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Research paper

Babesia divergens glycosylphosphatidylinositols modulate blood coagulation and induce Th2-biased cytokine profiles in antigen presenting cells

Françoise Debierre-Grockiego a,1, *, Terry K. Smith b,1, Stéphane Delbecq c, Céline Ducournau a, Louis Lantier a, Jörg Schmidt d, Virginie Brès c, Isabelle Dimier-Poisson a, Ralph T. Schwarz d, e, 2, Emmanuel Cornillot f, g, 2

a IST, Université Tours, INRA, 37000, Tours, France
b Biomedical Sciences Research Complex, University of St Andrews, St Andrews, Fife, Scotland, KY16 9ST, UK
c Vaccination Antiparasitaire, Université de Montpellier, 34093, Montpellier, France
d Institut für Virologie, AG Parasitologie, Philipps-Universität Marburg, 35043, Marburg, Germany
e Univ. Lille, CNRS, UMR 8576, Unité de Glycobiologie Structurale et Fonctionnelle, 59655, Villeneuve d’Ascq, France
f Institut de Biologie Computationnelle, 34095, Montpellier, France
g Institut de Recherche en Cancérologie de Montpellier (IRCM – INSERM U1194), Institut Régional du Cancer de Montpellier (ICM), Université de Montpellier, 34095, Montpellier, France

1. Introduction

Babesiosis caused by Babesia divergens, a protozoan parasite of the Apicomplexa phylum transmitted by the Ixodes ricinus tick, is an emerging disease in both human beings and animals [1]. Symptomatic patients present malaria-like febrile illness, but as babesiosis can be asymptomatic, it represents a major transfusion threat [2–4]. Only two standard antimicrobial combinations currently exist to treat human babesiosis: atovaquone and azithromycin,
effective and well tolerated, or clindamycin and quinine, especially useful in severe cases, but unfortunately poorly tolerated [1]. Erythrocyte exchange apheresis is required to complete the treatment [5].

During pathogenesis due to B. divergens, progression of the inflammation-coagulation auto-amplification loop leads to thrombosis, ischemia and in some rare cases, to death [6]. This might be due to Disseminated Intravascular Coagulation syndrome, thrombosis, ischemia and in some rare cases, to death [6]. This might be due to Disseminated Intravascular Coagulation syndrome, thrombosis, ischemia and in some rare cases, to death [6].

Regulation of pro-inflammatory Th1 and anti-inflammatory Th2 cytokine production by Babesia has been studied in vitro and in vivo. B. bovis merozoites (extracellular form) increased NO production and IL-1β, IL-12p40, TNF-α and IL-10 mRNA expression in bovine monocytes, but not in dendritic cells [9]. INF-γ, but no IL-10 was produced by blood mononuclear cells from B. divergens-infected sheep stimulated in vitro with merozoite protein extract [10]. High levels of the regulatory cytokine IL-10 were detected in the serum of B. microti-infected mice and of B. rossi-infected dogs [11,12]. In B. microti-infected mice, the progressive fall in parasitism from peak values at 14 days post infection was inversely proportional to the rise of IL-10 and parasite-specific IgG production, suggesting a role for IL-10-linked antibody responses in the reduction and clearance of the parasite [13].

To determine which parasite fraction is able to stimulate cells, a membrane-enriched fraction of B. bovis merozoites or supernatants from B. bovis-stimulated CD4+ T-cell lines containing IFN-γ and TNF-α have been tested on bovine macrophages and both induced production of NO, partially responsible for parasite replication inhibition and phagocytosis of infected erythrocytes in vitro [14,15]. Phosphatidic acid from a B. bovis attenuated strain and the combination of phosphatidylserine-phosphatidylinositol from attenuated and virulent strains were able to increase Th1 (TNF-α, IL-6), but not Th2 (IL-4) and regulatory (IL-10) cytokine production by mouse peritoneal macrophages in a TLR (Toll-Like Receptor)-2-dependent pathway [16]. Glycosylphosphatidylinositols (GPIs) are abundant glycolipids in the membranes of all apicomplexan parasites. GPIs have been determined as parasite toxins participating in pathogenicity due to their pro-inflammatory properties [17]. In the present study, we have investigated the role of B. divergens GPIs in the modulation of antigen presenting cells in terms of cytokine production, major histocompatibility molecule expression and apoptosis. In addition, direct effect of GPIs on the regulation of the coagulation system was explored ex vivo.

2. Materials and methods

2.1. Metabolic labelling of B. divergens GPIs

Merozoites of B. divergens strain Rouen 1987 were maintained in vitro in human erythrocytes (5% packed cell volume in Roswell Park Memorial Institute [RPMI] 1640 medium with 10% human serum). Metabolic labelling of B. divergens was performed in 20 mL glucose-free RPMI 1640 medium (Sigma) supplemented with 20 mM fructose, 25 mM Hepes and 0.5 mM d-[6-3H]-glucosamine hydrochloride (Hartmann Analytic GmbH) for 4 h at 37°C. After centrifugation, erythrocytes were lysed with a solution of NaCl at 0.2%, neutralized by the addition of same volume of NaCl at 1.6%. After centrifugation, the pellet was frozen at −80°C and washed in phosphate buffered saline (PBS). This step permitted the merozoites to detach from residual erythrocyte membranes. Glycolipids of free merozoites were extracted with chloroform-methanol-water (10:10:3, by volume) by sonication (ultrasound bath Branson 3200, 47 MHz) and recovered in the n-butyl alcohol phase after partitioning between water-saturated n-butyl alcohol and water (1:1, by volume) by centrifugation. Samples were separated by TLC on Silica Gel 60 plate (Merck) using a chloroform-methanol-water (4:4:1, by volume) solvent system. Silica plates were scanned for radioactivity using a Berthold LB 2842 linear analyser.

2.2. Purification of individual GPIs of B. divergens

Large amounts (1 × 1010) of non-labelled merozoites were collected and GPIs not linked to proteins were extracted with chloroform-methanol-water (10:10:3, by volume) by sonication, dried under a nitrogen stream and recovered in the n-butyl alcohol phase after partitioning between water-saturated n-butyl alcohol and water (1:1, by volume) by centrifugation. GPIs were precipitated under a stream of nitrogen to remove contaminating phospholipids [18]. GPIs were then separated by TLC on 0.5 mm silica gel 60 plate (Merck, GPIs from 5 × 109 parasites/plate) using a chloroform-methanol-water (4:4:1, by volume) solvent system, with spots of labelled GPIs used as tracers. TLC plates were scanned for radioactivity using a Berthold LB 2842 linear analyser and areas corresponding to individual GPIs were scraped off the plate, re-extracted with chloroform-methanol-water (10:10:3, by volume) by sonication (only half of the material is estimated to be recovered) and residual silica was removed by water-saturated n-butyl alcohol/water partition. GPIs were stored at −20°C in n-butyl alcohol until use. Absence of endotoxin in each GPI was checked with the Pierce® Limulus Amebocyte Lysate Chromogenic Endotoxin Quantitation kit according to the manufacturer’s instructions (Thermo Scientific).

2.3. Quantification and composition analysis of carbohydrates of B. divergens GPIs

The method is based upon quantification of the GlcN residues of the GPIs being converted to Man3-anhydromannitol (AHM) as described elsewhere [19].

2.4. Composition analysis of phosphatidylinositol moieties of B. divergens GPIs

Individual B. divergens GPIs were dried and dissolved in sodium acetate followed by the addition of sodium nitrite. The PI moieties released by deamination were partitioned into n-butyl alcohol. The n-butyl alcohol extracts were dried, suspended in chloroform-methanol and analysed by negative ion electrospray mass spectrometry (ES-MS, ABSciex 4000 QTrap). Daughter ion ES-MS-MS spectra were obtained with a collision voltage of 35–50 V [20].

2.5. Cell stimulation and cytokine/SEAP activity quantification

Antigen presenting cells were seeded at 2 × 105 in 96-well plates in 100 μL Dulbecco’s Modified Eagle Medium (DMEM) for RAW 264.7 macrophage cell line (ATCC® TIB-71™), at 105 in 24-well plates in 300 μL DMEM for peritoneal exudates cells (PECs) harvested from Swiss OF1 mice by washing with 5 mL DMEM (directive 2010/63/EU not applied [chapter 1, article 1.5.]) and at 106 in 24-well plates in 300 μL RPMI for SRDC dendritic cell line (Applied Biological Materials Inc.). HEK-Blue™ hTLR2 and hTLR4 cells (InvivoGen) were seeded at 2 × 105 in 96-well plates in 100 μL DMEM/1% fetal calf serum without selection antibiotics. The cells
were stimulated at 37 °C in 5% CO₂ atmosphere for 24 h with individual GPIs purified from 5 × 10⁸ (10⁸ for HEK-Blue™ cells) merozoites of *B. divergens* or with 200 ng/mL of lipopolysaccharide (LPS from *Escherichia coli* serotype 055:B5, Sigma). The amount of GPIs needed for one experiment was dried under a nitrogen stream to remove the solvent n-butyl alcohol. GPIs were suspended in culture medium (in 100 or 300 µl/well for RAW 264.7 and HEK-Blue™ or PECs and SRDC, respectively) by sonication. For the negative control, cells were incubated with same volume of n-butyl alcohol dried and suspended in medium by sonication. Levels of cytokines were quantified in the cell culture supernatants by using specific sandwich enzyme-linked immunosorbent assay (ELISA) from Affymetrix ebioscience or MACSplex Cytokine 10 Kit, mouse from Miltenyi Biotec GmbH, following the manufacturer’s instructions. SEAP (secreted embryonic alkaline phosphatase) reporter gene activity of HEK-Blue™ cells was measured at 630 nm after addition of QUANTI-Blue™ detection medium (InvivoGen) to supernatant.

2.6. Measurement of MHC expression and apoptosis

SRDC and PECs were stimulated as described above (2.5.). Supernatant was centrifuged at 300 × g to pool floating cells and attached cells after their detachment using accutase (Affymetrix ebioscience). After centrifugation at 300 × g, supernatant was removed for quantification of cytokines (section 2.5.) and pelleted SRDC were suspended and saturated for 30 min on ice in PBS containing 1% bovine serum albumin, 2% mouse serum and 0.1% azide (PBS-BSA-azide). After centrifugation, 3 × 10⁵ SRDC were incubated for 30 min on ice in the dark in PBS-BSA-azide with 0.5 µg fluorescein isothiocyanate (FITC) mouse anti-mouse H–2K [k] MHC class I antibody (clone 36-7-5, BD Pharmingen™), 0.5 µg FITC mouse anti-mouse I-El[k] MHC class II antibody (clone 14-4-4S, BD Pharmingen™), or 0.5 µg FITC mouse IgG2a, κ isotype control (clone G155-178, BD Pharmingen™). After centrifugation, SRDC were suspended in 300 µL PBS-2% paraformaldehyde and analysed by flow cytometry (BD FACSCalibur™ and CellQuest™ software, BD Bioscience). PECs were incubated for 15 min at 4 °C in the dark in 100 µL binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 5 mM CaCl₂) with 5 µL annexin V-FITC (BD Pharmingen™) and 5 µg/mL propidium iodide (Sigma). After centrifugation, PECs were suspended in 300 µL binding buffer and analysed by flow cytometry.

2.7. Measurement of coagulation parameters

Blood was taken from non-infected adult male Wistar rat anesthetized with 60 mg/kg pentobarbital. Nine volumes were mixed with one volume of trisodium citrate at 0.109 M (32%). Plasma was separated by centrifugation during 15 min at 2500 × g. Half of the plasma sample was incubated for 30 min at room temperature with GPIs of *B. divergens* (from 5 × 10⁸ merozoites for 200 µL plasma). Before adding GPIs, the solvent n-butyl alcohol was dried under a nitrogen stream and GPIs were suspended in 10 µL of Owren-koller buffer by sonication. Half of the plasma sample was incubated for 30 min at room temperature with 10 µL of Owren-koller buffer containing dried n-butyl alcohol alone. Negative and positive controls of coagulation, all reagents and Star 4 coagulation analyser were from Stago (France). Fibrinogen level was measured on sample diluted at 1/10 and 1/20 (final volume of 100 µl) with Fibri-Prest® Automate 2 (thrombin + heparine inhibitor) reagent, PT was measured on 50 µL sample with Neoplastine® Cl Plus (thromboplastin + calcium) reagent and APTT was measured on 50 µL sample with C.K. Prest® (cephalin + kaolin activator) or Cephascreen® (cephalin + polyphenol activator) reagent, according to the manufacturer’s instructions. All results are expressed in time of coagulation and given in seconds.

2.8. Statistics

The non-parametric one-way ANOVA test followed by the Dunnett’s multiple comparison test (for not significantly different variances determined with Bartlett’s test), the non-parametric Kruskal-Wallis test or the Friedman test followed by the Dunn’s multiple comparison test (for significantly different variances determined with Bartlett’s test) and the Wilcoxon test were used for statistical evaluation (GraphPad Prism 7).

3. Results

3.1. Analysis of carbohydrate and lipid composition of *B. divergens* GPIs

To detect the GPIs of *B. divergens* for the first time, merozoites were metabolically labelled with [³H]-glucosamine during *in vitro* culture within erythrocytes. Merozoites were separated from erythrocyte membrane by osmotic lysis and freezing. After their extraction with organic solvents, the different GPI species were separated by TLC and detected with a Berthold analyser. As shown in Fig. 1, two GPIs (3 and 8) were predominantly labelled, while eight GPIs were less expressed in *B. divergens* merozoites. In order to study biological effects of GPIs on mammalian cells and to determine their carbohydrate and lipid contents, GPIs were extracted from large amounts of merozoites. Contaminant phospholipids were completely eliminated by precipitation of GPIs [18]. After the separation of GPIs on preparative TLC, silica was scraped off according to the full width at half maximum of each peak. By this way, individual GPIs were highly purified. Based upon AHM and inositol quantification, GPI3 was the most abundant GPI species with more than 400—500 × 10⁴ copies per merozoite (Table 1). In contrast, GPI10 was the lowest represented GPI species with less than 3 × 10⁴ copies per merozoite. Except for GPI8, quantification was consistent with the counts of radioactivity on Fig. 1. The carbohydrate composition analysis by GC-MS of *B. divergens* GPIs revealed the presence of a maximum of two mannoses (Table 2). Galactose was detected on GPI1 and GPI2, but not on the other GPI species (Table 2). Mass spectroscopy analysis of the PI moieties showed that each GPI species exists in several forms with different fatty acid methyl esters (from 16:0 to 24:0) or diaclys (from 34:1 to...
48:0) at various ratios (Fig. 2A: ES-MS results for GPI3 and GPI8, Table 3: results for all GPI species). Altogether, composition analysis gives the proposed GPI-pathway illustrated in Fig. 3: GlcNAc-PI (GPI9), GlcN-PI (GPI8), GlcN-(acyl)PI (GPI10), Man-GlcN-(acyl)PI (GPI7), Man-Man-GlcN-(acyl)PI (GPI4, GPI5, GPI6, with remodelled long fatty acids), Man-Man-GlcN-PI (GPI3), (Gal)-Man-Man-GlcN-PI (GPI2), (Gal-Gal)-Man-Man-GlcN-PI (GPI1), with GPI8 and GPI3 the major GPI-intermediates. GPI4, GPI5, GPI6, GPI7 and GPI10 had an acylated inositol and the clear 640 m/z fragment ion strongly suggests

### Table 1
AHM and inositol quantifications of individual GPIs of *B. divergens*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>GPI1</th>
<th>GPI2</th>
<th>GPI3</th>
<th>GPI4</th>
<th>GPI5</th>
<th>GPI6</th>
<th>GPI7</th>
<th>GPI8</th>
<th>GPI9</th>
<th>GPI10</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHM quantification (× 10^6 copies per cell)</td>
<td>12.3 ± 1.6</td>
<td>39.2 ± 7.1</td>
<td>407.0 ± 18.0</td>
<td>12.1 ± 1.3</td>
<td>9.46 ± 0.49</td>
<td>7.92 ± 0.31</td>
<td>1.90 ± 0.16</td>
<td>4.38 ± 0.16</td>
<td>1.22 ± 0.09</td>
<td>1.07 ± 0.06</td>
</tr>
<tr>
<td>Inositol quantification (× 10^6 copies per cell)</td>
<td>1.54 ± 0.17</td>
<td>43.3 ± 6.3</td>
<td>511.0 ± 38.0</td>
<td>18.0 ± 0.9</td>
<td>14.9 ± 1.5</td>
<td>12.0 ± 1.0</td>
<td>2.06 ± 0.07</td>
<td>8.01 ± 0.62</td>
<td>1.44 ± 0.17</td>
<td>2.85 ± 0.38</td>
</tr>
</tbody>
</table>

Each GPI was submitted to base hydrolysis, deamination/reduction, methanolysis and TMS derivatisation for its subsequent analysis by GC-MS. Single ion monitoring of m/z 273 was selected to detect AHM and m/z 318 to detect both scyllo- and myo-inositol. The peak areas of the corresponding standards were used to calculate the molar relative response, allowing quantification of both AHM and myo-inositol in the samples. Values are means of molar ratios ± standard error of two replicates (equivalent to 2 × 10^8 parasites, each).

AHM: anhydromannitol.

### Table 2
Carbohydrate composition analysis of individual GPIs of *B. divergens*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>GPI1</th>
<th>GPI2</th>
<th>GPI3</th>
<th>GPI4</th>
<th>GPI5</th>
<th>GPI6</th>
<th>GPI7</th>
<th>GPI8</th>
<th>GPI9</th>
<th>GPI10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ino</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>1.9 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>1.2 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Gal</td>
<td>1.7 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Each GPI was submitted to base hydrolysis, deamination/reduction, methanolysis and derivatisation for its analysis by GC-MS. The peak areas of the corresponding standards were used to calculate the molar relative response, allowing determination of monosaccharide content. GlcNAc levels were calculated from AHM generated during deamination. Values are means of molar ratios ± standard error of two replicates.

Ino: inositol; Man: mannose; GlcNAc: N-acetyl-glucosamine; Gal: galactose.

Fig. 2. Spectra of the PI moieties released by deamination from individual *B. divergens* GPIs. ES-MS spectra of the PI moieties from GPI3 and GPI8 (A) and ES-MS-MS spectra of the PI moieties from GPI3-863 m/z and GPI10-1262 m/z (B). 384.6 m/z = GlcN-inositol-cyclic-phosphate, 402.6 m/z = GlcN-inositol-phosphate, 640.4 m/z = GlcN-(palmitoyl)inositol-phosphate. Fatty acids of all GPI species are listed in Table 3.
exclusive palmitoylation on the inositol, as there was no fragment ion at either 612 or 668 m/z, which would correspond to myristoyl or stearoyl, respectively (GPI10 spectra on Fig. 2B).

3.2. Responses of antigen presenting cells to B. divergens GPIs

3.2.1. Production of cytokines

In order to study the role of B. divergens GPIs in cytokine production, macrophages and dendritic cells were stimulated in vitro. For this, macrophages of the RAW 264.7 cell line, non-elicited peritoneal exudate cells (PECs, rich in macrophages), or dendritic cells of the SRDC cell line were stimulated during 24 h with the ten individual GPIs extracted from 5 × 10⁸ merozoites of B. divergens and cytokine levels were quantified in the cell culture supernatant by sandwich ELISA. B. divergens GPIs induced the production of the anti-inflammatory cytokines IL-5 and the regulatory cytokine IL-10 (Fig. 4). The range of produced cytokines was different between the three cell types and varied according to the GPI species. After their stimulation with B. divergens GPIs, PECs produced significantly higher levels of IL-5 (Fig. 4A upper histogram, \( p = 0.003 \) Kruskal-Wallis test), but not of IL-10 (Fig. 4A lower histogram, \( p = 0.109 \) Kruskal-Wallis test) compared to the control condition, certainly due to important variation. In the presence of B. divergens GPIs, RAW 264.7 macrophages showed the lowest increases in IL-5 levels (Fig. 4B upper histogram, \( p = 0.015 \) one-way ANOVA test), but the most significant global increase in IL-10 levels (Fig. 4B lower histogram, \( p = 0.0003 \) Kruskal-Wallis test). Increases of IL-5 levels produced by SRDC in response to the GPIs were highly significant (Fig. 4C upper histogram, \( p = 0.0001 \) Kruskal-Wallis test), while increases of IL-10 production by this cell type were modest (Fig. 4C lower histogram, \( p = 0.0196 \) Kruskal-Wallis test). Regarding individual GPIs, PECs were significantly stimulated by GPI7 to GPI10 to

<table>
<thead>
<tr>
<th>Table 3</th>
<th>MS analysis of the PI moieties and their corresponding fatty acid content released by deamination from individual B. divergens GPIs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>GPI1</td>
</tr>
<tr>
<td>Observed m/z</td>
<td>NA</td>
</tr>
<tr>
<td>Diacyl*</td>
<td>40:0</td>
</tr>
<tr>
<td>FAME</td>
<td>24:0 (9)</td>
</tr>
<tr>
<td></td>
<td>22:0 (4)</td>
</tr>
<tr>
<td></td>
<td>20:0 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GPI moieties were subjected to base hydrolysis, acidification, extraction and derivatisation prior to analysis of fatty acid methyl ester by GC-MS. Some of the PI moieties released by deamination were analysed by negative ion ES-MS. All of the molecular species refer to the corresponding peaks in the negative ion mode in Fig. 2. Observed [M – H]-ions, mass over charge from survey scans as described in the experimental procedure section.

*Peak identities refer to total number of carbon atoms and double bonds.

NA: not analysed.

FAME: fatty acid methyl ester (values in parenthesis are the ratio of FAME).
produce IL-5 and by GPI1, GPI3 and GPI9 to produce IL-10; RAW 264.7 macrophages were significantly stimulated by GPI3 to GPI8 to produce IL-5 and by all GPIs except GPI7 to produce IL-10; SRDC were significantly stimulated by GPI3 to GPI6, GPI9 and GPI10 to produce IL-5 and by GPI7 to produce IL-10 (Figs. 4A, B, C). Similar levels of IL-5 and IL-10 were obtained with higher amounts of GPIs (data not shown). In contrast to GPIs of other protozoan parasites studied until now, GPIs of B. divergens did not enhance the production of IL-1β, IL-12 and TNF-α by any of the cells tested, whereas bacterial lipopolysaccharide used as positive control did (IL-1β: 35.1 ± 1.7 pg/mL with butanol, from 30.4 ± 1.6 to 34.3 ± 1.7 pg/mL with GPIs, 128.1 ± 1.7 pg/mL with butanol, from 1.7 ± 0.6 to 5.5 ± 1.2 pg/mL with GPIs, 51.7 ± 2.3 pg/mL with GPIs; TNF-α: **p < 0.01). To further characterise the cytokine profile induced by B. divergens GPIs, a larger panel of cytokines was quantified by flow cytometry in the supernatant of RAW 264.7 macrophages. Levels of IFN-γ (Th1), IL-4 (Th2), GM-CSF and IL-17A (Th17) were under the detection threshold (data not shown). Compared to the control condition, the GPIs induced very low levels of IL-2, but significantly higher levels of IL-23, representative of a Th17 profile (Fig. 4E). Altogether, these results showed that the GPIs of B. divergens orientate antigen presenting cells towards a unique Th1/Th2/Th17 profile compared to the pro-inflammatory profile induced by GPIs of all other protozoan parasites explored until now.

3.2.2. TLR signalling
To determine whether B. divergens GPIs are ligands of TLRs, HEK293T cells modified to express alkaline phosphatase reporter gene after TLR2 or TLR4 ligation were stimulated with B. divergens GPIs. Alkaline phosphatase activity was detected in supernatants of HEK-TLR2 cells stimulated with GPI2 to GPI9 (Fig. 5A, p = 0.024 Kruskal-Wallis test) and in supernatants of HEK-TLR4 cells stimulated with GPI2 to GPI10 (Fig. 5B, p = 0.0483 Kruskal-Wallis test) with higher activities of the last cell line, suggesting a predominant signalling through TLR4.

3.2.3. Modulation of MHC expression and apoptosis
Expression of MHC molecules has been explored on SRDC stimulated with B. divergens GPIs by flow cytometry after labelling with specific antibodies. No difference in MHC class I molecule expression was observed in the presence or absence of GPIs.
(Fig. 6A), whereas *B. divergens* GPIs decreased MHC class II molecule expression at the surface of the cells (Fig. 6B). Although the difference was significant for GPI3 and GPI6, the global statistical analysis is not significant ($p = 0.22$ Kruskal-Wallis test). Because apoptosis is difficult to evaluate in immortalized cell lines, PECs primary cells have been chosen for the study of apoptosis in response to *B. divergens* GPIs, determined by flow cytometry after labelling with annexin V-FITC. The percentage of necrotic cells (annexin V-FITC and propidium iodide double positive cells) was less than 2% in all conditions (data not shown). The percentage of apoptotic PECs (annexin V-FITC positive cells and propidium iodide negative cells) was relatively elevated in the control culture (Fig. 6C), certainly due to the culture conditions (no serum to avoid interference with GPIs). All GPIs of *B. divergens* increased the basal percentage of apoptotic cells, but not significantly ($p = 0.09$ Friedmann test). Individually, apoptosis was significantly increased by GPI1, GPI2, GPI4 and GPI8 (Fig. 6C).

### 3.3. Ex vivo regulation of coagulation parameters by *B. divergens* GPIs

We finally asked us whether GPIs could regulate the coagulation system. Due to the difficulty to produce large amounts of GPIs, they were tested *ex vivo* and not administered *in vivo*. Blood was taken from rats to get enough volume of plasma and levels of fibrinogen, prothrombin time (PT) and APTT were measured after 30 min incubation with GPIs of *B. divergens*. As shown in Fig. 7A, the coagulation times reflecting the levels of fibrinogen were slightly increased in the presence of GPIs compared to the control ($p = 0.02$ Wilcoxon test), whereas PT (Fig. 7B) was not increased by the GPIs of *B. divergens* ($p = 0.06$ Wilcoxon test). APTT (Fig. 7C) was significantly increased by *B. divergens* GPIs *ex vivo* when tested with two different activators (polyphenol activator: $p = 0.002$ Wilcoxon test, kaolin activator: $p = 0.004$ Wilcoxon test). These results suggest a direct effect of GPIs on coagulation factors of the intrinsic pathway.

### 4. Discussion

By using metabolic labelling, ten different GPIs, including biosynthetic intermediates of *B. divergens* have been detected for the first time in parasites cultivated in erythrocytes. Only five GPIs have been detected in *B. bovis*, but the method used to visualize them after TLC (iodine vapors) was not sensitive enough to detect minor spots [21]. The GPI-profile of *P. falciparum* showed eight distinct peaks identified and all GPI species except one (PF) carry a fatty acid on the inositol ring [22]. Amongst other apicomplexan...
of an Apicomplexa [30,31], but this is the activator: right histogram) are expressed in time of coagulation and given in seconds.

Fibrinogen level ($\gamma$/C2) and PT ($\gamma$/C2) were suggested that the presence of only two mannoses in the core glycan observed in all other eukaryotes. As no homolog of the core structure, con

Fig. 7. Fibrinogen levels, PT and APTT coagulation parameters in response to GPIs of B. divergens. Rat plasma was incubated for 30 min with GPIs of B. divergens (from $5 \times 10^6$ merozoites for 200 ml plasma) or with the n-butyl alcohol solvent (But). Fibrinogen level (A), PT (B) and APTT (C, polyphenol activator: left histogram, kaolin activator: right histogram) are expressed in time of coagulation and given in seconds. Each box represents results obtained with the 10 different GPIs of B. divergens.

parasites, the GPI of the 17-kDa antigen from Cryptosporidium parvum contains an acylated inositol [23], whereas the inositol ring is not substituted in any of the mature GPIs of Toxoplasma gondii [24]. Myristic acid was the predominant modification of the inositol of GPIs purified from merozoite surface proteins-1 and -2 of the FCBR strain of P. falciparum [25], while palmitic acid and myristic acid represent 90% and 10%, respectively, of the acyl chain on inositol of GPIs from the FCR-3 strain of P. falciparum [26]. In a previous study on B. divergens, the GPI anchor of the Bd37 major surface antigen was identified to have a palmitic acid substitution on the inositol [27,28]. Our results confirm that palmitic acid is exclusively present on GPI4, GPI5, GPI6, GPI7 and GPI10 intermediates in B. divergens. In a previous work, we could find the sequences coding for only two mannosyltransferases (PfGM and PfIV, but not PfGB, the third mannosyltransferase) in the genome of B. microti, suggesting a di-mannose structure [28,29]. We could not rule out that one of the two mannosyltransferases or another enzyme could add a third mannose. However, a glycan structure of Man$_2$-GlcN has been identified in the main GPI species isolated from merozoites of B. bovis [21]. Here, we found the same particular structure, confirming that Babesia does not have the conserved GPI core glycan observed in all other eukaryotes. As no homolog of PfGB gene could be found in any piroplasmida genomes sequenced so far, we suggest that the presence of only two mannoses in the core glycan of the GPIs is a key feature of these parasites. Galactose is present in GPIs of Trypanosoma sp. and of Entamoeba histolytica [30,31], but this is the first time that this hexose is identified in GPIs of an Apicomplexa. Further analyses are needed to confirm the carbohydrate structure and to identify the final GPI anchor with ethanolamine phosphate, removed here through hydrolysis. The lipid moiety of GPI4 to GPI10 have palmitic, stearic, eicosanoic, docosanoic and tetracosanoic saturated fatty acids and oleic unsaturated fatty acid, whereas GPI1, GPI2 and GPI3 have only the longer chains (eicosanoic, docosanoic and tetracosanoic fatty acids) and no unsaturated fatty acids. In B. bovis, the structure of the main GPI also contains predominantly docosanoic and tetracosanoic fatty acids [21].

After infection with protozoan parasites, cells of the immune system produce Th1 cytokines playing an important role in the innate immune response and in the development of adaptive immunity. On the other hand, excess of Th1 cytokines is deleterious for the host. GPIs of P. falciparum have been defined as toxins eliciting hypoglycemia and excess of TNF-α production related to pyrexia and cachexia in mice [32]. GPIs of P. falciparum induced the production of IL-1β, TNF-α and NO by thioglycollate-elicited peritoneal macrophages [32,33]. Other studies have shown that GPIs of T. gondii [19,34], T. brucei [35] and T. cruzi [36] also induce Th1 cytokines (IL-12, TNF-α) by macrophages. Surprisingly, no Th1, but Th2/Th17 cytokines were secreted by antigen presenting cells stimulated by B. divergens. It would be interesting to expand the panel of cytokines analysed (i.e. families of IL-6 and TGF-β) to refine the profile. Studies on GPIs of T. brucei and P. falciparum have shown a dose-dependent effect on macrophages [35,37]. In the case of T. gondii GPIs, we have calculated that addition of individual GPI purified from 10$^5$ tachyzoites to a final volume of 200 μl of medium corresponds to a concentration of 1 μM [38]. Compared to LPS, 100-fold higher concentrations of T. gondii GPIs were required to induce similar TNF-α levels by RAW 264.7 macrophages (personal observation). Furthermore, the dose-response was not linear: around 500 pg/mL were produced with GPIs from 10$^6$ and 10$^7$ tachyzoites to jump to about 2000 pg/mL with GPIs from 10$^8$ and 10$^9$ tachyzoites, suggesting an all-or-nothing threshold to be passed for optimal cell response [34]. The same phenomenon seems to occur with B. divergens GPIs, since increased amounts did not lead to higher cytokine levels (data not shown).

Cytokine production was dependent on the complex B. divergens GPI species/target cell/cytokine. In a recent study, we have also shown that the cytokine pattern produced in response to GPIs of N. caninum depends on the GPI species, the type of cells (cell lines vs. primary cells) and the origin of the cells (murine vs. bovine) [39]. Thus, cells from natural host of B. divergens should be tested to further characterise their biological effects. Concerning the GPI structure, the present results contrast with those obtained with T. gondii and P. falciparum. Indeed, the six different T. gondii GPIs (GPI I to GPI VI) and the 8 different P. falciparum GPI species (Pfα to Pfβ) were all able to induce TNF-α production by RAW 264.7 macrophages [34,40]. However, induction of IL-5 or IL-10 production could involve different signalling pathways than TNF-α production. In T. brucei, it has been demonstrated that the galactose side chain of the glycosyl-inositol-phosphate (GIP) moiety is responsible for TNF-α production by macrophages [41]. B. divergens GPI, with two Gal residues was unable to induce IL-5 in any cell type studied. This is maybe related to the absence of TLR2/4 signalling triggered by this GPI. However, GPI2 with one Gal residue was able to signal through TLR2/4, but also did not induce IL-5 production. TNF-α production induced by P. falciparum GPIs is mediated mainly through TLR2 and to a lesser extent through TLR4 [42]. On the contrary, whole GPIs of T. gondii activated TLR4 in the CHO cell model, but their core glycans and diacylglycerols potentially separated by macrophage phospholipases, activated both TLR2 and TLR4 in macrophages [43]. Complementary experiments are required to determine whether B. divergens GPIs stimulate antigen presenting cells only after their cleavage by specific enzymes. In the past, we demonstrated that GPIs not linked to proteins are
expressed at the surface of T. gondii tachyzoites and clustered in lipid rafts [44]. Furthermore, GPIs not linked to proteins have been detected in the culture supernatant of the apicomplexan parasite Neospora caninum, suggesting their secretion by the tachyzoites [39]. Both surface and secreted GPIs not linked to proteins might also exist in B. divergens and, via their recognition by TLRs, contribute to the global response occurring at the parasite synapse when the merozoites are released from the erythrocytes.

The fatty acid moieties could also be responsible for distinct cell responses, but GPI1 and GPI2 have some chains in very close proportions. Lipids composition is the only difference between GPI4, GPI5 and GPI6. The three species induced similar cytokine profiles, but GPI4 significantly increases apoptosis and GPI6 significantly decreased MHC class II expression. A study on the effect of GPIs on MHC expression has demonstrated that GPIs of T. gondii increased expression of MHC class I and II molecules at the surface of bone marrow-derived macrophages [45]. The reduction of MHC class II expression confirms the opposite effect of GPIs of B. divergens on antigen presenting cells. Apoptosis of non-infected cells participates in pathogenesis of diseases due to virus, bacteria, or parasites. For example, host cell exosomes containing HIV Negative Factor or Ebola VP40, caused apoptosis of bystander lymphocytes, contributing to dysregulation of the immune system and higher viral replication [46,47]. In precedent studies, we have demonstrated that GPIs of P. falciparum increased apoptosis of primary rat cardiomyocytes after 48-h incubation [48], whereas GPIs of T. gondii were not able to induce apoptosis of HL60 cells [49]. In the present work, GPIs of B. divergens increased apoptosis. Soluble factors secreted in culture supernatants of macrophages infected with Mycobacterium tuberculosis were responsible for TNF-α-independent apoptosis of bystander Jurkat T cells [50]. In another study, the 38-kDa antigen secreted by M. tuberculosis induced macrophage ER-stress-induced apoptosis through activation of the TRL2/4-MAPK pathway and the production of reactive oxygen species [51]. Since B. divergens GPIs activated TLR2/4, but did not induce TNF-α production, apoptosis of PECs might be due to ER stress.

GPIs of B. divergens prolonged the activated partial thromboplastin time, suggesting a direct effect of GPIs on coagulation factors of the intrinsic pathway. In the same way, GPIs of other Babesia species could be responsible for the increase of APTT observed in vivo in serum of infected animals [8]. GPIs slightly increased coagulation time when the assay used to quantify fibrinogen levels was applied. Since an increase in these levels is not possible ex vivo, we can only conclude that GPIs does not degrade or interfere with fibrinogen. In vivo, GPIs might regulate other coagulation parameters through cell activation, but this could not be evaluated here.

Altogether, our study highlighted a unique biological profile of GPIs of B. divergens on antigen presenting cells compared to GPIs from all other protozoan parasites studied until now. This first study lays the foundations for understanding the roles played by GPIs in inflammation and coagulation during babesiosis. Th1/Th2 balance controls the fate (survival or death) of animals and human beings infected with protozoan parasites, especially the intracellular ones. The Th2 polarization of antigen presenting cells associated with the decrease in MHC class II expression induced by the GPIs of B. divergens are in favour of tolerance for the pathogen. Nevertheless, other molecules from Babesia or from the host might act in synergy with or inhibit the GPIs, as shown for fatty acids [40,52], leading to the production of higher Th1 cytokines during babesiosis.

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Author contributions

FDG, RTS and EC designed the research; FDG, TKS, SD, JS, VB and EC performed purification and biochemical studies; FDG, CD performed biological studies; TKS, SD, IDP, RTS and EC contributed to reagents/materials/analysis tools; FDG and TKS wrote the paper and all authors revised its final version.

Compliance with ethical standards

The authors declare that they have no conflicts of interest and no competing interests including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations and grants or other funding in relation with the contents of this article.

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