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BRIEF DEFINITIVE REPORT

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Maintenance of B cells during chronic murine *Trypanosoma brucei gambiense* infection

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Summary

African trypanosomosis is a debilitating parasitic disease occurring in large parts of sub-Saharan Africa. *Trypanosoma brucei gambiense* accounts for 98% of the reported HAT infections and causes a chronic, gradually progressing disease. Multiple experimental murine models for trypanosomosis have demonstrated inflammation-dependent apoptosis of splenic follicular B (FoB) cells and the destruction of B-cell memory against previously encountered pathogens. Here, we report that during murine infection with a chronic *T. b. gambiense* field isolate, FoB cells are retained. This coincided with reduced levels of IFN-γ and TNF-α during the acute phase of the infection. This result suggests that in chronic infections with low virulent parasites, less inflammation is elicited and consequently no FoB cell destruction occurs.

KEYWORDS

B lymphocyte, inflammation, Trypanosoma spp., trypanosomiasis

1 | INTRODUCTION

African trypanosomosis is a debilitating parasitic disease occurring in large parts of sub-Saharan Africa. African trypanosomes, the causative agents of the disease, are transmitted between mammalian hosts through the bite of the tsetse fly. Human African trypanosomosis (HAT), otherwise termed sleeping sickness, is caused by *Trypanosoma brucei* (*T. b.*) *rhodesiense* in East Africa and *T. b. gambiense* in West Africa. The disease typically involves two stages: the early hemolymphatic stage and the late meningoencephalitic stage. During the hemolymphatic stage parasites proliferate in the blood and lymphatic system. The meningoencephalitic stage begins when parasites penetrate the blood brain barrier and proliferate in the cerebral spinal fluid. When patients in the meningoencephalitic stage remain untreated, an encephalitic reaction can occur resulting in death.[1–4]

Trypanosoma brucei gambiense and *T. b. rhodesiense* cause clinically diverse infections. *T. b. gambiense* accounts for 98% of the reported HAT infections, and causes a chronic, gradually progressing disease.[5] The late meningoencephalitic stage is not reached before months or even years of infection. Asymptomatic *T. b. gambiense* infections have also been reported, hence *T. b. gambiense* infections are not 100% lethal.[6]

Trypanosoma brucei rhodesiense infections represent only 2% of the reported HAT incidences. These infections are acute and progress rapidly (within weeks) to the late meningoencephalitic stage. In contrast to *T. b. gambiense* infections, *T. b. rhodesiense* infections are assumed to be 100% lethal if left untreated.[3] Experimental mouse models have proven to be a valuable tool for the elucidation of many immuno-pathological processes occurring during murine trypanosome infections. Indeed,

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both murine *T. b. rhodesiense* and *T. b. brucei* (the nonhuman infective *T. brucei* strain) infections have been extensively characterized. However, research on murine models for *T. b. gambiense* has been limited as propagation of *T. b. gambiense* isolates in rodents is difficult. This has led to a limited availability of infective strains, most of them being acute infections leading to death of infected animals within days.[7]

Recently, Giroud et al.[7] isolated *T. b. gambiense* strains from cerebrospinal fluid of several HAT patients. These isolates were subsequently adapted to growth in mice and represent a new tool for the investigation of the pathology in chronic *T. b. gambiense* infections.

Investigation of trypanosome infections in mice and cattle indicates that B cells and antibodies play a crucial role in host protection,[8–12] but various mouse models have shown that trypanosome infections cause a sustained loss in splenic and bone marrow B-cell populations.[13–16] This loss of B cells coincided with the destruction of pre-infection induced B-cell memory to unrelated pathogens.[13] It is hypothesized that destruction of the B-cell compartment is an immune evasion strategy employed by trypanosomes to establish a chronic infection,[17] although it is not clear whether B-cell destruction also occurs in natural hosts for trypanosomosis.

Here, we use the clinical isolate *Tbg945*b to investigate B-cell destruction during chronic murine *T. b. gambiense* infection. We show that splenic B cells are maintained during chronic *T. b. gambiense* infections, and that this coincides with low levels of inflammation. In addition, the white pulp of *T. b. gambiense*-infected mice remained normal during the course of infection, with maintenance of B and T cell areas. Moreover, in contrast to previous results obtained after *T. b. brucei* infection, we demonstrated that *T. b. gambiense* infection do not abrogate vaccine-induced protective responses that were generated against nonrelated pathogens such as a highly virulent O18:K1 *Escherichia coli* (*E. coli*), using a heat-killed *E. coli*-based vaccine model.

2 | MATERIALS AND METHODS

2.1 | Mice, parasites and infections

Six- to 8-week-old female WT BALB/c mice were purchased from Janvier (Le Genest-Saint-Isle, France). Mice were housed at the animal facility of the Vrije Universiteit Brussel. Mice were infected by intraperitoneal (*i.p.*) injection of 5000 pleomorphic *Trypanosoma brucei brucei* AnTat1.1E (N. Van Meirvenne, Institute for Tropical Medicine, Belgium) or *T. b. gambiense* 945b (T. Baltz, Centre National de Recherche Scientifique, Université Bordeaux) parasites.

2.2 | Ethics statement

All the experiments were performed according to directive 2010/63/EU of the European Parliament for the protection of animals used for scientific purposes and approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (protocol #14-220-23 and #12-220-2).

2.3 | Flow cytometry

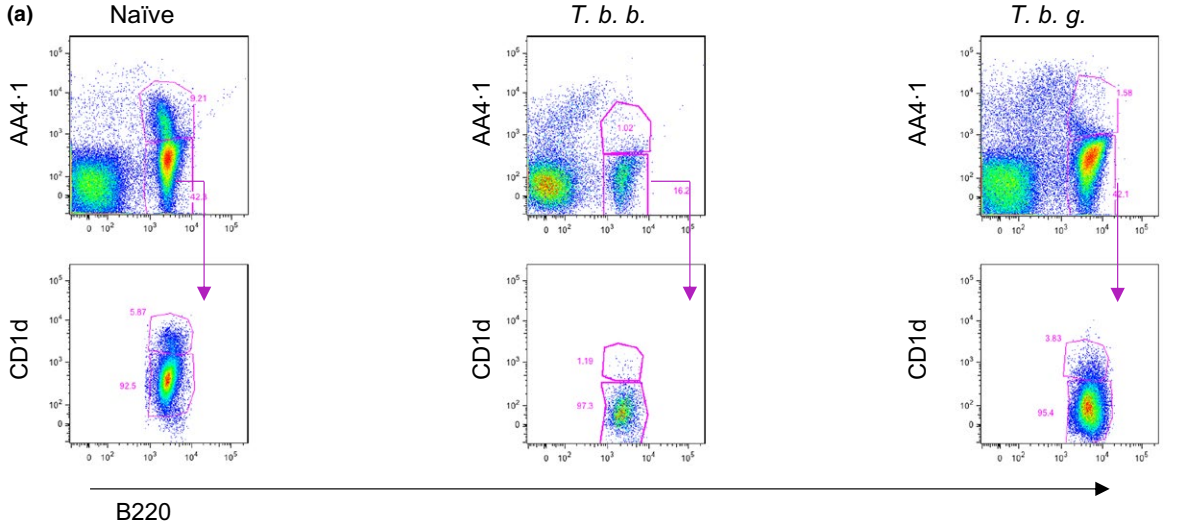
Spleen, tibia and femur were harvested from CO_2 euthanized non-infected control and trypanosome-infected mice. Spleen cells were obtained by homogenizing the organs in 10 mL RPMI medium containing 5% foetal calf serum (FCS) and filtered (70 μm pore filter). Bone marrow cell suspensions were obtained by flushing tibia and femur with 10 mL RPMI medium containing 5% FCS. Next, cell suspensions were centrifuged (400 x g, 7 minutes, 4°C) and the pellet was treated with RBC lysis buffer (0.15 mol/L NH_4Cl, 1.0 mmol/L $KHCO_3$, 0.1 mmol/L Na_2-EDTA). Cells were washed with FACS medium (5% FCS in RPMI) and nonspecific binding sites were blocked by incubating 20 minutes at 4°C with an Fc-blocking antibody (anti-CD16/32, clone 2.4G2). Subsequently, cell suspensions were stained with fluorescent conjugated antibodies for 30 minutes at 4°C. Fluorescent antibodies: CD23-FITC (clone B3B4), CD45R (B220)-FITC (clone RA3-6B2), IgM-PE (clone II/41), CD93–PE (clone AA4.1), IgM-PE-Cy7 (clone II/41), CD93-APC (clone AA4.1), CD1d-PE (clone 1b1), CD43-PE (clone 1B11), CD45R (B220)-APC-Cy7 (clone RA3-6B2), CD19-APC-Cy7 (clone 1D3) (BD Biosciences Franklin Lakes, New Jersey, USA). Analyses were performed using a FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) and data were processed using FLOWJO software (Tree Star Inc., Ashland, OR, USA). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue.

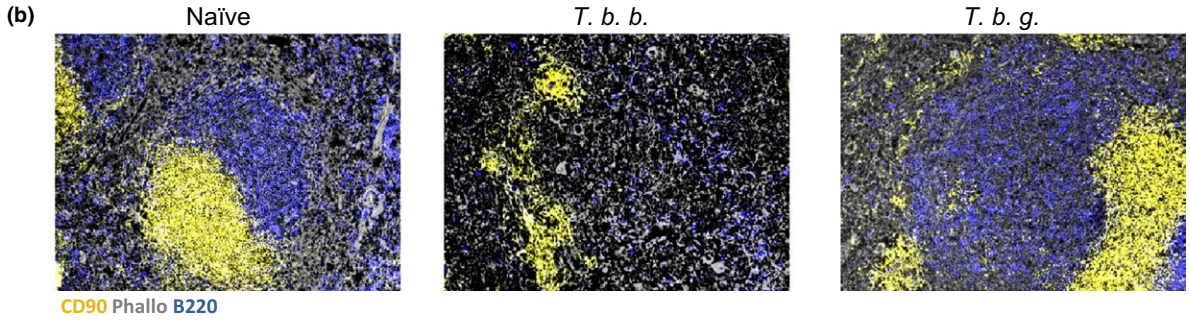
2.4 | *E. coli* vaccination and induction of peritonitis

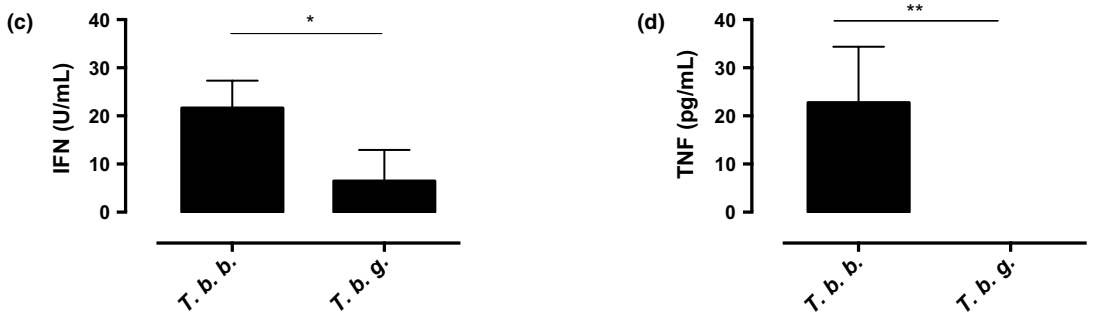
BALB/c mice were vaccinated using 10^8 CFU of heat-killed (HK) 018:K1 *E. coli*[18,19] (Re Heat-killed (HK) *E. coli* bacteria were

FIGURE 1 Maintenance of FoB cells during chronic *Trypanosoma brucei gambiense* infection. (a) Splenic B-cell subsets in naïve mice (first panel), *Trypanosoma brucei brucei*-infected mice day 30 *p.i.* (second panel) and *T. b. gambiense*-infected mice day 30 *p.i.* (third panel). Immature B cells ($B220^+$ $AA4.1^+$ $CD1d^-$), marginal zone B cells ($B220^+$ $AA4.1^-$ $CD1d^+$), and follicular B cells ($B220^+$ $AA4.1^-$ $CD1d^-$) can be determined. (b) Immunofluorescence analysis of spleen of naïve mice (first panel), spleen of *T. b. brucei*-infected mice 30 days *p.i.* (middle panel) and spleen of *T. b. gambiense*-infected mice 30 days *p.i.* (third panel). $CD90.2^+$ T cells (yellow), $B220^+$ B cells (blue) and phalloidin (grey) were visualized with a 20× magnification. Data represent 5 mice per group. (c) Serum concentration of IFN-γ. Data represent mean±SEM of 5 mice per group. (d) Serum concentration of TNF-α. Data represent mean±SEM of 5 mice per group. (e) Mice were vaccinated with 10^8 CFU of heat-killed *Escherischia coli* and four weeks later infected with 5000 *T. b. gambiense* parasite by intraperitoneal injection. Four weeks post-infection, mice were treated with Berenil and challenged three weeks later with 3.5×10^4 CFU of *E. coli*/mouse *i.p.* (■). Control groups consisted of nonvaccinated *Bordetella pertussis* challenged mice (×), and DPTa vaccinated mice that were challenged with *B. pertussis* 24 days after the second DTPa boost (□). *P<.05, **P<.01, ***P<.001

obtained by a 60-minutes incubation at 70°C. We confirmed that the HK bacteria did not grow after inoculation of an overnight culture in Luria-Bertani medium. After a further 28 days mice were infected *i.p* with 5000 *T. b. gambiense* parasites/mouse. Forty days post-infection, unvaccinated as well as both infected and uninfected mice were treated *i.p.* with diminazene aceturate (Berenil, 40 mg/kg, Sigma Aldrich, St. Louis, MO, USA) in PBS. Mice were challenged intraperitoneally (*i.p.*) with 3.5×10^4 live *E. coli* bacteria 30 days post-Berenil treatment) and survival (≥6 mice/group) was monitored.







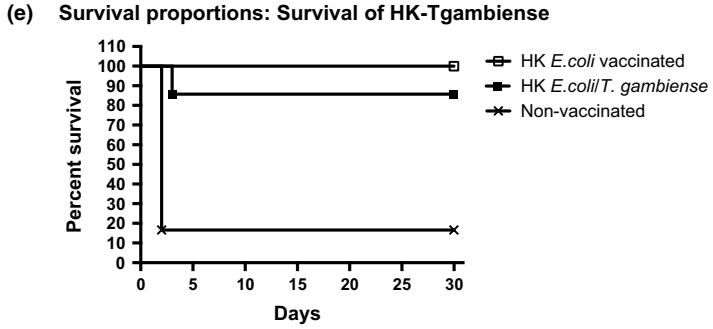


TABLE 1 Number of immature, marginal zone B (MZB) cells and follicular B (FoB) cells in spleen of naïve and day 30 infected mice with *Trypanosoma brucei brucei* or *Trypanosoma brucei gambiense*

	Spleen B cell number				
	Naive	*T. b. brucei*		*T. b. gambiense*	
Immature B cells	$1.43\times10^7\pm0.58\times10^7$	$2.54\times10^6\pm1.79\times10^6$	*	$4.48\times10^6\pm1.06\times10^6$	*
MZB cells	$2.45\times10^6\pm0.83\times10^6$	$6.30\times10^4\pm5.68\times10^4$	***	$8.45\times10^5\pm7.72\times10^5$	*
FoB cells	$3.55\times10^7\pm1.06\times10^7$	$7.03\times10^6\pm4.43\times10^6$	***	$3.16\times10^7\pm0.65\times10^7$	ns

Data represent mean±SD of 5 mice per group. Next to every number the *P* value is indicated on difference compared with naïve value. **P*<.05, ***P*<.01, ****P*<.001.

2.5 | Immunohistofluorescence

Spleens were fixed for 3 hours at 4°C in 1% paraformaldehyde (pH 7.4) and incubated overnight at 4°C in a 20% PBS-sucrose solution under agitation. Tissues were embedded in the Tissue-Tek OCT compound (Sakura) Tacoma, WA, USA, frozen at −80°C, and cryostat sections (10 mm) were prepared. Tissues sections were rehydrated in PBS, then incubated successively in a PBS solution containing 1% blocking reagent (PBS-BR 1%) and in PBS-BR 1% containing Alexa Fluor 488 phalloidin (Molecular Probes Eugene, Oregon, USA) and any of the following mAbs: Anti-Human/Mouse CD45R (B220) eFluor® 570 (Ebioscience, San Diego, CA, USA) and Anti-Mouse CD90.2 (Thy-1.2) Biotin-coupled mAbs were amplified using Streptavidin, Alexa Fluor® 350 conjugate (Life Technologies, Waltham, MA, USA) in PBS-BR 1%. Slides were mounted in Fluoro-Gel medium (Electron Microscopy Sciences, Hatfield, PA, USA). Labelled tissues sections were visualized under a Zeiss fluorescent inverted microscope (Axiovert 200) equipped with high resolution monochrome camera (AxioCam HR, Zeiss, Jena, Germany). All images were acquired with 20× objective at maximal camera resolution.

2.6 | Statistical analysis

Statistical analysis was performed using Student's *t* test with GraphPad Prism software (GraphPad 6, San Diego, CA, USA). Values are expressed as mean±standard deviation (SD) or mean±standard error of the mean (SEM). Values of $P \leq .05$ are considered to be statistically significant.

3 | RESULTS

3.1 | Maintenance of splenic B cells during chronic *T. b. gambiense* infection coincides with low levels of inflammation

Trypanosoma brucei brucei infection in BALB/c mice is characterized by an initial high parasitemia peak ($2–5\times10^8$ parasites/mL) at day 7 post-infection, followed by some smaller peaks.[20] Parasites are detectable in the circulation throughout the entire infection. After approximately 40 days, BALB/c mice succumb to infection. In contrast, *T. b. gambiense* 945b infection of BALB/c mice is characterized by low arbitrary parasitemia peaks ($1.2\times10^6–1.2\times10^7$), and death within 6–8 months post-infection.[7] The effect of the acute *T. b. brucei* and chronic *T. b. gambiense* infection on splenic B-cell subsets was investigated. Splenic B-cell subsets can be discriminated based on B220, CD1d and AA4.1 expression (Fig. S1). After 30 days of infection, *T. b. brucei* induced disappearance of immature ($B220^+$ $CD1d^-$ $AA4.1^+$), marginal zone B (MZB, $B220^+$ $CD1d^+$ $AA4.1^-$) cells and follicular B (FoB, $B220^+$ $CD1d^-$ $AA4.1^-$) cells (Fig. 1a). Although *T. b. gambiense* infection induced disappearance in immature B cells and MZB cells, FoB cells were strikingly retained (Fig. 1a, Table 1).

Murine *T. b. brucei* infections are characterized by a strong type 1 immune response during the first week post-infection.[21,22] More specifically, IFN-γ is identified as a crucial driver of this acute pro-inflammatory reaction.[23] In addition, IFN-γ mediates FoB cell depletion through up-regulation of death receptors on FoB cells and induction of apoptosis.[24] Interestingly, serum levels of IFN-γ and TNF-α are much lower in *T. b. gambiense* infections compared with *T. b. brucei* infections after 1 week post-infection (Fig. 1c, d), suggesting that the reduced pro-inflammatory environment during chronic infection might lead to maintenance of FoB cell subsets.

3.2 | Splenic micro-architecture is maintained during *T. b. gambiense* infection

Previous results of Radwanska et al. showed that *T. b. brucei* infections lead to rapid destruction of splenic micro-architecture, disrupting follicular structure.[13–15] As maintenance of follicular B cells was observed during *T. b. gambiense* infection by flow cytometry, the organization of B and T cells in the splenic white pulp was examined. At d30 *p.i.*, it was indeed confirmed that splenic follicular structure was maintained during *T. b. gambiense* infection in sharp contrast to experimental *T. b. brucei* infections (Fig. 1b).

3.3 | Maintenance of cross-protective memory response against unrelated pathogen such as virulent *Escherichia coli* following *T. b. gambiense* infection

Previous results by our laboratory demonstrated that *T. b. brucei* infection is capable of abrogating the efficacy of the vaccine-induced protective responses against nonrelated pathogens such as gram-negative *Bordetella pertussis* bacteria.[13–15] Using a mouse vaccination model against another highly pathogenic gram-negative bacterium, the O18:K1 *E. coli* strain, we show here that mice vaccinated against *E. coli* and subsequently infected or not with *T. b. gambiense* remain protected against a lethal challenge with live *E. coli* bacteria, while unvaccinated mice succumb from infection within two days

post-challenge (Fig. 1e). These results indicate that *T. b. gambiense* does not abrogate vaccine-induced memory.

4 | DISCUSSION

Multiple murine models for trypanosomosis are characterized by the disappearance of splenic B-cell populations and the destruction of memory against previously encountered pathogens.[13–15] More specifically, an IFN-γ-induced acute pro-inflammatory response leads to apoptosis of splenic B-cell subsets.[24] Here, we report that during murine infection with a chronic *T. b. gambiense* field isolate FoB cells are retained. This coincided with reduced levels of IFN-γ and TNF-α during the acute phase of the infection. This result suggests that in chronic infections with low virulent parasites, less inflammation is elicited and consequently no FoB cell destruction occurs. However, the low-grade inflammation that accompanies the initiation of a *T. b. gambiense* infection apparently still leads to transient MZB cell and immature B-cell depletion. As a consequence, *T. b. gambiense*, in contrast to *T. b. brucei*, does not abrogate the protective immune response against nonrelated pathogens such as gram-negative bacteria. This raises the question whether B cell disappearance in more acute and virulent mouse models is a consequence of the strong inflammatory response in these models. Could destruction of the B-cell compartment be an artefact of the murine *T. b. brucei* trypanosomosis model? A recent field trial on antimeasles vaccinated *T. b. gambiense*-infected patients showed that antimeasles antibodies were lower in HAT patients compared with healthy controls. After HAT treatment, these antibody titres remained lower than in controls, but exceeded the cut-off values presumed to provide protection.[25] It should be mentioned that during trypanosome infections antibody quantification is a suboptimal tool for the investigation of B-cell memory, as trypanosomes elicit polyclonal B cell activation and hence cross-reactive antibodies. A functional characterization of the antimeasles antibodies is therefore necessary. Whether *T. b. gambiense* infection reduces B-cell lymphopoiesis in bone marrow or induces splenic extramedullary lymphopoiesis, as observed during experimental *T. b. brucei* infection, is currently being investigated.[26] Furthermore, B-cell destruction needs to be investigated in *T. b. rhodesiense* infections, as these are more virulent and elicit more inflammation and pathology.

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DISCLOSURES

The authors report no conflict of interest.

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