

Tsetse fly saliva accelerates the onset of a *Trypanosoma brucei* infection in a mouse model associated with a reduced host inflammatory response.

Running title: Tsetse fly saliva accelerates trypanosome infection onset

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Abstract

Tsetse flies (*Glossina sp.*) are the vectors that transmit African trypanosomes, protozoan parasites that cause human sleeping sickness and veterinary infections in the African continent. These bloodfeeding dipteran insects deposit saliva at the feeding site that enables the blood feeding process. Here we demonstrate that tsetse fly saliva also accelerates the onset of a *Trypanosoma brucei* infection. This effect was associated with a reduced inflammatory reaction at the site of infection initiation (reflected by a decrease of IL-6 and IL-12 mRNA) as well as lower serum concentrations of the trypanocidal cytokine TNF. VSG (variant-specific surface glycoprotein)-specific antibody isotypes IgM and IgG2a, implicated in trypanosome clearance, were not suppressed. We propose that tsetse fly saliva accelerates the onset of trypanosome infection by inhibiting local and systemic inflammatory responses involved in parasite control.

Introduction

African trypanosomes are extracellular protozoan flagellates that infect a broad range of vertebrate hosts, including humans, and rely for their transmission on the obligate bloodfeeding tsetse flies (*Glossina* sp.) (62). In the mammalian host, the parasites establish a controlled growth to ensure survival and optimal transmission. In order to escape the adaptive immune system, trypanosomes undergo antigenic variation by altering their major surface antigen, the Variant-specific Surface Glycoprotein (VSG) (6). Beside this immune evasion mechanism, trypanosomes have been shown to modulate immune functions of macrophages (8, 43), T- (56) and B-lymphocytes (2). The main host immune effectors involved in parasite control are considered to be trypanosome-specific antibodies (30) and the cytokine TNF (33). Concerning trypanosome-specific antibodies, increased VSG-reactive IgM and IgG2a antibody isotypes have been associated to improved control of trypanosome infections (34, 55). TNF, as a host cytokine, was released from activated macrophages in response to stimulation by soluble VSG (sVSG) and membrane-bound VSG (mfVSG) (11, 35). TNF was demonstrated to have trypanocidal properties for certain trypanosome stocks and to be associated with the occurrence of immune pathology in infected animals (33). As such, direct or indirect modulation of parasite-specific antibody induction and TNF release might influence the trypanosome growth and severity of infection.

Focussing on the early stage of trypanosome infection in the mammalian host, the effect of tsetse fly salivary components on the parasitemia onset and the involved host anti-parasite immune effectors has been poorly investigated. Studies in other blood-sucking arthropods, like ticks and sand flies, have demonstrated that salivary proteins are potent modulators of host innate and adaptive immune responses. Especially in ticks, characterised by an extended feeding time, a

broad repertoire of immune modulatory activities has been described. These modulatory activities include the predominant induction of a Th2 response with an overall inhibition of proinflammatory and Th1 cytokines (23, 24, 38, 54), suppression of the effector functions of antigen presenting cells (5, 26, 28, 59) , modulation of T-cell (28, 59) and B-cell responses (16, 17) as well as the inhibition of granulocyte infiltration (39, 53) and natural killer (NK) mediated cytotoxicity (25). Illustrating the importance of salivary components for pathogen transmission, the tick protein Salp15 was shown to significantly increase the infectivity of *Borrelia burgdorferi* spirochetes in mice (50). Also the salivary extracts from old and new world sandflies have been described to modulate several effector functions of the host immune system, promoting the initial *Leishmania* infection at the inoculation site (3, 15, 32, 58, 60).

A general immunological effect observed in several studied vector/host-interaction models is an anti-inflammatory action of the salivary components and the occurrence of a Th2-associated cytokine response in exposed hosts (23, 24, 37, 38, 54). Consistent with this, we have demonstrated that tsetse fly saliva also biases the immune system to a Th2 response (4), possibly reflecting the anti-inflammatory potential of tsetse fly saliva. As a pro-inflammatory response in an early stage of trypanosome infection has been shown to be crucial for an efficient control of parasitemia (21, 33, 44), a putative anti-inflammatory property of tsetse fly saliva might enhance trypanosome progression at the early stage of infection. Moreover, tsetse fly saliva was shown to suppress IgG responses against the heterologous antigen ovalbumin (4). As such, the salivary components might also influence the host anti-trypanosome antibody induction responsible for parasite clearance. This paper describes the effect of *Glossina morsitans morsitans* saliva on the onset of a *Trypanosoma brucei brucei* infection and the associated immune responses in mice.

Material and methods

Animals

6-8 weeks old female Toll-like receptor-2/4 (TLR-2/4) deficient mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for the experimental infection experiments. 6-8 weeks old female in-house bred F1 (BALB/c × C57BL/6) mice were used for anti-saliva immunisation and natural trypanosome infection via tsetse flies. Mouse care and experimental procedures were performed under approval from the Animal Ethical Committee of the Vrije Universiteit Brussel. Tsetse flies (*Glossina morsitans morsitans*) were available from the insectaria at the Prins Leopold Institute of Tropical Medicine Antwerp (ITMA), originating from puparia collected in Kariba (Zimbabwe) and Handeni (Tanzania). Flies were fed on rabbits and maintained at 26°C and at a relative humidity of 65%. Animal ethics approval for the tsetse fly feeding on live animals was obtained from the Animal Ethical Committee of the Institute of Tropical Medicine, Antwerp (Belgium).

Parasites

Pleiomorphic AnTat1.1E *Trypanosoma brucei brucei* parasites were used for the experimental infection experiments using an intradermal injection method. Parasites were grown in mice and purified from their heparinized blood, using diethylaminoethyl-cellulose (DE52, Whatman) anion exchange chromatography (27). Parasites were collected in PSG buffer (PBS pH 7.4 supplemented with 10% glucose), centrifuged at $850 \times g$ and resuspended in sterile PBS or RPMI1640 prior to injection.

For natural infection experiments, tsetse flies were infected with the AnTAR 1 *Trypanosoma brucei brucei* parasite strain that was shown to efficiently develop into a mature salivary gland infection.

Soluble Variant Surface Glycoprotein (sVSG) purification

The soluble form of the GPI-anchored VSG was purified from isolated AnTat1.1E parasites following a stress-induced activation (50mM NaH₂PO₄, pH 5.5) of the endogenous phospholipase (PLC) releasing the VSG from its membrane anchor. sVSG was further purified using a combination of ion exchange and gel filtration chromatography (7).

Tsetse fly saliva isolates

Three days after the last blood meal, 10 to 15 days old non-infected tsetse flies were dissected under a binocular microscope and salivary glands were isolated using forceps and collected in ice-cold, sterile PBS (pH 7.4). Saliva was allowed to outflow into the buffer solution followed by separation from the salivary glands by centrifugation for 1 min at 12000 × g. The supernatant (= saliva) was sterilised by filtration through a 0.2µm pore sized filter. Protein concentrations were assessed by the BCA protein assay reagent kit (Pierce Biotechnology) and aliquots stored at – 20°C. The approximate yield of eluted saliva proteins was approximately 5µg per salivary gland.

Natural and experimental exposure to tsetse fly saliva

Natural exposure to tsetse fly saliva was performed by allowing 15 flies per mouse ($n=6$) to feed twice a week over a period of five weeks. An additional six F1 mice were experimentally immunized by priming with 5 μ g saliva in FCA and boosting twice with three weekly intervals by administering the same dose in Freund's incomplete adjuvant (FIA) subcutaneously at the tail base. Control mice were subjected to the same injection schedule with only adjuvant in PBS. From all exposed mice, sera were collected 14 days after the last boost for analysis of the immune response.

Intrapinna trypanosome infections

Experimental trypanosome infections were initiated intradermally in the presence and absence of 5 μ g saliva (equivalent of one salivary gland content). The inoculation consisted of a single injection of 25 μ l buffer containing 2000 AnTat1.1E trypanosomes with or without 5 μ g saliva between the ventral and dorsal ear dermis (intrapinna), using a gauge 30 insulin microsyringe. Saliva was added to the trypanosome inoculum just prior to injection and inoculated doses were confirmed retrospectively by parasite counting. Parasitemia was analysed microscopically using a Bürker hematocytometer in 1/200 diluted blood samples taken from the tail vein.

Tsetse fly transmitted trypanosome infections

Freshly emerged tsetse flies were infected by feeding on AnTAR 1 infected mice at the peak of parasitemia. In order to obtain a pleomorphic trypanosome population at high titer, these mice were immune suppressed with cyclophosphamide (20mg/kg). 28 days after the infecting bloodmeal, flies were screened for a mature salivary gland infection by induced probing on pre-

warmed glass slides followed by a microscopic analysis for the presence of metacyclic trypanosomes in the saliva. Infection of tsetse flies with *T. brucei* parasites was performed in compliance with the regulations for biosafety and under approval from the Environmental administration of the Flemish government.

To initiate a natural infection, one individual tsetse fly with a mature salivary gland infection was allowed to feed per mouse. To avoid interrupted tsetse feeding, mice were anesthetized prior to the tsetse exposure. Parasitemia of the injected mice was analysed microscopically in blood samples taken from the tail vein.

Dermal and white blood cell RNA extraction

In order to analyse local inflammatory responses upon infection in the ear dermis, total RNA was extracted from the ear dermal layers using Trizol reagent (Gibco-Invitrogen) following the manufacturer's recommendations. Systemic immune responses during infection were assessed at the mRNA level in the white blood cell (WBC) fraction of total heparinized blood. The WBC fraction was obtained by erythrocyte lysis that was achieved by a 1/5 dilution of the blood in erythrocyte lysis buffer (10mM Tris-HCl pH 7.5, 0.83% NH₄Cl). After 5 minutes, the lysis was stopped by adding 3 volumes RPMI 1640 medium (Gibco-Invitrogen) and cells were pelleted by centrifugation for 8 minutes at 410 × g. RNA was extracted from the cell pellet using Trizol reagent.

Quantitative reverse-transcription polymerase chain reaction (RT-QPCR) analysis

1 μ g of total RNA was reverse-transcribed using oligo(dT)₁₂₋₁₈ and Superscript II reverse transcriptase (Gibco-Invitrogen). Quantitative real-time PCR was performed in a Bio-Rad (Hercules, CA) iCycler, with Bio-Rad iQ SYBR Green Supermix. PCR conditions were as described before (48, 49). Used primers were: IFN- γ sense (5'-GCTCTGAGACAATGAACGCT-3'), IFN- γ antisense (5'-AAAGAGATAATCTGGCTCTGC-3'), IL-6 sense (5'-GTCAGATACCTGACAACAGG-3'), IL-6 antisense (5'-GTCTTCTGGAGTACCATAGC-3'), iNOS sense (5'-TCCACCAGGAGATGTTGAAC-3'), iNOS antisense (5'-TGGAGCCAAGGCCAAACACAG-3'), IL-12p35 sense (5'-GGAGGTTTCTGGCGCAGAGT-3'), IL-12p35 antisense (5'-GATGACATGGTGAAGACGGCC-3'), TNF sense (5'-GTCTACTCCCAGGTTCTCTTC-3'), TNF antisense (5'-CCTTCACAGAGCAATGACTC-3'), IL-10 sense (5'-GGACTTTAAGGGTTACTTGG-3'), IL-10 antisense (5'-ACTCAATACACACTGCAGGTG-3'), S12 sense (5'-CCTCGATGACATCCTTGGCCTGAG-3'), S12 antisense (5'-GGAAGGCATAGCTGCTGGAGGTGT-3'). The amplicon sizes were respectively 229, 368, 458, 401, 302, 344 and 367 bp. For all primers sets, each PCR cycle consisted of 1 min denaturation at 94°C, 45 s annealing at 54°C and 1 min extension at 72°C. Gene expression was normalized using ribosomal protein S12 as an extensively validated house keeping gene (20, 61) and expressed as relative to the naive condition.

Serum cytokine analysis

Cytokine responses (IFN- γ , TNF- α and IL-10) were analyzed in sera from mice infected at different time points after infection, by cytokine-specific sandwich ELISAs (Pharmingen, R&D Systems) using horseradish peroxidase based detection.

Antigen-specific antibody titers and Ig-isotypes

Murine anti-trypanosome antibody responses were assessed in solid phase ELISA. For antibody titer measurement and isotype determination, Immunosorb plates (Nunc) were coated with 200ng AnTat1.1E soluble VSG (sVSG) per well in 0.1M NaHCO₃ (pH 8.3) and blocked with 10% fetal bovine serum (FBS) in PBS. Serial ½ serum dilutions were applied to antigen coated wells followed by specific immunoglobulin (IgG1, 2a, 2b, 3 and IgM) detection using an isotyping kit (EmTec).

Graphs and statistical analysis

All graphs were prepared by the use of the GraphPad Prism 4.0 software (GraphPad Software). The same software was used for statistical analysis (two-tailed unpaired *t* tests, one-way ANOVA) of the data. Data are represented as mean ± SEM.

Results

Tsetse fly saliva accelerates the early onset of trypanosome infection

Trypanosome infections were initiated intrapinna in the presence and absence of tsetse fly saliva, followed by the analysis of parasite concentrations in the blood. Experimental infections were conducted in TLR-2/4^{-/-} mice to exclude the effect of bacterial endotoxins that are present in the saliva isolates due to the harvesting procedure (data not shown). In four independent experiments,

a significant infection accelerating effect of tsetse fly saliva was observed in the early stage of infection (Fig. 1A). This observation includes a reproducible earlier detection of parasites within 4 days of infection in the tail vein blood samples and statistically significant higher parasite concentrations up to the first parasitemia peak (day 5: $P<0.05$, day 6: $P<0.05$, day 7: $P<0.01$) in mice where the trypanosomes were co-injected with tsetse saliva. The differences in absolute peak parasitemia levels were not always statistically significant and no long lasting effect of saliva on the parasite levels was observed after the first peak (Fig. 1A). As such, survival times were not significantly different between the two experimental series (Fig. 1B).

Saliva suppresses early inflammatory responses in the dermal infection site

The early onset of a local inflammatory response upon intradermal trypanosome inoculation with or without saliva was analysed by RT-QPCR on RNA extracts from the ear dermis. Inflammatory cytokine mRNA levels were upregulated within three hours of trypanosome infection. Upon co-administration of the parasites with saliva, local transcription of early inflammatory cytokines IL-6 and IL-12p35 was suppressed after respectively one ($P<0.1$, $P<0.05$) and three hours ($P<0.01$, $P=0.17$) of infection (Fig. 2). Dermal transcription of IFN- γ , iNOS, TNF and IL-10 was slightly upregulated upon infection but not significantly altered within 3 hours of infection upon co-administration of saliva.

Saliva affects systemic cytokine transcription and translation during infection

Systemic cytokine responses were analysed in the blood at the transcriptional and translational level after 3 and 6 days of infection, corresponding respectively to the early and progressed stage

of systemic parasitemia (see Fig. 1A). Cytokine transcription was assessed using RT-QPCR on RNA extracts from the white blood cell (WBC) fraction (Fig. 3A). Several transcripts encoding type I and type II cytokines were significantly upregulated during infection as compared to the naive condition. *Il-6* and *Il-12p35* transcripts that were suppressed by saliva in the dermal infection site within a few hours of infection (see Fig. 2), were not influenced systemically at days 3 and 6 post infection (Fig. 3A). Although infections initiated with saliva resulted in a significantly more severe parasite burden, systemic transcription of several inflammatory cytokines by WBC was reduced in the early stage of infection. TNF mRNA (day 3 and 6 p.i.: $P<0.05$) and IL-10 mRNA levels (day 3 p.i.: $P<0.05$, day 6 p.i.: $P<0.01$) were lower before the first parasitemia peak upon infection with saliva. Transcripts encoding IFN- γ (day 3 p.i.: $P<0.05$, day 6 p.i.: $P<0.1$) and iNOS (day 6 p.i.: $P<0.1$) also tended to be suppressed.

The reduced TNF mRNA levels upon saliva-facilitated infection were also associated with lower serum TNF concentrations (Fig. 3B; day 3 p.i.: $P=0.17$, day 6 p.i.: $P<0.1$). The transcriptional and translational differences for TNF coincide with the increased early outgrowth of the parasite population. IL-10 and IFN- γ concentrations were not affected in the serum.

Saliva does not inhibit anti-trypanosome antibody titers

Antibody induction against the major membrane component of the infecting trypanosome clone, AnTat1.1E VSG, was analysed in the early stage of trypanosome infection with or without tsetse fly saliva. IgM was the only AnTat1.1E sVSG-reactive antibody isotype that was significantly induced in both experimental groups prior to the first peak of parasitemia, as measured in ELISA at day 6 post infection (Fig. 4). Associated to the higher parasite concentrations observed in the

saliva-facilitated infection, an increased anti-sVSG IgM antibody titer was detected in mice where trypanosomes were co-injected with tsetse fly saliva. Moreover, sVSG-reactive IgG2a antibodies could be detected upon co-injection of trypanosomes with saliva, while this antibody isotype was undetectable at day 6 after infection without saliva (Fig. 4).

Repetitive saliva exposure promotes the onset of trypanosome infection

To evaluate the effect of repeated confrontation with saliva and saliva-induced immune responses on the onset of a trypanosome infection, mice were pre-exposed to repetitive tsetse fly bites. This pre-exposure resulted in an accelerated onset of a subsequent AnTAR1 infection initiated by the bite of a tsetse fly (Fig. 5A; day 6 p.i.: $P<0.01$, day 7 p.i.: $P<0.05$). Consistent with the natural exposure, experimental immunisation of mice against tsetse fly saliva also resulted in a significantly earlier onset of parasitemia upon natural trypanosome challenge as compared to control mice (Fig. 5B; day 6 p.i.: $P<0.01$, day 7 p.i.: $P<0.1$, day 8 p.i.: $P<0.05$). Immunogenicity of saliva components was analysed in western blot and ELISA, showing that saliva-specific antibodies, including IgE isotypes, were raised upon exposure to saliva as described earlier (4). Post-first peak parasite concentrations in the blood and survival times of infected mice were not altered in the different experimental groups.

Discussion

The interaction of African trypanosomes with the host immune system is complex and involves several aspects such as the antigenic plasticity of the parasite as well as the modulation of immune responses. In order to maintain a controlled growth, trypanosomes co-ordinately alter

their major surface antigen (VSG) coat and modify functions of antigen presenting cells (8, 43) as well as T- (56) and B-lymphocytes (2). During a trypanosome infection, an equilibrated balance between inflammation and anti-inflammation was shown to be crucial for successful parasite control while limiting the outcome of immune pathology (33, 44). Information on the immunology of African trypanosomiasis is mainly available from experimental trypanosome infections that exclude the vector-host interaction. The presented data demonstrate that tsetse fly salivary components play a significant role in the early infection stage by accelerating the early outgrowth of the trypanosome population. This effect was not resulting from differences in infectious dose or improved trypanosome viability in a saliva environment. As such, the faster onset of the trypanosome infection could be the result of the interference of tsetse fly saliva with host haemostatic reactions (e.g. vasoconstriction) and host immune reactions such as the recruitment and activation of host immune cells or the release of inflammatory mediators at the site of infection.

Vaso-active salivary factors, present in several bloodsucking arthropods (40, 52), might be responsible for increasing the parasite's efficiency to invade the lymphatic and blood vessels. In the case of *Plasmodium*, mosquito mediated transmission resulted in an increased efficiency of the parasite to leave the dermal infection site as compared to an experimental syringe injection (1). Moreover, the *Lutzomyia* sandfly promotes *Leishmania* transmission by the presence of the vasodilatory peptide maxadilan with immune modulatory properties in the saliva (29, 40, 41). In our study, trypanosome parasites reached earlier detectable levels in the bloodstream in the saliva co-injected mice, indicating that salivary components might enhance vessel invasion from the dermal infection site.

Since trypanosomes are extracellular parasites, they are in principle more sensitive to antibody-mediated immune effects including complement mediated lysis (13, 46) and phagocytosis of

opsonized parasites (9, 55). Although the involvement of antibodies in parasite clearance during an early stage of infection might be limited, the influence of tsetse fly saliva facilitated infection on trypanosome-specific antibody induction was assessed by evaluating the level of sVSG specific antibodies in sera of infected mice. The higher parasite burden observed in the saliva/trypanosome co-injected mice is associated with higher titers of AnTat1.1E sVSG specific IgM and IgG2a antibodies, most probably reflecting the increased confrontation of the host immune system with the trypanosomal VSG rather than a systemic saliva-effect. The higher VSG-specific antibody titers upon saliva-facilitated infection as compared to the control infection might explain the equally efficient clearance of the first parasitemia peak despite the more severe parasite burden. Collectively, the infection accelerating effect of saliva is not dependent on the inhibition of VSG-specific antibody induction.

Modulation of effector functions of antigen presenting cells (APCs) is another general feature of several arthropod salivary gland extracts. For instance, ticks and sandflies suppress the production of cytokines, NO and reactive oxygen intermediates by APCs (26, 45, 59) and thereby might be involved in suppressing anti-pathogen responses. A major trypanosome component reported to trigger APCs is the variant-specific surface glycoprotein (VSG), in particular its GPI anchor. This molecule can trigger inflammatory TNF, IL-6 and IL12 release from antigen presenting cells through MyD88-dependent signalling, indicating an interaction with pattern recognition receptors (11). Within three hours of intradermal *T. brucei brucei* infection, local transcription of inflammatory genes was induced at the dermal site. Initiation of infection in the presence of tsetse fly saliva significantly suppressed local transcription of genes encoding IL-6 and IL12p35. Transcripts encoding other inflammatory molecules such as TNF, IFN- γ and iNOS tended to be upregulated in the ear dermis in the early infection stage (1-3 h) but were not significantly inhibited by saliva. Systemically, reduced transcription of IFN- γ , TNF and IL-10 in

white blood cells was observed after three and six days of trypanosome infection initiated with saliva. Additionally, reduced TNF mRNA levels in the WBC compartment were corroborated by altered serum TNF concentrations at days 3 and 6 post infection as measured by ELISA. As TNF was shown to exert trypanolytic activity for the AnTat1.1 *T. brucei* stock and to be directly involved in trypanosome control (33), the observed suppression correlates with the increased parasite burden upon saliva-facilitated infection. Together, these data indicate that the infection promoting effect of tsetse fly saliva is associated with a suppression of local inflammatory responses (IL-6 and IL12p35 mRNA) as well as a systemic effect on TNF production. Here, the systemic effect might result from the direct translation of the local anti-inflammatory action of saliva, indirectly from the higher parasite burden or from a combination of both. However, as the affected cytokines are mainly produced by APCs in response to trypanosome antigens, our observations suggest that the saliva-facilitated parasite onset modulates the activation of these cells in the early infection stage.

Besides affecting the responsiveness of APCs to inflammatory trypanosome components, tsetse fly saliva might inhibit APC activation by interfering with inflammatory triggers released upon tissue damage at the dermal site of infection. Based on the molecular identification of salivary components and suggestive functional information, tsetse fly saliva might interfere with ATP-mediated triggering of purinergic receptors on APCs and the subsequent induction of inflammatory cytokines (10). In this context, the enzymatic degradation of extracellular nucleotides has been proposed as a mechanism of anti-inflammation exploited by both endo- and ectoparasites, including bloodsucking arthropods (14, 51). Tsetse fly saliva has been demonstrated to contain components with apyrase (36), putative 5'-nucleotidase (GenBank accession No.: **AAK63848**) and adenosine deaminase activity (31). With this array of enzymes,

saliva could convert a potential inflammatory nucleotide (ATP) to anti-inflammatory degradation products, such as adenosine and inosine (18, 19).

Another important immunological aspect of the vector-host interaction is the occurrence of hypersensitivity reactions at the site of the bite that might result from repetitive exposure to insects. In the case of sandflies, saliva enhances the severity of *Leishmania* infection, while DTH responses, raised by repeated exposure to the vector (22, 57) or immunisation against individual salivary components (41, 60), can abrogate the *Leishmania* infection transmitted by the sandfly.

Tsetse fly saliva also promotes the onset of trypanosome infection while repeated exposure to tsetse fly bites induces saliva-specific IgE antibodies that might be involved in local hypersensitivity reactions (4, 12). Moreover, DTH responses have been observed earlier in tsetse fly exposed rabbits (12). To evaluate the effect of repeated confrontation with tsetse fly saliva on the onset of a naturally transmitted trypanosome infection, mice were repeatedly exposed to tsetse feeding and immunised against total saliva in adjuvant. Upon challenge with infected tsetse flies, the onset of infection was faster in exposed and saliva-immune as compared to control mice. This indicates that anti-saliva immune responses can be beneficial for the initiation of trypanosome infection. This differs from *Leishmania* transmission by the sandfly, where DTH responses against saliva or individual constituents can abrogate the infection (22, 41, 57, 60). However, the intracellular nature of the *Leishmania* parasite implies that the efficient penetration of APCs becomes a sensitive point in the onset of infection (47, 57), while trypanosomes as extracellular parasites appear unaffected by local hypersensitivity reactions (42) and might even benefit from the increased vaso-permeability to leave the initial site of infection more efficiently. This does not exclude that other exposure schemes or immunisation against individual salivary proteins might still result in protection against trypanosome transmission by tsetse flies. However, the

presented saliva-exposure experiments did not confer protective immunity against natural trypanosome challenge.

Together, the presented data demonstrate that tsetse fly saliva exerts an immune modulatory effect in the murine host associated with an infection promoting effect during the early stage of a *T. b. brucei* infection. Moreover, repeated exposure to saliva can make mice more sensitive to infection in the early stage. Evaluation of the possible epidemiological importance of both effects would depend on an extensive experimental assessment of the minimal infective dose as a crucial parameter.

Acknowledgements

This project was supported by fundings from the VUB (Vrije Universiteit Brussel) and an IWT (Instituut voor de aanmoediging van innovatie door wetenschap en technologie in Vlaanderen) PhD grant (IWT1149). S.M. is an NFWO postdoctoral research fellow. The work was performed within the frame of an interuniversity attraction pole (IUAP) programme with the partnership of the Prins Leopold Institute of Tropical Medicine (ITM).

This work was supported by the kind technical assistance of Ella Omasta, Jos Van Hees and Marie-Thérèse Detobel.

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Figure legends:

Fig.1: Initiation of *T. b. brucei* AnTat1.1E infections in the presence and absence of *G. morsitans morsitans* saliva: effect on the parasitemia (A) and survival (B) of TLR-2/4^{-/-} mice. Arrows in (A) indicate the analysis of early inflammatory responses at the dermal infection site by RT-QPCR (empty arrow: one and three hours after infection initiation) and systemic immune responses measured by ELISA in serum and by RT-QPCR in RNA extracted from the white blood cell fraction (filled arrows: three and six days after infection initiation). The presented data are representative of four independent experiments with at least four mice per group.

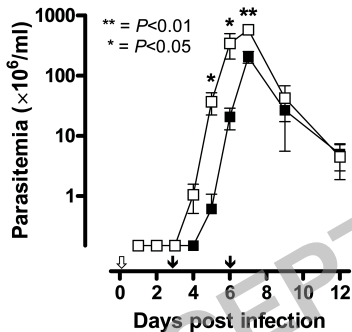
Fig. 2: Local effects of tsetse fly saliva on dermal inflammation at one and three hours after infection of TLR-2/4^{-/-} mice: relative S12-normalized mRNA levels encoding IL-6, IL-12, IFN-γ, iNOS, TNF and IL-10 in dermal RNA extracts from intrapinna infected TLR-2/4^{-/-} mice. The presented data are representing two independent infection experiments with respectively two and four mice per group per time point.

Fig. 3: Effect of tsetse fly saliva on the systemic immune response at three and six days after infection in TLR-2/4^{-/-} mice: (A) relative S12-normalized IL-6, IL-12, IFN- γ , iNOS, TNF and IL-10 mRNA levels measured in RT-QPCR in RNA extracts from the white blood cell fraction and (B) serum IFN- γ , TNF and IL-10 cytokine concentrations measured by specific capture ELISAs. The presented data are representative of two independent infection experiments with 4 mice per group per time point.

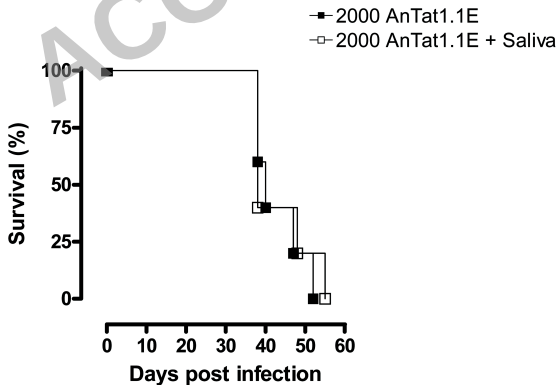
Fig. 4: Isotype specific titration (serial dilutions in X-axis: dilution = $50 \times 2^{n-1}$) of AnTat1.1E sVSG-reactive antibodies (IgM and Ig2a) six days after intradermal infection of TLR-2/4^{-/-} mice ($n=4$) with *T. b. brucei* AnTat1.1E in the presence ($\square$) and absence ($\blacksquare$) of tsetse fly saliva.

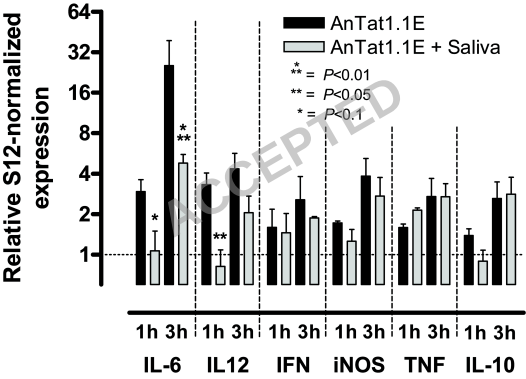
Fig. 5: Effect of repetitive exposure to tsetse saliva on the initiation of a tsetse fly transmitted *T. b. brucei* AnTAR1 infection: parasitemia onset in F1 (BALB/c $\times$ C57BL/6) mice confronted with saliva by (A) repeated exposure to tsetse flies (B) and by immunisation of saliva in adjuvant. The presented data of the two independent settings are the result of single infection experiments with 6 mice per group.

A

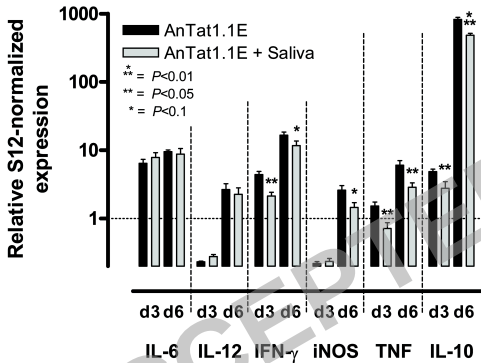


B

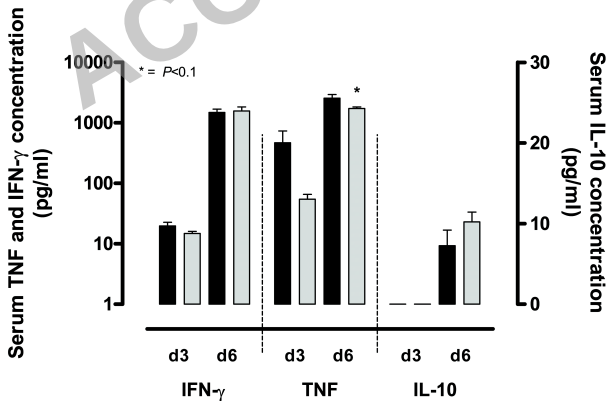




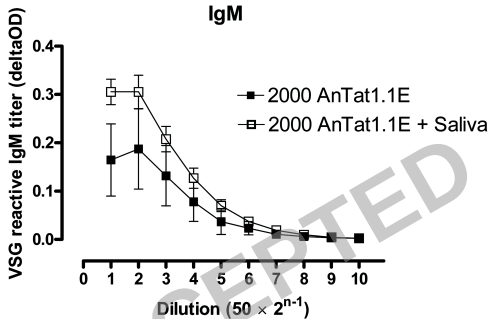
A



B



IgM



IgG2a

