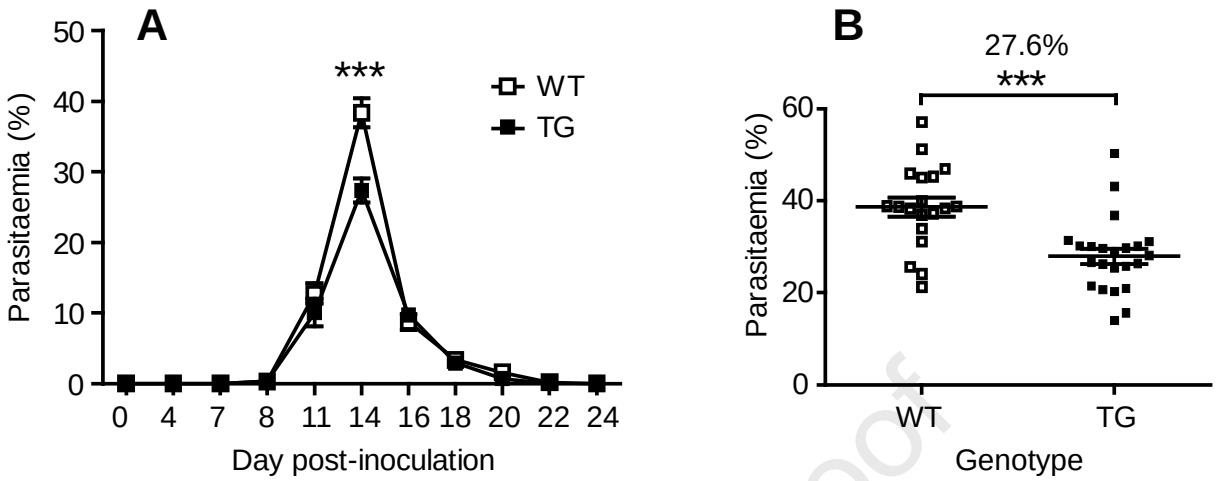


Figure 2

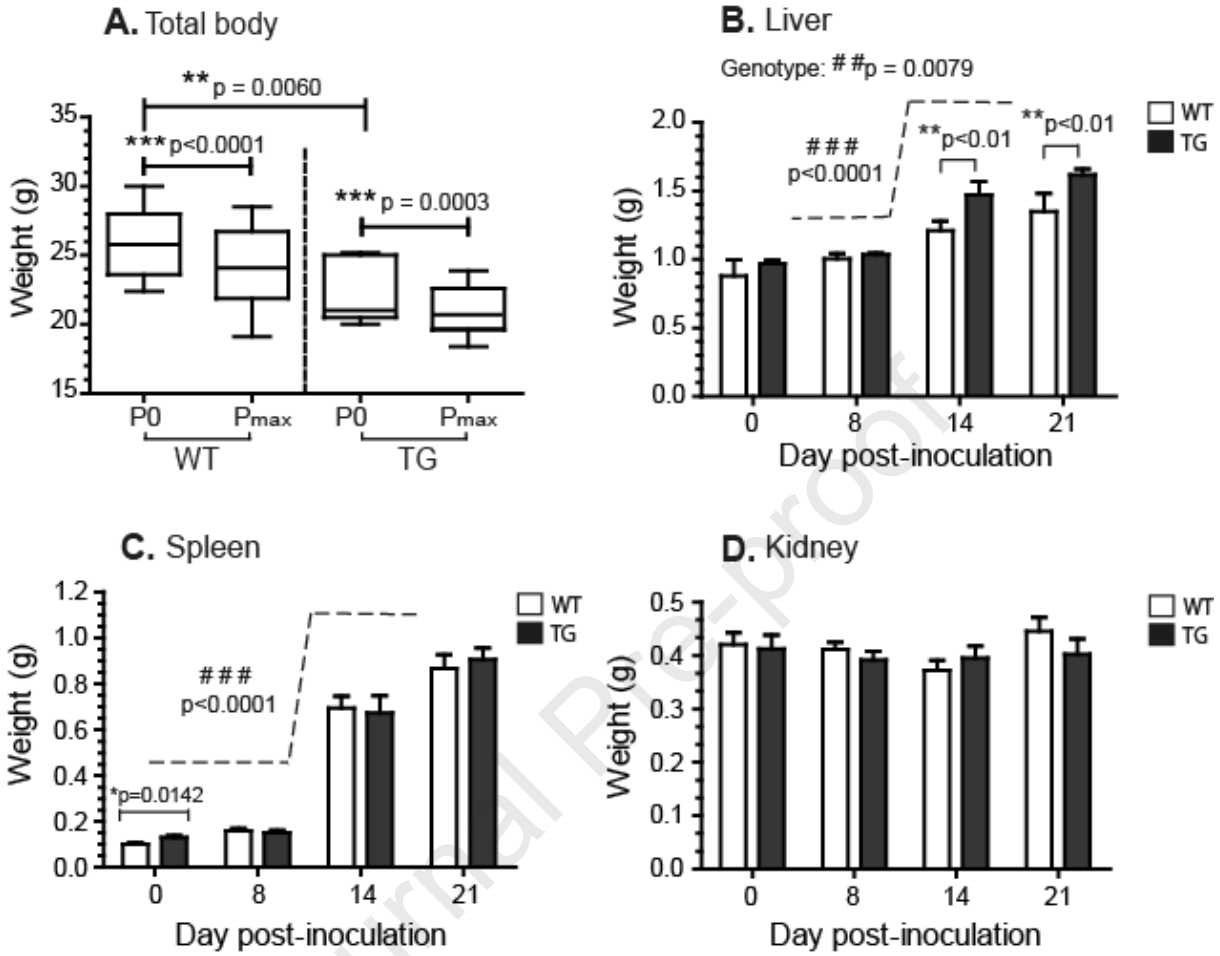


Figure 3

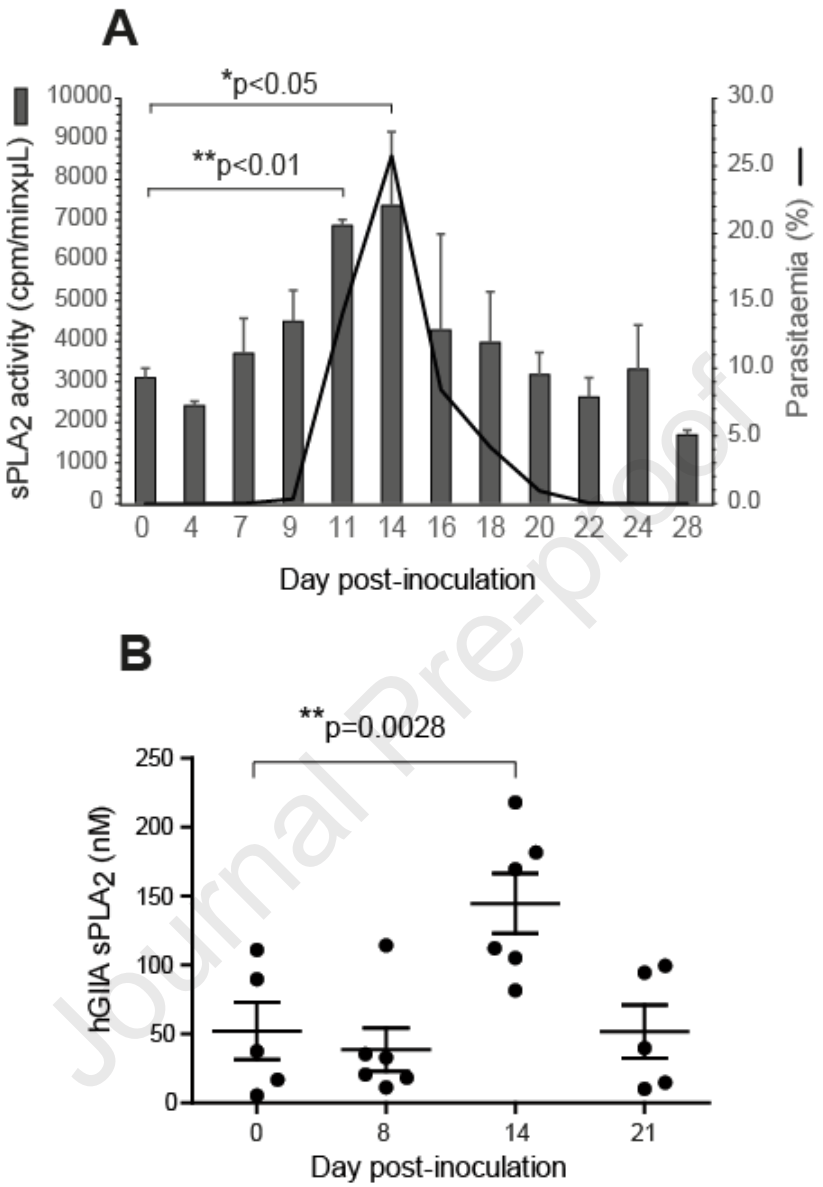
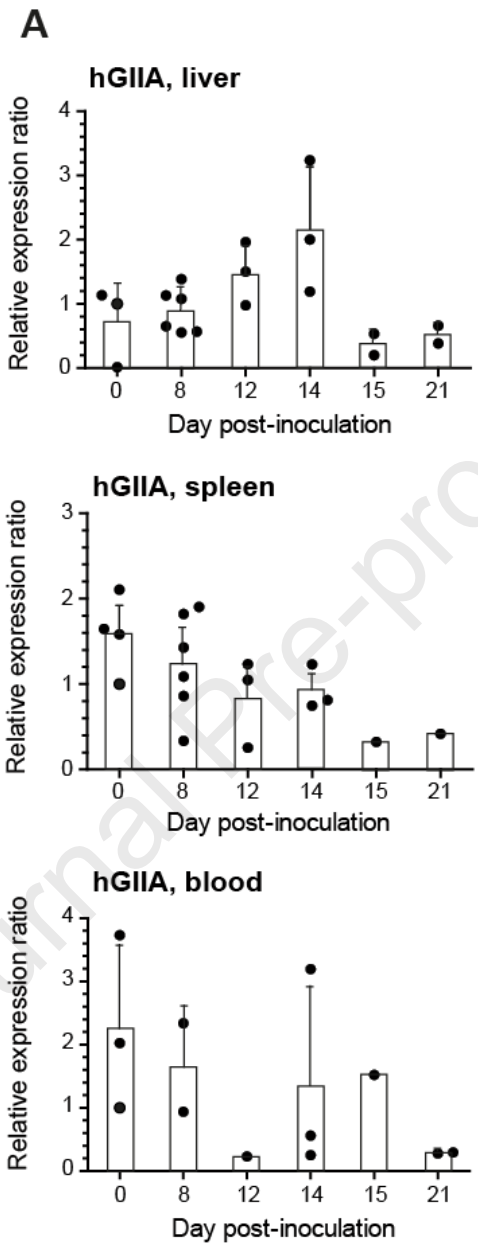
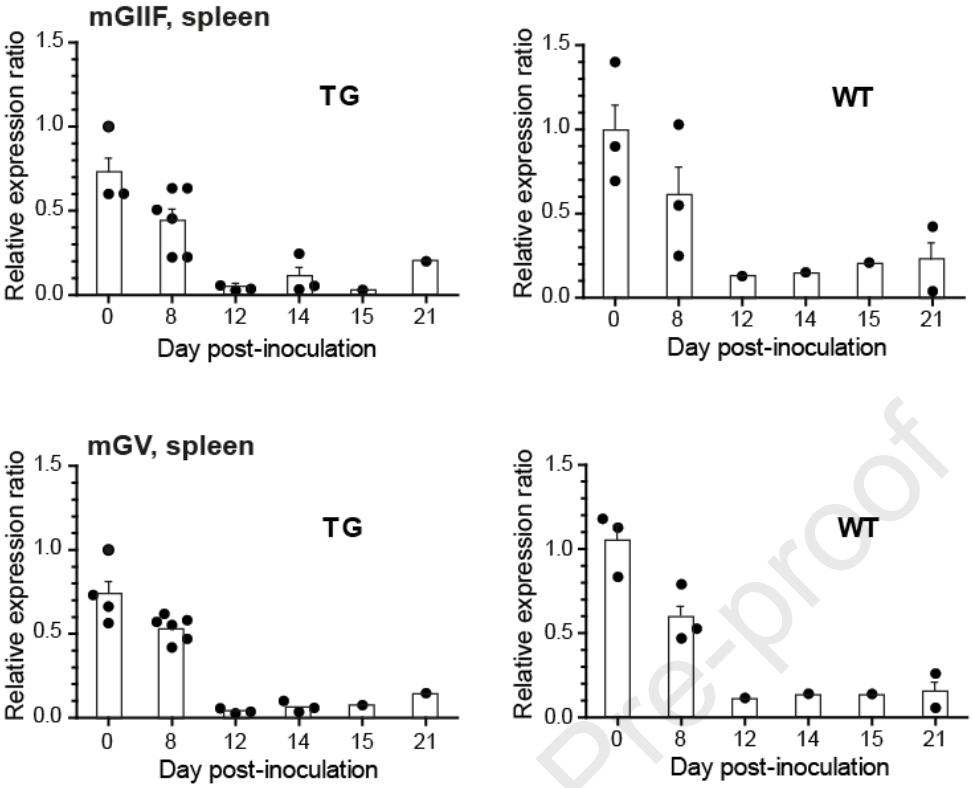


Figure 4



B



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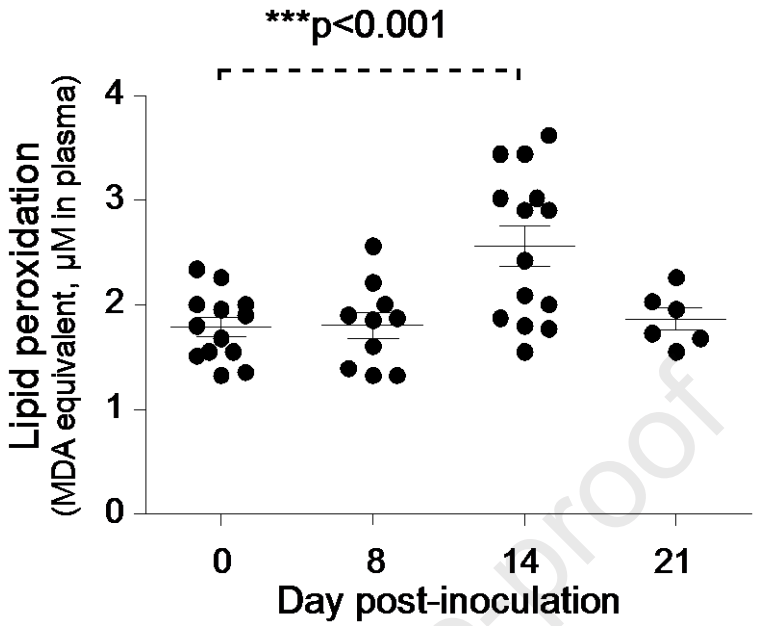


Figure 6

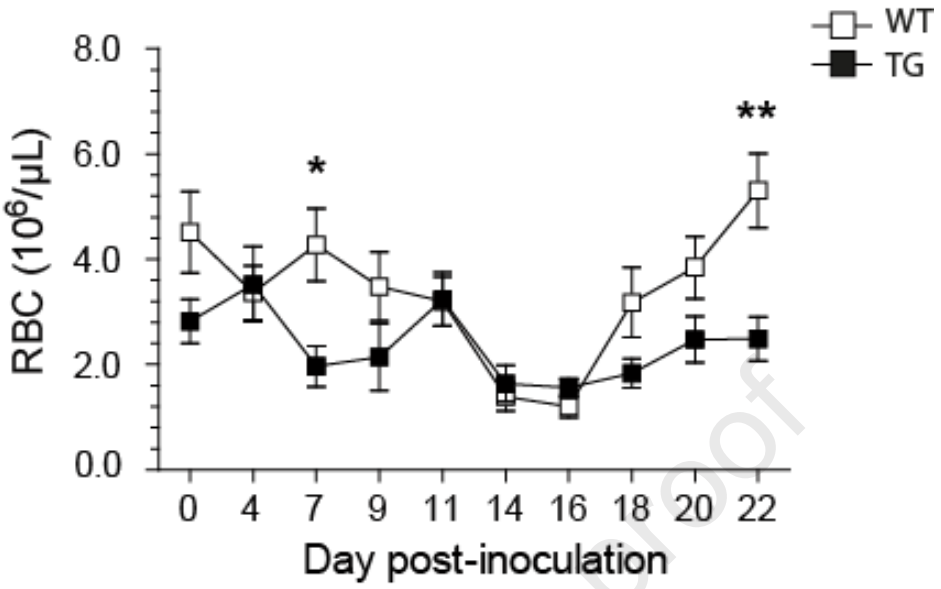


Figure 7

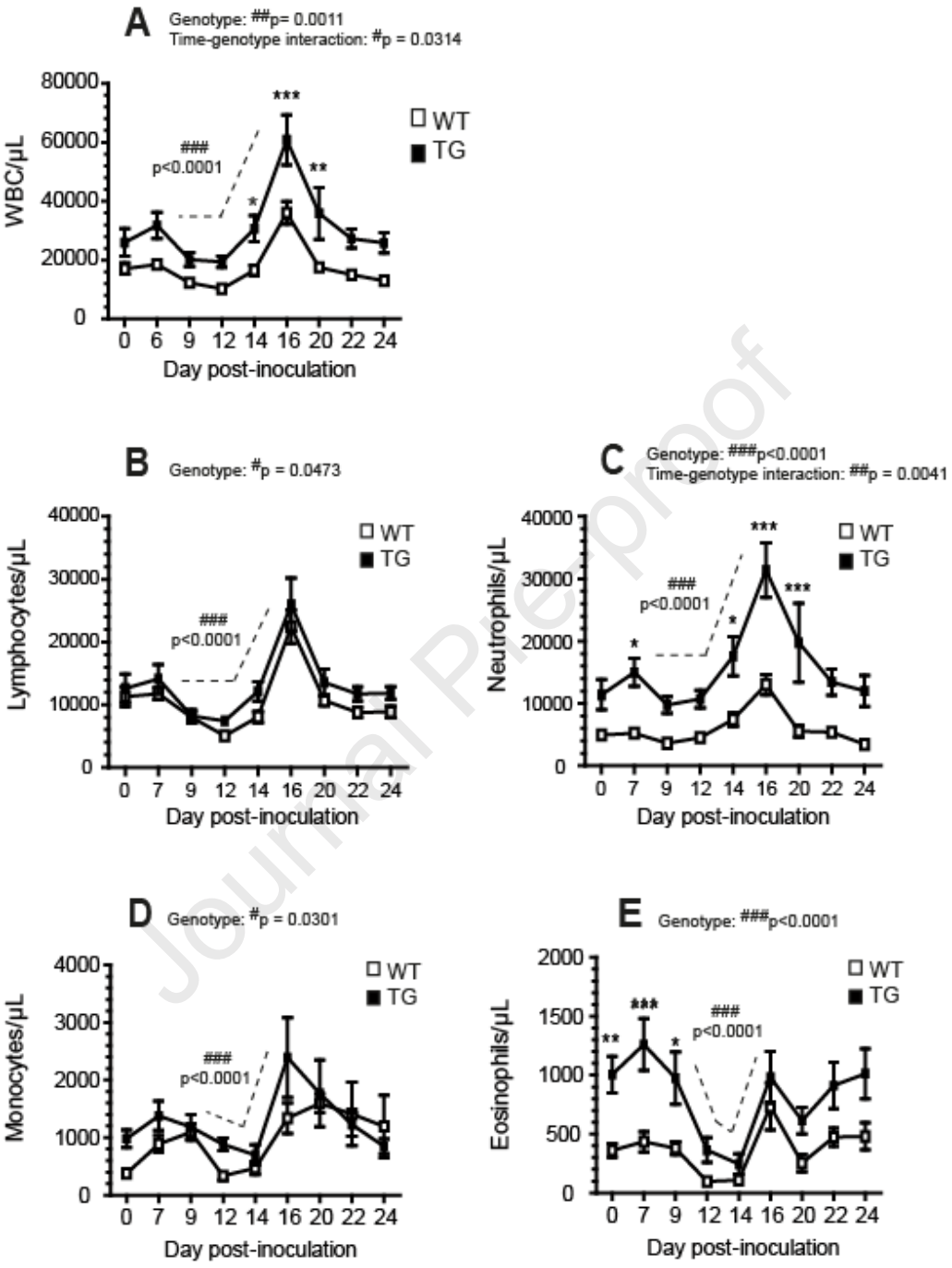
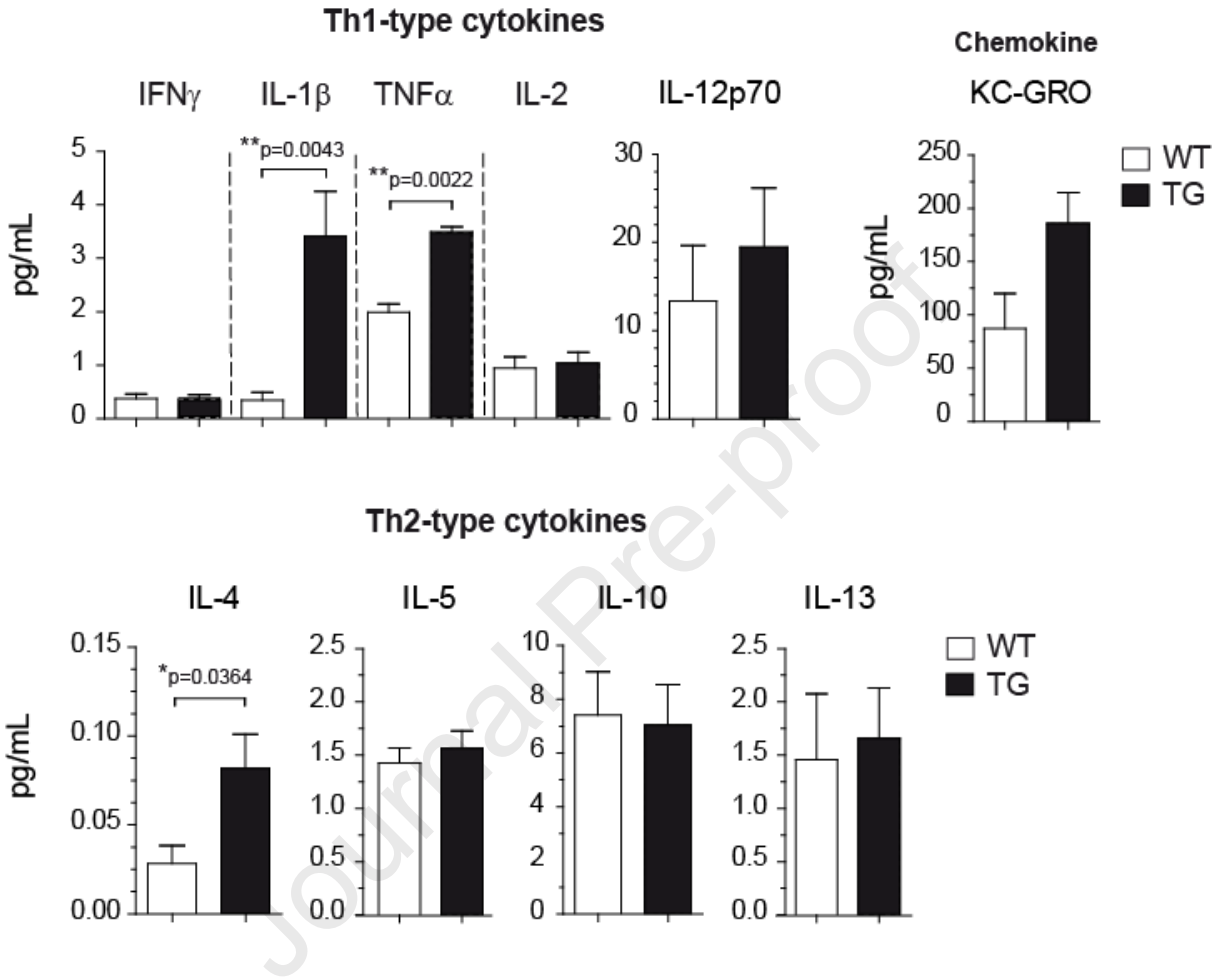


Figure 8

A. Before parasite inoculation



B. After parasite inoculation

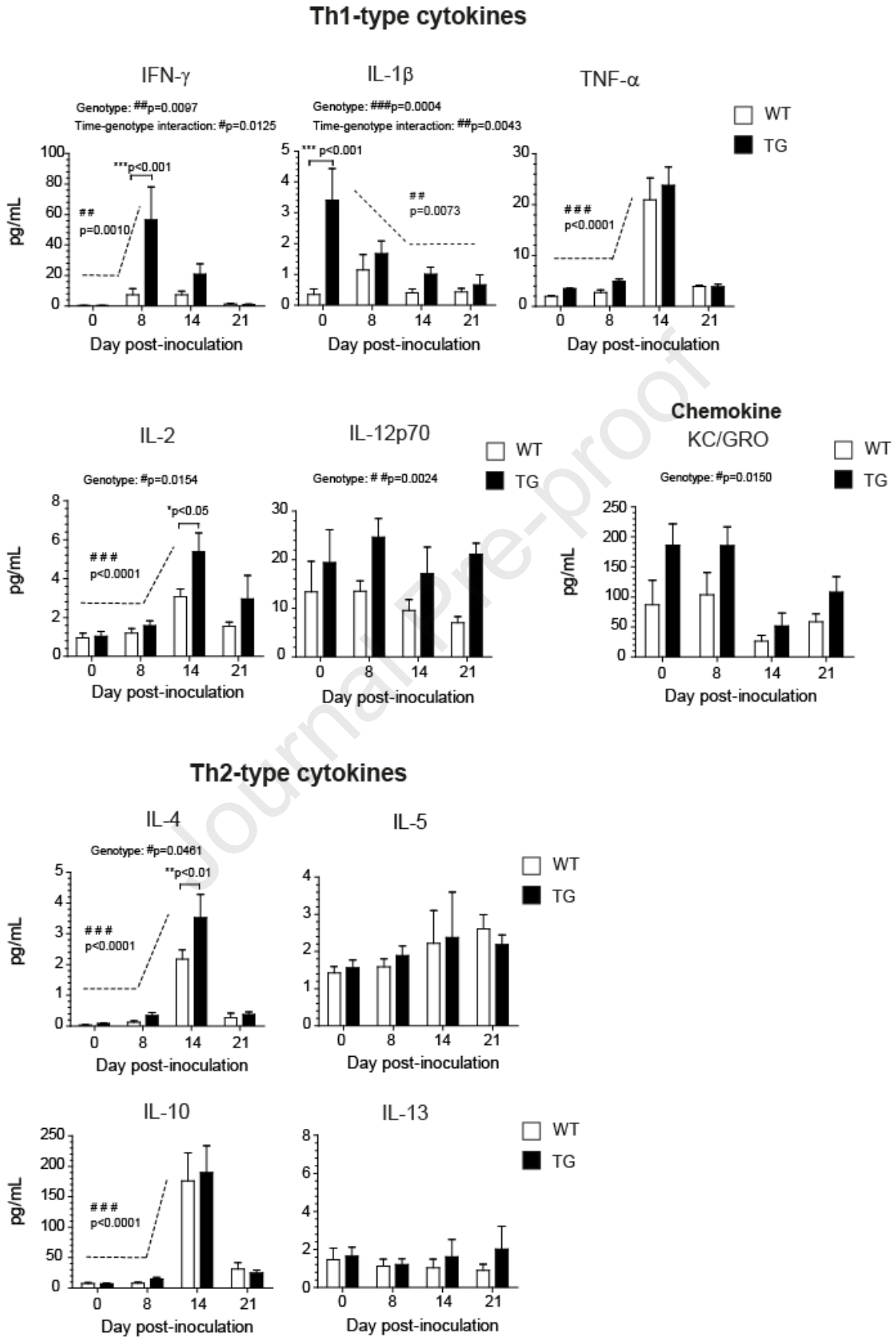
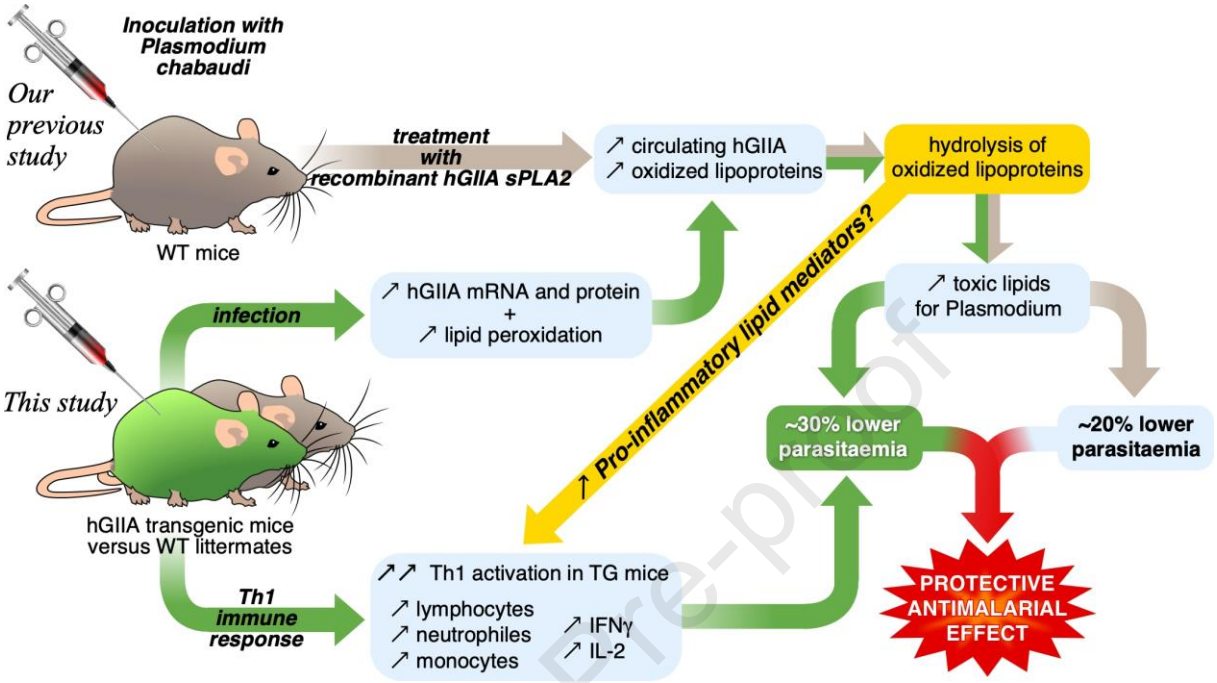


Figure 9



HIGHLIGHTS

1. Mice overexpressing hGIIA $sPLA_2$ (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 parasiticidal lipids from oxidized lipoproteins
5. Likely mechanism 2: hGIIA boosts host innate immunity against *Plasmodium*