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Tsetse fly saliva biases the immune response to Th2 and induces anti-vector antibodies that are a useful tool for exposure assessment

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Abstract

Tsetse flies (*Glossina* sp.) are blood-feeding dipteran insects that transmit African trypanosomes, parasites that are responsible for human sleeping sickness and veterinary infections. Increasing attention is being paid to the effects of tsetse fly saliva deposited at the feeding site, which enables the blood-feeding process and putatively promotes parasite transmission. Here we demonstrate that saliva induces strong humoral responses against the major 43–45 kDa protein fraction (tsetse salivary gland proteins 1 and 2 – Tsal1 and Tsal2) in mice and humans and suppresses murine T and B cell responses to heterologous antigen. The saliva-induced immune response is associated with a Th2-biased cytokine profile and the production of mainly IgG1 and IgE antibody isotypes. Functionally, the antibodies raised in mice exposed to tsetse fly bites or induced after experimental saliva immunisation do not affect the fly's blood-feeding efficiency nor its survival. We propose that anti-saliva as well as anti-Tsal1/2 antibody responses can be used in epidemiological studies as a tool to analyze human exposure to tsetse flies.

Keywords: *Glossina*; Vector–host interaction; Immune modulation; T-helper cell type 2; Antibodies

1. Introduction

Tsetse flies (*Glossina* sp.) are obligate haematophagous insects, transmitting the protozoan parasite *Trypanosoma* sp., responsible for disease in livestock and Human African Trypanosomiasis (HAT), also known as sleeping sickness. New infections are initiated when metacyclic trypanosomes are inoculated into the vertebrate skin by the vector during the blood-probing process (Youdeowei, 1975). In natural tsetse populations, *Trypanosoma* sp. prevalence has been estimated to be around 5–10% in endemic regions of African trypanosomiasis. For *Trypanosoma brucei* sp., the causative agents of HAT and nagana in cattle, infection prevalence in flies is much lower (0.1%) (Otieno and Darji, 1979; Morlais et al., 1998; Msangi et al., 1998). As a result, hosts are mostly exposed to the bites of uninfected tsetse flies. It is known that during this interaction, tsetse flies expose the host to a complex mixture of antigens including physiologically active salivary components which overcome host haemostatic responses (Parker and Mant, 1979; Mant and Parker, 1981; Cappello et al., 1996; Champagne, 2004) and are predicted to play a crucial role in the blood-feeding process by ensuring persistent blood flux at the feeding site and preserving mouthpart and crop function by avoiding clot formation. These include a tsetse thrombin inhibitor (TTI, 3.5 kDa) (Cappello et al., 1996), an 11.3 kDa inhibitor of thrombin serine protease and esterase activities (Parker and Mant, 1979) and a >30 kDa protein fraction exerting apyrase activity (Mant and Parker, 1981).

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In addition, several tsetse salivary gland proteins with a yet unknown function have been characterised at the molecular level. These include two related secreted proteins (TSGF-1, 56.6 kDa and TSGF-2, 58.3 kDa) with growth factor and adenosine-deaminase motifs (Li and Aksoy, 2000), tsetse antigen 5 (TAg5, 28.9 kDa) and tsetse salivary gland proteins 1 and 2 (Tsal1, 45.6 kDa and Tsal2, 43.9 kDa) which have been identified as major soluble proteins from *Glossina morsitans morsitans* salivary glands (Li et al., 2001; Haddow et al., 2002). The two abundant homologous gene products Tsal1 and Tsal2 have also been found in *Glossina fuscipes* and *Glossina austeni* and share limited sequence homology with components of the genome of *Anopheles* (GenBank Accession No. EAA03868), the salivary proteome of the house mosquito *Culex* (GenBank Accession No. AAR18449) and the new world sandfly *Lutzomyia* (GenBank Accession No. AAS16916) (Valenzuela et al., 2004). TAg5 shares similarity with the Crisp-Antigen 5 (CAP) family of proteins (Li et al., 2001), including antigen-5 which is an important venom allergen of hornets, wasps and fire ants (Fang et al., 1988; King and Spangfort, 2000). As such, TAg5 might be involved in the hypersensitivity reactions that have previously been observed in tsetse fly-exposed rabbits (Ellis et al., 1986).

The knowledge of the immunological aspects of tsetse fly feeding is very limited. Beside the occasional occurrence of skin reactions, exposure of rabbits to *Glossina palpalis palpalis* or *Glossina morsitans centralis* induces humoral immune responses against several tsetse fly salivary components (Ellis et al., 1986; Matha and Weiser, 1988). In some reported cases, repeated exposure of rabbits to *Glossina* resulted in decreased tsetse feeding efficiencies and decreased longevity (Parker and Gooding, 1979; Matha et al., 1986). However, the authors attributed these effects to locally mediated effects and not to circulating antibodies. The immunogenicity of *Glossina* saliva in other hosts, such as humans, has not been documented so far.

Studies of other haematophagous arthropods demonstrate that salivary components and saliva-specific immune responses play significant roles in the efficiency of blood feeding and disease transmission. For example, components of sandfly saliva bias the host immune response towards a T helper cell type 2 (Th2) environment, favouring the progression of *Leishmania* infection (Titus and Ribeiro, 1988; Lima and Titus, 1996; Belkaid et al., 1998). In that context, the vasodilatory peptide maxadilan with anti-inflammatory properties (Morris et al., 2001) and Salp15 as suppressor of T helper cell activation (Anguita et al., 2002) were identified as active immune modulatory and putative transmission-promoting constituents of sandfly saliva. Pre-exposure of mice to sandflies (Kamhawi et al., 2000; Thiakaki et al., 2005) as well as immunisation against individual salivary components (Morris et al., 2001; Valenzuela et al., 2001) were shown to provide partial or complete protective immunity against natural *Leishmania* transmission. This protection is associated with the induction of a strong delayed-type hypersensitivity (DTH) response at the site of parasite delivery. Additionally, the antibody responses to sandfly salivary antigens could be used to monitor host exposure to this medically important vector and provide a suitable risk marker for *Leishmania* transmission in endemic areas (Rohousova et al., 2005). Moreover, natural or experimental host anti-sandfly immunisation reduced blood-feeding efficiency and increased mortality in the post-bloodmeal vector population (Ghosh and Mukhopadhyay, 1998).

In the case of *Glossina*, comparable studies of the immune modulatory potential of saliva have yet to be undertaken. Host anti-*Glossina* immunity has been addressed only in experimental settings, while the effects on vector fitness remain unclear. Therefore, this study focuses on the immune response raised against tsetse fly saliva in mice and humans as well as the impact of naturally and experimentally induced anti-vector immune responses on the blood-feeding efficiency and survival of tsetse flies. Additionally, this study evaluated whether anti-saliva humoral responses as well as responses against individual recombinant proteins can be used to analyze tsetse fly exposure in epidemiological studies and as such function as a risk measure for trypanosomiasis.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female in-house bred F1 (BALB/c × C57BL/6) mice were used for all experiments. Mouse care and experimental procedures were performed under approval from the Ethical Committee of the Vrije Universiteit Brussel. Tsetse flies (*G. m. 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 Commission of the Institute of Tropical Medicine, Antwerp (Belgium).

2.2. Tsetse fly saliva isolates

Three days after the last blood meal, 10- to 15-day-old tsetse flies were dissected and salivary glands were isolated under a binocular microscope using forceps and collected in ice-cold, sterile PBS (pH 7.4). Centrifugation for 1 min at 20,000*g* separated the salivary gland tissue in the pellet and soluble salivary components in the supernate (= saliva), with an approximate yield of 10 μg per fly (5 μg/gland). Saliva was subsequently sterilised by filtration through a 0.2 μm pore sized filter and lipopolysaccharides were removed using Remtox beads. Protein concentrations were assessed by the BCA protein assay reagent kit (Pierce Biotechnology) and aliquots were stored at −20 °C.

2.3. N-terminal protein sequencing

Saliva isolates were subjected to SDS–PAGE under reducing conditions, using a 10% polyacrylamide gel, followed by an electrotransfer to polyvinylidene difluoride (PVDF) membranes (Sequi-Blot, BioRad) according to standard protocols (Towbin et al., 1979). Blots were stained using amido black, allowing the selection of protein bands of interest for identification of the first 10 N-terminal amino acids on an ABI 473A Edman protein sequencer (Altabioscience, Birmingham, UK).

2.4. Recombinant Tsal1 (rTsal1) and Tsal2 (rTsal2)

The full-length Tsal1 and Tsal2 coding sequences devoid of the leader sequences were amplified (forward primers: 5′-GCGCCCATGGATTGTTCGTTAAAAATACCAG-3′ and 5′-GCTCGGATCCACAATGTTCCATTAACATACC-3′/reverse primers: 5′-GCGCGGATCCATTAAATTTTAACAAATTATTAATTTC-3′ and 5′-GATCGCTCGAGTCAATATTTTAAAAGGCCTTTG-3′) from a *G. m. morsitans* salivary gland cDNA library constructed in pcDNA3.1(+) (Invitrogen). Amplicons were cloned into the respective prokaryotic expression vectors pQE60 (Qiagen) using *Bam*HI/*Nco*I restriction sites and pET17b (Novagen) using *Bam*HI/*Xho*I restriction sites. Tsal1 was expressed in Top10F′ *Escherichia coli* and purified using Ni-NTA affinity chromatography (Qiagen) from the insoluble fraction after resolubilisation in 8 M urea. To remove urea, the rTsal1 protein sample was dialysed against PBS. Tsal2 was expressed in *E. coli* BL21 (DE3) pLysS cells and purified from the insoluble fraction after resolubilisation in 0.1 M urea using an anti-T7Tag affinity column (T7Tag-purification kit, Novagen). Protein concentrations were assessed by the BCA protein assay reagent kit and aliquots were stored at −20 °C.

2.5. Intrapinna saliva inoculations

In order to assess local antigen-specific cellular and cytokine responses induced by tsetse fly saliva, the intrapinna injection was used for intradermal inoculation of saliva, allowing the subsequent isolation of the draining lymph node (Belkaid et al., 1996). The inoculation consisted of a single injection of 5 μg saliva (equivalent of one salivary gland content) in 25 μl PBS between the ventral and dorsal ear dermis using a gauge 30 insulin microsyringe. Cervical lymph nodes were isolated from three mice per experimental group at days 7 and 14 after injection.

2.6. Saliva immunisations

Host anti-vector immune responses were analysed both upon natural exposure of F1 mice to tsetse fly bites and upon intrapinna and s.c. inoculation of saliva extracts either admixed or not admixed with adjuvant.

Natural anti-saliva immunisations were performed by allowing five flies per mouse to feed weekly over a period of 6 weeks. Mimicking a natural exposure, eight F1 mice were immunised intrapinna with three doses of 5 μg saliva in PBS with three weekly intervals. In a trial to target the tsetse fly fitness, another eight F1 mice were primed with 5 μg saliva in FCA and boosted twice at three weekly intervals by administering the same dose in Freund's incomplete adjuvant (FIA) s.c. at the tail base. From all immunised mice, sera were collected 14 days after the last immunisation.

2.7. Tsetse fly feeding efficiency and survival

For each immunisation group, 32 newly emerged tsetse flies were individualised in numbered cages and fed three subsequent times on anaesthetised naive or on one of the three different saliva-immunised groups of F1 mice (saliva/PBS and saliva/adjuvant immunised and exposed to tsetse fly bites). In order to quantify blood meal weights and the body mass increase, individual fly weights were measured to an accuracy of 0.1 mg before and after blood feeding using an analytical balance (Sartorius).

2.8. Ovalbumin and saliva co-immunisation

Co-immunisations were performed as described above into the ear dermis by injecting 5 μg of the heterologous antigen OVA (ovalbumin, Sigma) with or without 5 μg tsetse fly saliva twice with a 3 week interval. Seven days after the last immunisation, cervical lymph nodes and spleens were isolated from three mice per experimental group for antigenic restimulation purposes. Sera were collected for determination of antigen-specific IgG titers.

2.9. T cell proliferation and cytokine assays

Draining lymph node cells (dLNCs) from the intrapinna saliva-inoculated mice were counted and brought to a concentration of 2×10^6/ml in RMPI 1640 supplemented with 10% FCS. For T cell proliferation analysis, 4×10^5 cells per well of a 96-well plate were seeded whereas 10^6 cells/well of a 48-well plate were seeded for the analysis of the cytokine response. Restimulation of the cells in appropriate wells was performed with a final concentration of 10 μg/ml saliva. Cell proliferation was measured by adding 5 μCi/ml (1 μCi/well) [^{3}H]thymidine after 72 h stimulation, followed by a transfer to glass fibre filters (Wallac) 18 h later using a cell harvester (Inotech) and subsequent measurement of [^{3}H]thymidine incorporation (1450 Microbeta, Wallac). IFN-γ, IL-4 and IL-10 production by dLNCs was analysed in cellular supernates, 90 h after stimulation, by cytokine-specific sandwich ELISAs (Pharmingen) using horseradish peroxidase based detection.

In the co-immunisation experiment, both dLNCs and splenocytes (SPCs) were OVA restimulated (10 μg/ml) for T cell proliferation and cytokine analysis purposes.

2.10. Antigen-specific antibody titers and Ig-isotypes

Murine anti-saliva and anti-OVA antibody responses were assessed in Western blot and solid phase ELISA. For Western blot analysis, saliva (5 μg) or OVA (2 μg) was run under denaturing conditions on a 10% SDS–PAGE followed by electrotransfer to a nitrocellulose membrane. Antigen detection was performed with a 1/200 dilution of the murine serum, followed by a detection using peroxidase-conjugated goat anti-mouse IgG (Sigma) and chromogenic substrate. For densitometry analysis of anti-OVA IgG responses, blots were scanned at 300 dpi in greyscale and saved as TIFF-files. Band intensities were quantified as integrated peak O.D. measurements using the Imagemaster 1D Elite v3.01 software package (Amersham Pharmacia Biotech).

For antibody titer measurement and isotype determination, Immunosorb plates (Nunc) were coated with 200 ng antigen (saliva, recombinant Tsal1 or Tsal2) per well in 0.1 M $NaHCO_3$ (pH 8.3) and blocked with 10% FCS in PBS. Serial half serum dilutions were applied to both antigen and FCS-coated wells followed by specific immunoglobulin (IgG1, 2a, 2b, 3) detection using an isotyping kit (EmTec). Antigen-specific IgE detection was performed using a sheep anti-mouse IgE antibody (Calbiochem).

2.11. Anti-tsetse saliva immune screening of human plasma samples

In order to determine human Ig responses to tsetse fly exposure, a panel of plasma samples from 141 HAT patients and 36 non-infected persons were examined. These individuals were recruited in Tororo (Uganda) as part of a study described elsewhere (Maclean et al., 2006). In addition, a further 17 plasmas from unexposed humans resident in Europe were incorporated as negative controls in our immune screening. The use and collection of these samples followed protocols approved by Ethical Committees in Uganda (Ministry of Health) and the UK (Grampian Research Ethics Committee). Plasma samples were subjected to Western blot analysis and tested in a total saliva, rTsal1- and rTsal2-specific human IgG detection ELISA. FCS-coated plates served as negative controls to evaluate the specificity of the antibodies for the relevant antigens. European plasma samples were used to demonstrate that the signals obtained against total saliva, rTsal1 and rTsal2 in ELISA are reflecting specific antibodies raised upon tsetse fly exposure.

For ELISA and Western blot analyses, antigen coating and blotting conditions were the same as described above. Human plasma samples were 1/200 diluted in 10% FCS/PBS for both immune analyses. Antigen-specific IgGs were detected with a peroxidase-conjugated goat anti-human Fc antibody (Sigma). ELISA results were expressed as the differences between the measured O.D.s (λ: 450 nm) and the average O.D. of the European negative control samples. Comparison between the responses to the antigen (either total saliva, rTsal1 or rTsal2)-coated wells and FCS-coated wells allowed subdivision into two responder types: (i) an antigen-specific response (ΔO.D. > 0.05) more than double the ΔO.D. on the overcoat or differing at least 0.1 O.D. (specific); and (ii) no significant binding to the antigen as compared with the FCS coat (negative).

2.12. Graphs and statistical analysis

All graphs were prepared using GraphPad Prism 4.0 software (GraphPad Software). The same software was used for statistical analyses (normality tests, two-tailed unpaired *t* tests, one-way ANOVA) of the data. Data are represented as means ± SEM. Values of $P \leqslant 0.05$ are considered to be statistically significant.

3. Results

3.1. Tsetse fly saliva induces a Th2 response with antibodies against Tsal1 and Tsal2

Upon repeated exposure of mice to tsetse fly bites or experimental immunisation with saliva (with or without adjuvant), high anti-saliva IgG titers were measured in ELISA, while Western blot analysis indicated that raised IgGs in the three immunisation groups reacted by consensus to the most abundant protein band (43–45 kDa, Fig. 1A). Three doses of saliva without adjuvant elicited, almost exclusively, Ig responses against this 43–45 kDa fraction. Anti-saliva IgGs, raised by repeated tsetse fly exposure or immunisation with adjuvant, reacted additionally with salivary components of high (130–170 kDa) and intermediate molecular weights (57–58 and 64–66 kDa). The 45 kDa immune-reactive band consisted of the two homologous tsetse salivary proteins 1 and 2 (Tsal1, Tsal2) as was evidenced by N-terminal protein sequencing by Edman degradation of their 10 first amino acids: DCSLKIPESV and GQCSINIPDD corresponding, respectively, with the N-terminus of Tsal1 and Tsal2. ELISA using total saliva, rTsal1 and rTsal2 as coating antigens, indicated that saliva-specific immune responses could be assessed using both recombinant proteins (Fig. 1B).

Isotype analysis revealed that the saliva-specific Ig response to tsetse exposure was mainly of the IgG1 and IgE isotype, while IgG2a titers were very low (Fig. 2A). This indicates that saliva primes the host to develop a Th2 adaptive immune response. Intradermal (intrapinna) inoculation of saliva extracts corroborated this observation, as saliva-specific cytokine responses in the draining lymph node were characterised by high IL-4 and IL-10 and low IFN-γ responses (Fig. 2B), with IL-4 being a known inducer of IgG1 and IgE antibody-subclass switching.

3.2. Anti-saliva immunity has no effect on tsetse fly blood-feeding efficiency and survival

Average engorged blood masses (Fig. 3A) did not significantly differ ($P > 0.05$) between the flies that were fed on

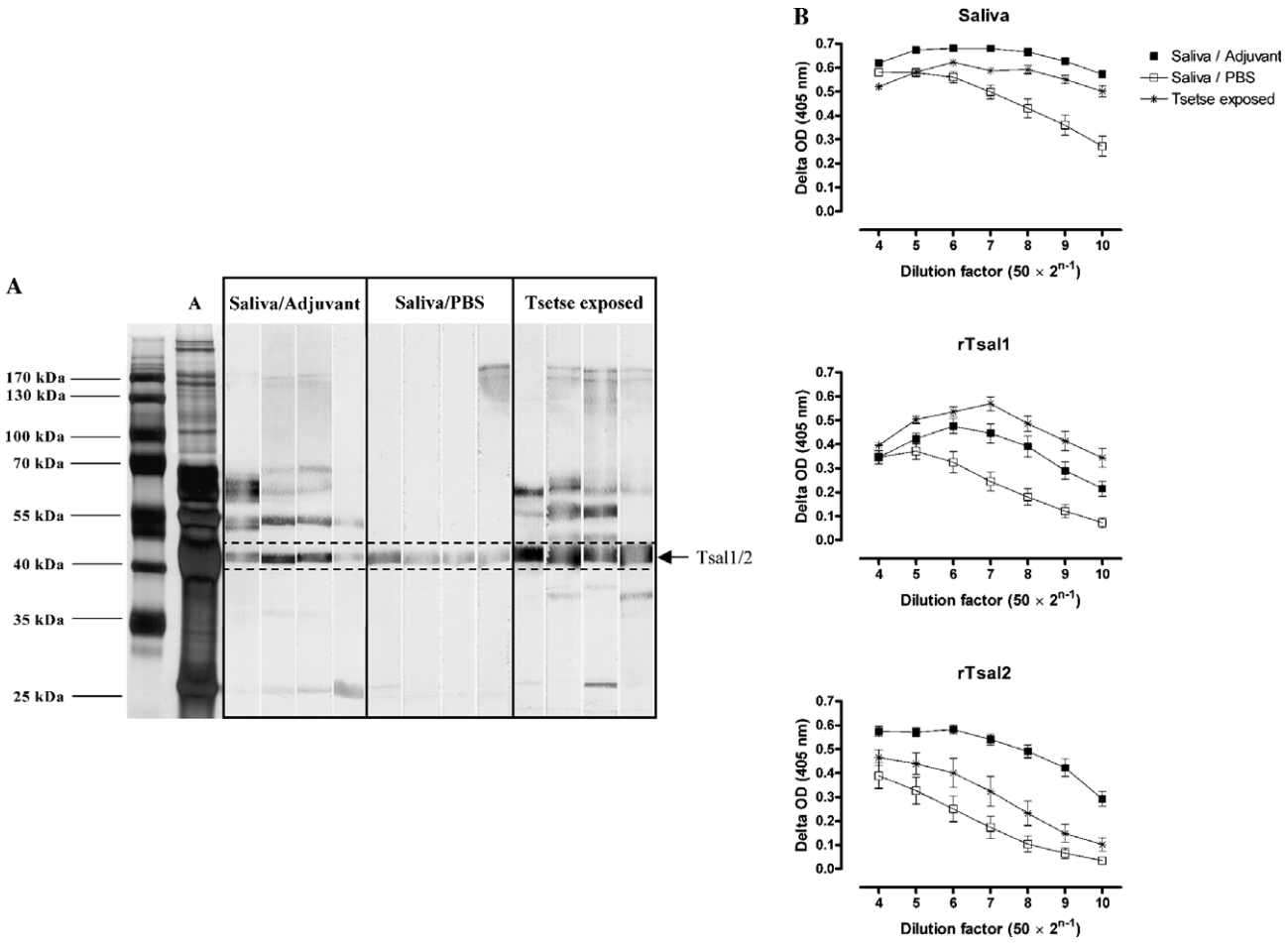

Fig. 1. (A) Western bot analysis of saliva-specific IgG responses in serum from experimental tsetse saliva immunised (with or without adjuvant) and naturally, tsetse fly-exposed F1 mice ($n = 8$/group). Lane A represents the silver-stained protein profile of total saliva. (B) Saliva, recombinant Tsal1 (rTsal1)- and Tsal2 (rTsal2)-specific IgG responses in corresponding titrated sera (X-axis, dilution $= 50 \times 2^{n-1}$) measured in a murine IgG ELISA.

saliva immunised (blood meal mass: 25.2 ± 0.9 mg), on saliva/adjuvant immunised (24.4 ± 1.0 mg), on tsetse fly exposed (25.0 ± 1.1 mg) or on the naive murine hosts (24.6 ± 1.0 mg). During three subsequent feeding occasions, blood-feeding efficiencies in the different experimental fly series were not significantly different ($P > 0.05$). In all groups, mortality was very low and only unfed flies died. The individual fly bodyweights (Fig. 3B) after three blood meals on a naive or an immunised host were not significantly different: 20.2 ± 0.4 mg versus 21.1 ± 0.4 mg (saliva immunised), 19.7 ± 0.6 mg (saliva/adjuvant immunised) and 20.6 ± 0.5 mg (exposed to tsetse fly bites). These data clearly demonstrate that tsetse fly saliva, despite its immunogenicity, does not induce harmful anti-vector immune responses in mice. Enhancing the anti-saliva immunisation with Freund's adjuvant did not affect vector fitness. These observations were corroborated by the high reproductive performance and very low mortality rates of the tsetse fly colony (Prins Leopold Institute of Tropical Medicine Antwerp, ITMA) which is maintained by feeding on rabbits that throughout this process raise saliva-, Tsal1- and Tsal2-specific humoral responses (data not shown). Here, mortality rates in a population of 2,200 female flies, fed four times a week on a battery of 16 rabbits naturally immunised by weekly exposure to approximately 1,000 bites, were $0.34 \pm 0.11\%$ with an excellent reproductive performance of 1.01 ± 0.05 pupa per fly per week.

3.3. Tsetse saliva suppresses humoral and cellular immune responses against heterologous antigen

A significant suppression of the IgG response against the heterologous antigen OVA ($P = 0.0377$), when co-immunised with tsetse fly saliva, was demonstrated in three independent experiments both by Western blot (Fig. 4A) and ELISA (Fig. 4B). Both analyses indicated a three to fourfold decrease of OVA-specific IgG titers upon saliva co-administration. The same relative decrease in antibody titers has been observed using another heterologous antigen, a 32 amino acid anti-thrombin peptide linked to the carrier neutravidin (data not shown). Also the OVA-specific in vitro proliferation of lymph node and spleen cells was significantly reduced ($P = 0.0262$ and $P = 0.0063$, respectively) in the saliva co-immunisation group (Fig. 4C).

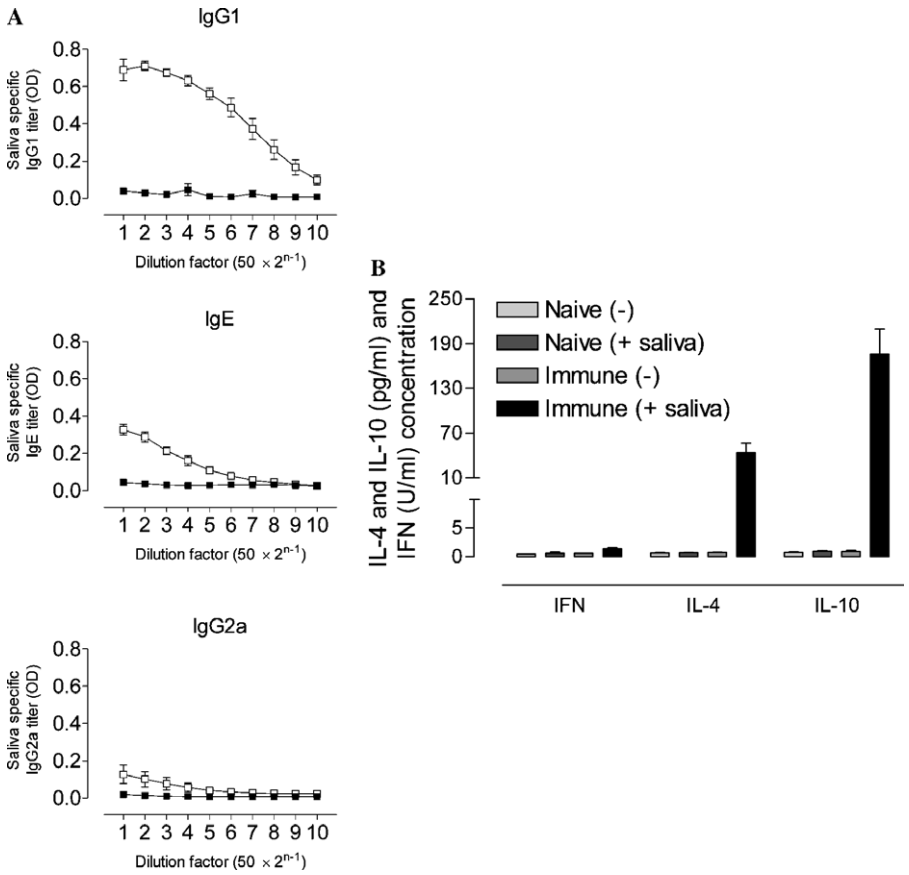

Fig. 2. (A) Isotype-specific titration of saliva-specific antibodies (IgG1, IgE and Ig2a) after natural exposure of F1 mice ($n = 8$) to tsetse fly bites. Serial dilutions (X-axis, dilution $= 50 \times 2^{n-1}$) were analysed for naive (■) and immune sera (□). (B) IL-4, IL-10 and IFN-γ cytokine ELISA of supernates from in vitro saliva-restimulated versus unstimulated draining lymph node cells from naive and saliva-injected mice ($n = 3$/group) at 7 days p.i. The results presented are representative of at least two separate experiments.

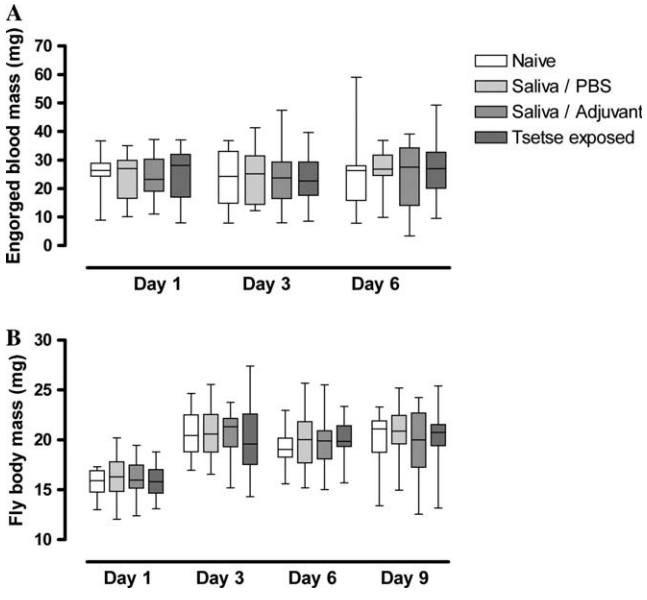

Fig. 3. Engorged blood meal weights (A) and body mass increase (B) of tsetse flies fed ($n = 32$) on naive or immune (saliva/PBS, saliva/adjuvant and tsetse fly exposed) mice ($n = 8$/group) on three feeding occasions depicted as box plots with median, minimum, maximum and 25/75 percentiles. The results presented are representative of two separate experiments.

These data indicate that tsetse fly saliva has suppressive effects on B and T cell responses to heterologous antigen.

3.4. Immunological assay for tsetse fly exposure in epidemiological studies

As described above, exposure of mice to tsetse fly bites by consensus resulted in humoral responses against the tsetse salivary proteins Tsal1 and Tsal2. Plasma samples from Ugandan individuals also scored positive for saliva-specific IgGs in Western blot, although qualitative differences were observed (Fig. 5A). Serum IgGs from saliva-immune individuals sporadically reacted with salivary components with high (130–170 kDa), intermediate (57–58 and 64–66 kDa) and low molecular weights (28 kDa) while Tsal1/2 was recognised by consensus as a major protein band in total saliva (Fig. 5A). As a BLAST analysis only revealed limited homology between Tsal1/Tsal2 and salivary components of other haematophagous insects in the Old World, it was envisaged that IgG responses to recombinant Tsal1 and Tsal2 in ELISA (Fig. 5B) may provide a selective measure of exposure to tsetse challenge in human populations. More than 95% of the Ugandan plasma samples scored positive against saliva, while ELISA of anti-rTsal1 and anti-rTsal2

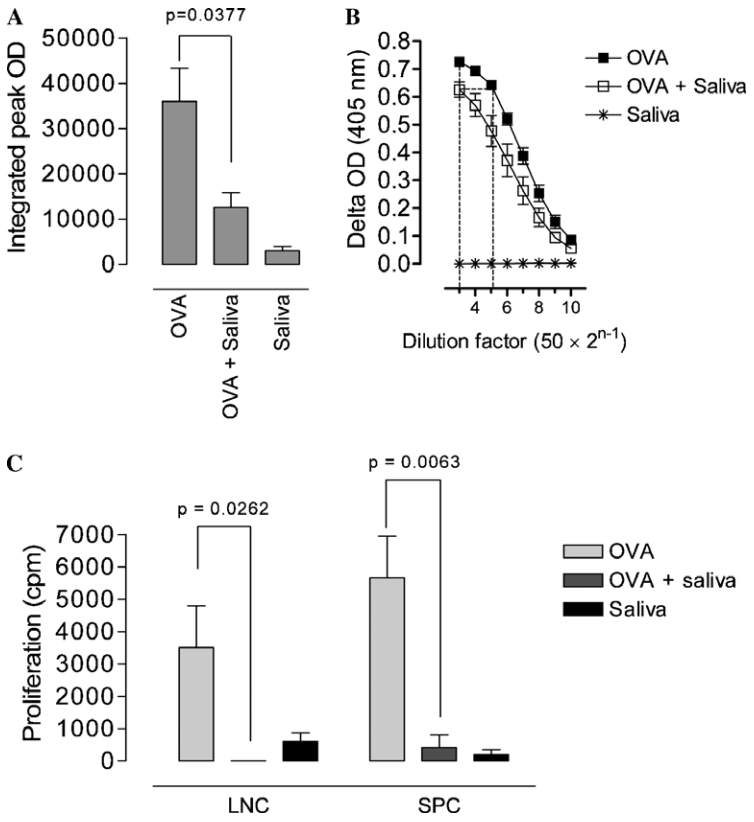

Fig. 4. Influence of saliva on immunisation with ovalbumin: (A) densitometric quantification of OVA-specific IgG responses in Western blot (serum dilution: 1/200) presented as integrated peak O.D. measurements and (B) corresponding OVA-specific IgG responses in titrated serum samples measured in ELISA. The dotted lines illustrate a fourfold drop at a 1/200 diltution in OVA-specific IgG titers upon saliva co-immunisation. (C) In vitro splenocyte (SPC) and lymph node cell (LNC) proliferation upon OVA restimulation. The results presented are representative of at least two separate experiments with three to six mice per group.

Ig responses revealed around 80% specific responders (Fig. 6). Less than 5% of the Ugandan plasma samples scored negative on the three coating antigens, independent of whether they were collected from HAT patients or *Trypanosoma*-negative Ugandan individuals. European control samples all scored negative for specific saliva recognition. Recombinant Tsal1 as coating antigen remained specific for tsetse fly exposure, while rTsal2 yielded limited false positive responses (29.4%) for the European samples in the human IgG ELISA (Fig. 6). To further illustrate that exclusively upon tsetse fly challenge Tsal1/2-specific antibodies are induced, sera from rabbits, repeatedly exposed to the bites of African anopheline mosquitoes, were analysed. This revealed no rTsal1 and rTsal2 cross-reacting antibodies whereas these sera clearly cross-detected the 28.9 kDa band in saliva (data not shown). Together, these observations indicate that the anti-saliva Ig response and especially anti-rTsal1 IgGs may provide a specific measure for tsetse fly exposure in the human population in a sleeping sickness endemic region.

4. Discussion

Haematophagous insects have co-evolved with their hosts to overcome haemostatic and potentially detrimental immune responses. Salivary gland extracts from several blood-sucking arthropods have been proven to contain very potent anti-haemostatic components and are often reported to bias the immune response towards anti-inflammation. The anti-haemostatic potential of tsetse fly saliva has been revealed by the discovery of the very potent TTI peptide and demonstration of the presence of an apyrase, responsible for the inhibition of platelet aggregation (Mant and Parker, 1981; Cappello et al., 1996). However, information on the immunogenicity or immunomodulatory potential of tsetse fly salivary components remains scanty. The results presented here suggest that tsetse fly saliva induces a Th2 response as was evidenced by cytokine responses in lymph nodes draining the inoculation site and by saliva-specific antibody isotypes in serum. Indeed, a single inoculation of saliva in the murine host, followed by antigenic restimulation of the dLNCs 7 days later, resulted in significantly high IL-4 and IL-10 and comparatively low IFN-γ production. In serum from tsetse fly-exposed hosts, these Th2-biased cytokine profiles are associated with relatively higher saliva-specific IgG1 and IgE titers. All together, these data demonstrate the immunogenic nature of tsetse fly saliva and its ability to predominantly induce Th2 responses. These findings are consistent with several reports on immunomodulation by saliva from other blood-sucking

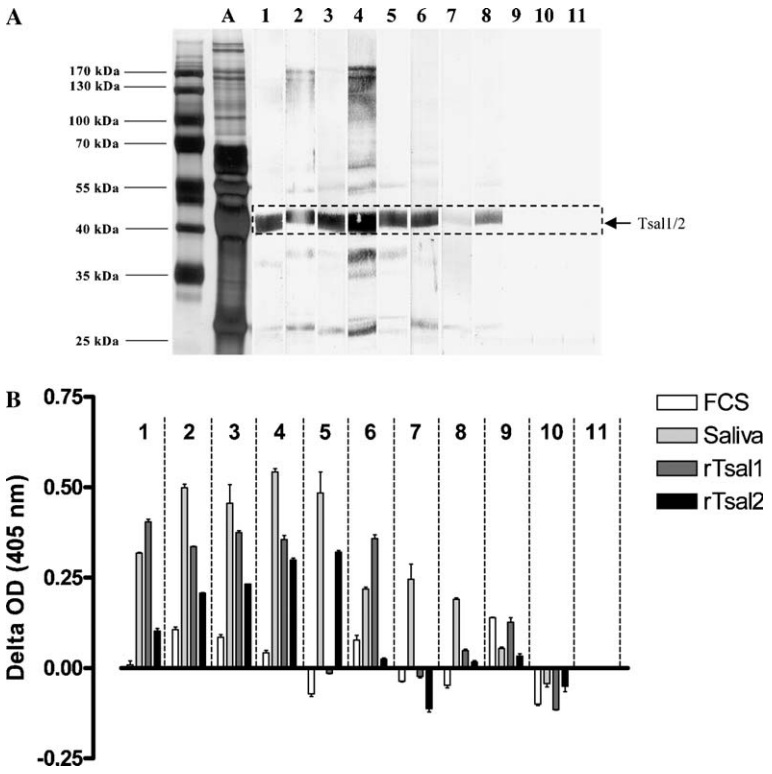

Fig. 5. Immune screening for tsetse fly exposure: (A) Western blot analysis for the qualitative assessment of saliva-specific IgGs in human plasma samples from human African trypanosomiasis (HAT)-negative [1,2], HAT-positive Ugandan [3–8] and European individuals [9,10]. Lane A represents the silver-stained protein profile of total saliva. Strip 11 is a negative control with only detection antibody. The 43–45 kDa band in total saliva, visualised by Western blot, represents Tsal1/2. (B) Analysis of anti-saliva humoral responses as well as responses against the individual recombinant proteins Tsal1 and Tsal2 in ELISA: represented values are delta O.D.s (O.D. – average O.D. from European controls), allowing subdivision into different responder types: specific for all antigen coats (saliva, rTsal1 and rTsal2) [1–4], anti-rTsal1 IgG-negative [5], anti-rTsal2 IgG-negative [6] and anti-rTsal1/2 IgG-negative [7,8]. Included negative controls were European plasma samples [9,10] and a detection antibody control [11].

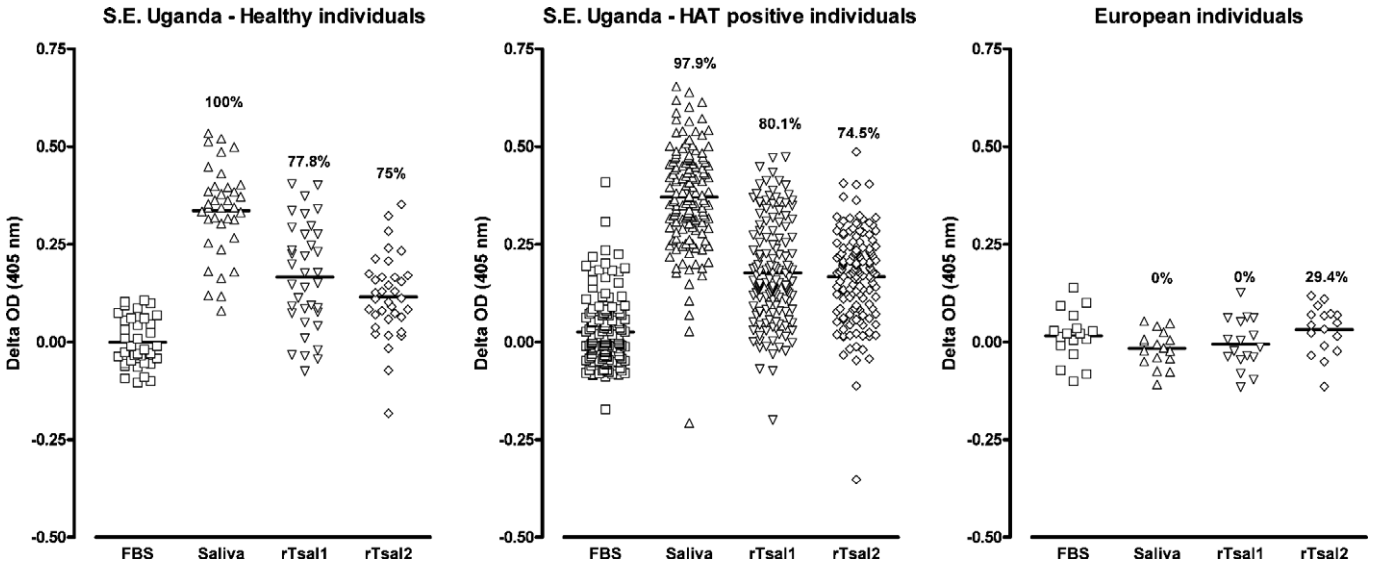

Fig. 6. ELISA of human IgG responses against tsetse fly saliva components: anti-FCS (negative control), anti-saliva, anti-rTsal1 and anti-rTsal2 IgG responses in 36 healthy Ugandan, 141 Ugandan human African trypanosomiasis (HAT)-positive and 17 European individuals. Values are delta O.D.s (O.D. – average O.D. from European controls). In the scatter plots, means are indicated as horizontal bars and percentages for specific responder samples are indicated above each data set.

arthropods (e.g. ticks, mosquitoes and sand flies). Several vector–host interaction models are characterised by an induction of a Th2 response with an overall inhibition of proinflammatory and Th1 cytokines (Kopecky et al., 1999; Kovar et al., 2001; Mejri et al., 2001; Schneider et al., 2004), effector functions of antigen presenting cells

(Theodos and Titus, 1993; Hall and Titus, 1995; Soares et al., 1998; Cavassani et al., 2005) as well as the inhibition of innate immune responses, e.g. natural killer (NK)-mediated cytotoxicity (Kubes et al., 2002) and granulocyte infiltration (Ribeiro et al., 1990; Montgomery et al., 2004). Determination whether naive helper T cells differentiate into Th1 or Th2 phenotypes is influenced by many factors including cytokines such as IL-12, IFN-γ and IL-4 (Hsieh et al., 1993; Seder et al., 1993), antigen dose (Constant et al., 1995; Hosken et al., 1995), accessory molecules (King et al., 1995; Webb and Feldmann, 1995), the affinity of the major histocompatibility complex–T cell receptor interaction (Pfeiffer et al., 1995; Tao et al., 1997; Holzer et al., 2003) and the specific epitopes presented to T-cells (Pfeiffer et al., 1995; Tamura et al., 2004). As the vector–host interaction is generally associated with the suppression of inflammatory responses, accompanied by low IFN-γ secretion, tsetse fly saliva might enhance trypanosome transmission by avoiding trypanocidal cytokine production which occurs in a type I environment (Hertz et al., 1998). Beside the preferential induction of a Th2 immune response, our data also demonstrated that tsetse fly saliva is able to suppress T and B cell responses against heterologous antigen. Here, the immunogenicity of the heterologous antigen OVA was shown to be significantly reduced upon administration in saliva, both at the level of antigen-specific T cell responses in lymph nodes and spleen ($P = 0.0262$ and $P = 0.0063$, respectively) and OVA-specific IgG titers ($P = 0.0377$). Together, these data indicate that saliva influences host anti-vector immunity, at the level of both helper T cell and B cell responsiveness. As such, tsetse fly saliva putatively avoids detrimental anti-vector immune responses in a continuously exposed host population. Our experiments indeed show that naturally as well as experimentally induced anti-vector immune responses in mice do not interfere with tsetse fly feeding efficiency and survival. Efficient maintenance of the tsetse fly colony on rabbits indicates that this observation also holds true for other hosts. This apparently contradicts two reports (Parker and Gooding, 1979; Matha et al., 1986) on the reduced fitness and survival of tsetse flies fed on repeatedly exposed rabbits. Supporting our conclusions, these authors suggest that locally mediated effects rather than antibodies are responsible for these observed anti-vector effects. However, although saliva inhibits B cell responsiveness to heterologous antigen, several salivary components were demonstrated to be immunogenic in the performed immunisation experiments. This demonstrates that the raised Igs lack the potential to neutralise key enzymatic activities, e.g. involved in the blood-feeding process, necessary to maintain an efficient vector–host interaction. This is corroborated by the observation that purified anti-saliva IgGs do not interfere with the anti-haemostatic activity of tsetse fly saliva in vitro (Parker and Gooding, 1979). Moreover, the biologically important TTI peptide was shown to be non-immunogenic (Parker and Mant, 1979; Ellis et al., 1986). This indicates that protection against potentially detrimental anti-vector responses could be guaranteed by immune modulation, by the preferential induction of IgG responses against non-functional protein domains and by the lack of immunogenicity of functional epitopes.

Qualitative analysis in Western blot of the anti-vector humoral response showed that tsetse fly-exposed mice, as well as most of the exposed humans, produced immunoglobulins to the 43–45 kDa protein band. This protein band was identified by Edman degradation to be composed of at least two homologous tsetse saliva proteins, Tsal1 and Tsal2. Screening of the human plasma samples also indicated sporadic immune reactivity against other salivary components with high (130–170 kDa) and intermediate molecular weights (57–58 and 64–66 kDa). An earlier report on saliva-specific antibody responses and hypersensitivity reactions in rabbits (Ellis et al., 1986) reported that sera from rabbits with immediate hypersensitivity more consistently recognised the 160 kDa protein than chronically exposed rabbits. As such, individual differences in reactivity of the human plasma samples might reflect differences in exposure to tsetse fly bites. However, as the 42–45 kDa protein band is commonly recognised in all serum samples from *Glossina*-exposed mice, rabbits (Ellis et al., 1986) and humans, recombinant Tsal1 and Tsal2 may be used as an alternative for total saliva in the immune analysis of exposure to tsetse flies. Although the saliva is collected from *G. m. morsitans* and sequences for Tsal1 and Tsal2 recombinant protein production are obtained from the same species, we demonstrated that saliva-, rTsal1- and rTsal2-based immune screening of Ugandan samples efficiently cross-detected *Glossina fuscipes fuscipes* exposure, responsible for *Trypanosoma brucei rhodesiense* transmission in that area (Hide, 1999). In the same analysis, rTsal1 was shown to be more reliable as coating antigen since, unlike Tsal2, no false positive reactions were observed with samples from non-exposed individuals. Together with the observation that no Tsal1/2 cross-reacting antibodies are raised in a host exposed to the bites of Anopheline mosquitoes, to which many African humans are frequently exposed, these data indicate that the detection of anti-rTsal1 IgGs may provide a specific read-out for tsetse fly exposure. Additionally, the proposed immune screening for tsetse fly-specific IgGs in HAT patients was equally efficient as in non-infected individuals, indicating that the generalised immune suppression described for trypanosomiasis (Beschin et al., 1998) does not prevent anti-vector responses.

Together, the data indicate that screening for rTsal1-specific as an alternative for saliva-specific IgGs can be useful in epidemiological surveys of tsetse fly challenge and thereby help to determine the risk for human populations in a specific area to be exposed to the vector and as such to become infected with trypanosomes.

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