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African trypanosomosis permanently obliterates DTPa vaccine induced memory so that post-treatment *Bordetella pertussis* challenge fails to trigger a protective recall response.

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Abstract: Salivarian trypanosomes are extracellular parasites causing anthroponotic and zoonotic infections. Anti-parasite vaccination in considered the only sustainable method for global trypanosomosis control. Unfortunately, not a single field applicable vaccine solution has been successful so far. Active destruction of the host's adaptive immune system by trypanosomes is believed to contribute to this problem. Here we show *Trypanosome brucei brucei* infection results in the lasting obliteration of immunological memory, including vaccine-induced memory against non-related pathogens. Using the well-established DTPa vaccine model in combination with a *T. b. brucei* infection and diminazene diaceturate anti-parasite treatment scheme, our result demonstrate that while the latter ensured the full recovery from the *T. b. brucei* infection, it failed to restore an efficacious anti-pertussis vaccine recall response. DTPa vaccine failure coincided with a shift in the IgG1/IgG2a anti-pertussis antibody ratio in favor of the latter, and a striking impact on all spleen immune cell populations. Interestingly, an increased plasma IFNγ level in DTPa vaccinated trypanosome infected mice did result in a temporary antibody-independent improvement of early-stage trypanosomosis control. In conclusion, our results are the first to show that trypanosome inflicted immune damage is permanent, and is not restored by successful anti-parasite treatment.

Keywords: Trypanosomosis; treatment; DTPa; *Bordetella pertussis* 3

1. Introduction

Salivarian trypanosomes are extracellular flagellated single cell eukaryotic parasites that are known to colonize the mammalian bloodstream and lymphatics as well as the brain. More recently the fat tissue was identified as a major parasite survival location [1]. *Trypanosoma brucei rhodesiense* and *T. b. gambiense* are the agents of sleeping sickness, i.e. Human African Trypanosomosis (HAT) [2]. Animal trypanosomes (AT) are not infective to humans, as they are susceptible to lysis by the human serum protein APOL1 [3-5]. Species belonging to this category are *T. b. brucei* and the closely related *T. evansi*, *T. congolense*, *T. vivax* and *T. simiae* [6]. The 'AT' classification of *T. evansi* has however been called into question, as this parasite has been reported as the causative agent of several cases of atypical Human trypanosomosis (aHT) in Asia [7-9]. *T. evansi* is also the most widely distributed pathogenic trypanosome, resulting from the fact that it can be mechanically transmitted by a range of biting flies and other blood consuming vectors such as vampire bats. Hence, infections have been reported on all continents, including Europe [6]. Mechanical transmission also occurs in case of *T. vivax* [10] while *T. congolense* is spread both through mechanical transmission [11], and the tsetse vector. Tsetses also transmit *T. b brucei* animal trypanosomosis and *T. b. gambiense/rhodesiense* sleeping sickness [12,13]. In trypanosomo-

sis, the host-anti-parasite response relies in large on the capacity to generate anti-trypanosome antibodies. The success of colonizing a broad range of hosts, including humans, infers however that trypanosomes acquired adaptations allowing to circumvent multiple immune killing mechanisms. These mechanisms have been best studies for *T. brucei*, with the cloned *T. b. brucei* AnTat 1.1 parasite serving as one of the most widely implemented *in vivo* and *in vitro* laboratory model [14,15]. Assessing the host-parasite interaction of *T. brucei* at a molecular level revealed already more than 40 years ago that these parasites use antigenic variation of the Variant Surface Glycoprotein (VSG) coat as a first line of defense against antibody-mediated killing [16-20]. This involves chromosomal recombination, expression side activation/silencing and access to an extensive reservoir of over 1000 VSG genomic VSG genes and pseudogenes, allowing trypanosomes to 'eternally' outrun the mammalian adaptive immune system [21]. Interestingly, the elaborate VSG switching is not the only mechanism involved in parasite evasion of the host immune system, as there are severe limitations to the efficacy of antigenic variation. Indeed, as the trypanosome (i) does not have accesses to an unlimited reservoir of immunologically distinct VSGs deprived of shared conserved T cell epitopes [22], and (ii) there are non-variable surface molecules that are needed by the parasite for nutrient binding and uptake, the parasite had to acquire additional defense mechanisms. This secondary level of protection is obtained by rapid lateral surface movement and endocytosis of antibody-complexed surface molecules. This allows not only the removal of VSG-bound antibodies, but also reduces the efficacy of complement mediated killing after C3b surface deposition [19,23]. This is important as IgM-mediated complement cascade activation and CR3-mediated parasite phagocytosis by macrophages is considered a crucial anti-trypanosome clearance strategy [24]. An additional defense against C3b surface binding is provided by shedding of soluble (s)VSG molecules, resulting in complexing the opsonin C3b away from the parasite membrane [25]. As trypanosomes also cause the rapid reduction in C1, C1q and C3 serum concentrations, it is clear that these parasites have adopted a range of strategies to reduce the risk of IgM/C3b-mediated elimination [26]. Finally, trypanosomes also severely compromise the hosts' antibody production capacity by exerting a detrimental effect on the B cell compartment itself [15,27-29]. The cytotoxic effect of NK cells and contact-dependent interactions between B cells and trypanosomes that induces apoptosis of Transitional B cells after caspase activation in conjunction with CD95 surface upregulation, have both suggested to impair the host immune defense. Caspase-3 dependent apoptosis of MZB cells coinciding with the downregulation of anti-apoptotic marker Bcl-2 as well as TNFRSF13C, encoding the surface expressed B cell maturation and survival receptor BAFF-R, delivers an additional damaging effect to the antibody production capacity [15,27,30-32].

As trypanosomes have a severe detrimental impact on the mammalian adaptive immune response, there has been no progress made towards a field applicable vaccine [17]. The problem of trypanosomosis is exacerbated by the fact that field data has shown that infections result in failures of unrelated veterinary vaccines such as those for classic swine fever [33], Foot and Mouth disease [34], *Pateurella multocida* [35] and *P. haemolytica* [36], and cause overall susceptibility to secondary infections [37]. While this pathology aspect of trypanosomosis is not well-studied in HAT, a study addressing measles vaccine interference in HAT patients showed a significant infection-associated reduction in vaccine induced antibody titers, although the functional consequence of the latter was not addressed [38]. Interestingly, antibody titers did not recover after therapeutic anti-HAT treatment. In a more experimental vaccine setting, the first data that showed the detrimental impact of trypanosomosis on heterologous vaccine induced memory in mouse models, involved the commercial Boostrix® DTPa vaccine [30]. This human combination vaccine is routinely used to raise protection against diphtheria, tetanus, and pertussis. It consists of diphtheria toxoid, tetanus toxoid and three purified antigens of *Bordetella pertussis*, i.e. pertussis toxoid (PT), pertussis filamentous haemagglutinin (FHA) and pertactin (PRN), adsorbed onto aluminum salts. Using an antigen cocktail rather than a whole-

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cell approach allows to reduce inflammatory side effects. While this vaccine has been shown to be very effective in safeguarding mice against a nasal *B. pertussis* challenge, protection is totally lost in mice that are suffering from an experimental *T. brucei* infection [30].

Taken the vast size of the wildlife trypanosome reservoir, only a vaccine-based approach will be able to lift the global burden of this disease. Indeed, while active case detection and targeted treatment of Human African Trypanosomosis (sleeping sickness) has been successful in recent years [39], zoonotic trypanosomosis control requires a sustainable vaccination approach, as eradication of the reservoir is considered to be impossible. The same counts for the protection of livestock animals in which prolonged drug treatment causes undesirable side effects. These including the risk of drug resistance buildup in the parasite population, and the toxicity problems related to the presence of drug residues in animal consumption products such as milk and meat, special when extremely dangerous treatment regiments are used [40]. Hence, understanding the mechanism of trypanosomosis induced vaccine failure as a result of the permanent destruction of the immune memory compartment needs to be thoroughly addressed. Here, this pathology aspect was tackled by readdressing the DTPa/*T. brucei* heterologous vaccine setting, showing that curative anti-parasite treatment does allow full naïve immune recovery in mice, but fails to restore pre-existing vaccine induced protective memory responses.

2. Materials and Methods

Mice and parasites infections.

Female 7 to 9-week-old BALB/c were purchased from Koatech, Korea. The pleomorphic AnTat 1.1E (EATRO 1125 stock) *T. brucei brucei* was used as previously described, infecting mice by i.p. injection of 5000 parasites/mouse [41]. Every 2 to 3 days, the number of parasites present in the blood was counted using a hemocytometer and light microscope and a 2,5 µl blood sample collected from the tail vein diluted 1/200 in DPBS (Invitrogen, USA). Parasitemia was recorder for a total period of 15 days. All experimental mouse procedures were approved by the GUGC Institutional Animal Care and Use Committee (IACUC) (file # 2018-012).

DTPa vaccine procedure and in vivo B. pertussis challenge.

Mice were vaccinated according to the previously published protocol [30]. In short, vaccination was done using 1/4th of a human dose of the commercially available DTPa vaccine (Boostrix®) administered sub-cutaneous (s.c.) in the scruff of the neck. After 21 days, mice received a booster injection (s.c.) with the same amount of vaccine. After a further 14 days, mice were infected i.p. with 5000 *T. brucei* parasites/mouse. Two weeks post-infection, mice received a curative 40 mg/kg dose of diminazene diaceturate (Veriben ® CEVA, France) and were allowed to recover for 6 weeks, prior to intranasal challenge with a dose of 5x10^6 *B. pertussis* bacteria/mouse (ATCC 9797 reference strain) in 10 µl DPBS (Invitrogen, USA). Control mice received the intranasal *B. pertussis* challenge in the absence of a trypanosome challenge, or in the absence of vaccination and parasite infection. Lung bacterial load clearance was monitored after 18 hours, and 3, 6, 9 days post-challenge. Mice were sacrificed and whole lungs were isolated and homogenized in 5 ml DPBS (Invitrogen, USA). Serial 10-fold dilutions were prepared and aliquots of 200 µl were plated onto the Bordet-gengou agar plates (Merck, DE, Cat. No. B4551). The number of colony forming units (CFU's) was counted after 72 hours of incubation at 36°C. All experimental mouse procedures were approved by the GUGC Institutional Animal Care and Use Committee (IACUC) (file # 2018-012).

Quantification of IFNγ by ELISA.

Cytokine quantification was done using the Mouse IFNγ MAX™ Deluxe set (Biolegend, USA, Cat. No. 430804). In short, heparinized plasma was collected at several timepoint throughout the experiment and stored in aliquots at -20°C. After thawing, plasma was diluted ½ in the assay diluent provided by the manufacturer and processed according to the kit's protocol.

Quantification of anti-pertussis and anti-VSG antibody titers by ELISA

B. pertussis was cultured at 37°C in Stainer–Scholte broth (SS) medium, seeding bacteria at approx. OD = 0.2 and harvesting cells 30h later at OD = 1. Whole cells were pelleted by centrifugation for 10 minutes at 5000 x g and resuspended in ice-cold TE buffer. Cells were centrifuged for 10 min at 5000 x g and resuspended in 1 ml 0.04M lysozyme/TE buffer followed by incubation for 35 min at 37°C. Samples were sonicated using 5 cycles of 10-sec burst/30-sec cooling on ice, diluted in 4 mL PBS pH 7.2 and centrifuged for 30 min at 16000 x g, at 4°C. Protein concentration in the supernatant was determent by Bradford assay (Thermo Scientific, USA, kit Cat. No. 23200) and stored at -20°C. Lysate was coated in 96-well Half Area Clear Flat Bottom Polystyrene High Bind Microplate (Corning, NY, USA) at 4°C overnight, coating 0.1 μg/50 μl/well, using a 0.05M bicarbonate 9.6 pH coating buffer (3.7 g Sodium Bicarbonate ($NaHCO_3$) / 0.64 g Sodium Carbonate (Na_2CO_3) / 1L H_2O). Heparinized plasma was collected from infected mice at several timepoints throughout the experiment and stored in aliquots at -20°C. After thawing, plasma was diluted 1/200 and subsequently diluted further as a 1:2 serial dilution up to 1/51.200, using DPBS (Invitrogen, USA). Plasma IgG1 and IgG2a titers were determined using horseradish peroxidase-labeled specific secondary antibodies (Southern Biotech, USA, kit Cat. No. 5300-05). Anti-VSG titers were measured as described before [42], using purified AnTat 1.1 VSG as coating (0.1 μg/50 μl/well, using a 0.05M bicarbonate 9.6 pH coating buffer) and 96-well Half Area Clear Flat Bottom Polystyrene High Bind Microplate (Corning, NY, USA). Total plasma Ig titers were determined using horseradish peroxidase-labeled specific secondary antibodies (Southern Biotech, USA, kit Cat. No. 5300-05). Plasma was diluted 1/200 as start concentration and subsequently diluted further as a 1:2 serial dilution up to 1/51.200 using DPBS (Invitrogen, USA).

Cell isolation and flow cytometry analysis

Spleen cells were isolated at different time points. Single-cell suspensions were prepared by homogenizing spleens in 6 mL of DMEM (Capricorn Scientific, DE) supplemented with 10% FBS (Atlas Biologicals, USA) and 1% penicillin/streptomycin using gentleMACS™ Dissociator (Miltenyi Biotec, DE). After passing the homogenate through a 70 μm cell strainer (SPL Life Sciences, Korea), cells were centrifuged at 314 x g for 7 minutes at 4°C, followed by re-suspension and incubation in RBC lysis buffer (Biolegend, USA) at 4°C for 5 minutes. After washing (314 x g 7 minutes at 4°C), cells were kept on ice in FACSFlow Sheath Fluid (BD Biosciences, USA) containing 0.05% FBS (Atlas Biologicals, USA) and Fc block (CD16/CD32 Fcγ III/II, Biolegend, CA, USA) (1/1000 dilution) for 30 minutes in the dark at 4°C. Subsequently, 10^5 cells per sample were incubate for 30 minutes in the dark at 4°C, with antibody cocktails specificity for different splenocytes populations, followed by flow cytometry analysis using BD Accuri™ C6 Plus flow cytometer (BD Biosciences, 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, with the total live cell number determined for each cell preparation in combination with microscopy live cell counts for every individual cell preparation.

Antibodies and detection reagents

The following antibodies (Biolegend, USA) were added to 100 μl aliquots of 10^5 Fc-blocked splenocytes prepared as described above to make final 1/600 dilution: anti-CD1d-PE(clone 1B1), anti-CD11b-FITC (clone M1/70), anti-B220-FITC and anti-B220-PE-Cy7 (clone RA3-6B2), anti-CD93-APC and anti-CD93-PE-Cy7 (clone AA4.1), anti-CD138-PE-Cy7 (clone 281-2), anti-GL7-PE (clone GL7), , anti-Ter119-PE (clone TER-119), anti-Ly6G-Alexa488 (clone 1A8), anti-Ly6C-PE (clone HK 1.4).

Quantification and statistical analysis

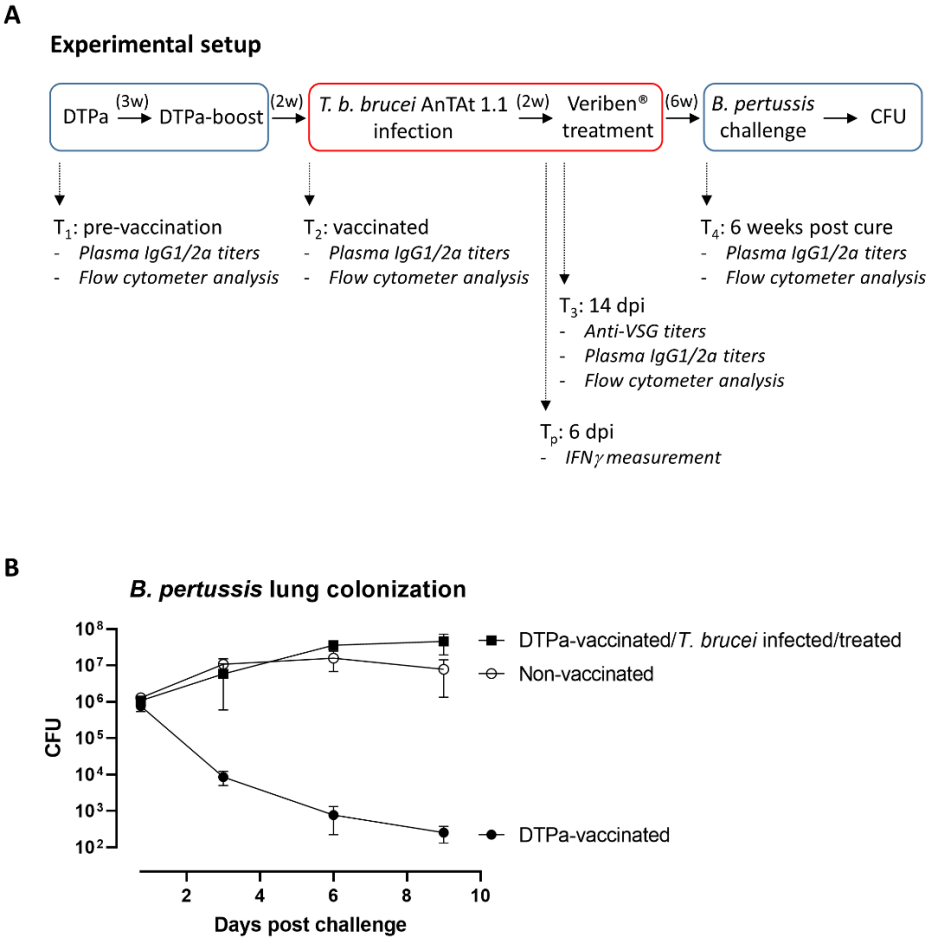
GraphPad Prism v.8.3 (GraphPad Software Inc., USA) was used for final data presentation and statistical result analysis. Unless otherwise stated, data were compared with naïve using student's t-test. Means are given as ± standard deviation (SD).

3. Results

3.1. T. b. brucei permanently destroys DTPa vaccine induced protection against Bordetella pertussis.

In previous research, we have shown that active *T. brucei* infections suppresses protection of a commercial DTPa vaccine against *B. pertussis* [30]. This observation was in line with other reports showing the detrimental impact of ongoing trypanosome infections on commercial veterinary vaccines [33-36]. However, none of these studies addressed whether the trypanosomosis-associated damage to the immune system was permanent, or merely the result of immunosuppression caused by the ongoing parasite infection. Hence, we adopted a protocol of trypanosome infection followed by treatment, and assessed the capacity of host immune system to recover from *T. b. brucei* induced immune dysfunction. Mice were vaccinated and boosted with the DTPa vaccine over a period of 5 weeks prior to exposure to a *T. b. brucei* AnTat 1.1 infection. Two weeks into the trypanosome infection, mice were cured using a standard treatment of diminazene diaceturate (40 mg/kg) and allowed to recover for 6 weeks. Subsequently, mice received an intranasal challenge with $5x10^6$ *B. pertussis* bacteria, after which lung CFUs were estimated (Fig. 1a). Results show that under control conditions, the DTPa vaccine does offer adequate protection against *B. pertussis*, with lung CFUs rapidly declining over a 9-day monitoring period (Fig. 1b). In contrast DTPa-vaccinated mice that had been exposed to *T. brucei* infection 8 weeks earlier subsequently cured, and allowed to recover for a 6-week period, completely failed to mount a protective vaccine recall response.

Figure 1. (a) Experimental layout. Mice were vaccinated and boosted with DTPa with a 3-week interval, followed by intraperitoneal challenge with 5000 *T. b. brucei* AnTat 1.1 parasites. Two weeks



into the infection all mice were treated with diminazene diaceturate (40 mg/kg). After a 6-week recovery period, mice were challenged intranasal with $5x10^6$ *B. pertussis* bacteria (ATCC 9797 reference strain) in 10 µl DPBS. Subsequently lung bacterial load clearance was monitored by CFU determination. Anti-pertussis plasma antibody titer determination (IgG1 and IgG2a) as well as flow cytometry analysis of the spleen was executed at T_1, T_2, T_3 and T_4. A plasma ELISAs for IFNγ was performed at the peak of the *T. b. brucei* infection T_P (peak timepoint = 6 dpi). Control groups included naïve mice, non-vaccinated *T. b. brucei* infected mice and DTPa-vaccinated mice that were not challenged with trypanosomes. (b) Experimental infection with *T. b. brucei* leads to the permanent abrogation of DTPa vaccine protection against *B. pertussis*. Bordet-Gengou agar plate lung homogenate CFUs were determined after 18h as well as after 3, 6 and 9 days of infection. CFU's were measured after 72 h incubation and are represented as the mean ± SD of 3 individual mice per time point.

3.2. T. b. brucei infection alters the IgG1/IgG2a ratio of anti-pertussis antibody titers in DTPa-vaccinated mice.

The protective effect of DTPa against *B. pertussis* has been associated with the strong IgG1 inducing potential of the vaccine, mostly reported by the high IgG1/IgG2a ratio of DTPa-induced antibodies [43,44]. Here, we recorded plasma anti-pertussis antibodies in vaccinated mice, compared to vaccinated mice infected with *T. b. brucei* AnTat 1.1. Results show that following vaccination and boost (T_2 compared to T_1), all mice had significant IgG1 titers against the soluble fraction of total bacterial *B. pertussis* lysate, with endpoint titers reaching 1/6400 (Fig. 2a). Vaccination induced inferior IgG2a titers, reaching endpoint titers that were four times lower compared to the IgG1 titers, reaching only 1/1600 (Fig. 2b). Two weeks into the *T. b. brucei* infection (T_3), DTPa-induced IgG1 titers showed a downwards trend in ELISA OD readings, being significant in the plasma dilution from 1/400 down to 1/6000. The endpoint tires of anti-pertussis antibodies were however the same in both infected and non-infected DTPa-vaccinated mice, being 1/3200. This corresponds to a two-fold reduction compared to the pre-trypanosome challenge timepoint. *T. b. brucei* infection itself did not induce any cross-reactive anti-pertussis IgG1 antibodies (Fig 2c.). Also for the anti-pertussis IgG2a antibody levels, a two-fold reduction was observed when comparing endpoint titers between pre-infection (T_2) and 14 dpi data, with titers reaching only 1/800 at T_3. However, in contrast to the IgG1 results, ELISA OD readings at plasma dilutions 1/200 and 1/400 show a clear trypanosomosis-associated induction of cross-reactive IgG2a antibodies. In this case, also non-vaccinated infected mice (T_3, 14 dpi) showed significant IgG2a antibody levels against the soluble fraction of total bacterial *B. pertussis* lysate, reaching the same 1/800 endpoint titers as the DTPa-vaccinated infected mice. These data indicate that the IgG2a titers measured in this assay resulted from polyclonal activation of the adaptive immune system, caused by infection-induced inflammatory pathology. Indeed, (Fig. 2d). Six weeks post-treatment (T_4) antibody titers gradually declined once again two-fold in DTPa-vaccinated control mice due to natural antibody clearance, now exhibiting endpoint tires of 1/6400. In contrast mice that had been exposed to a *T. b. brucei* infection, and subsequently cured, showed a much greater reduction in IgG1 levels both in terms of absolute ELISA OD readings as well as endpoint tires, with the latter only reaching 1/400 (Fig. 2e). In contrast, cross-reactive *T. b. brucei* induced IgG2a antibody levels remained high in ELISA OD readings at low plasma dilutions (1/200 and 1/400), while endpoint tires itself dropped to the same levels as those for IgG1, being 1/400 (Fig. 2f).

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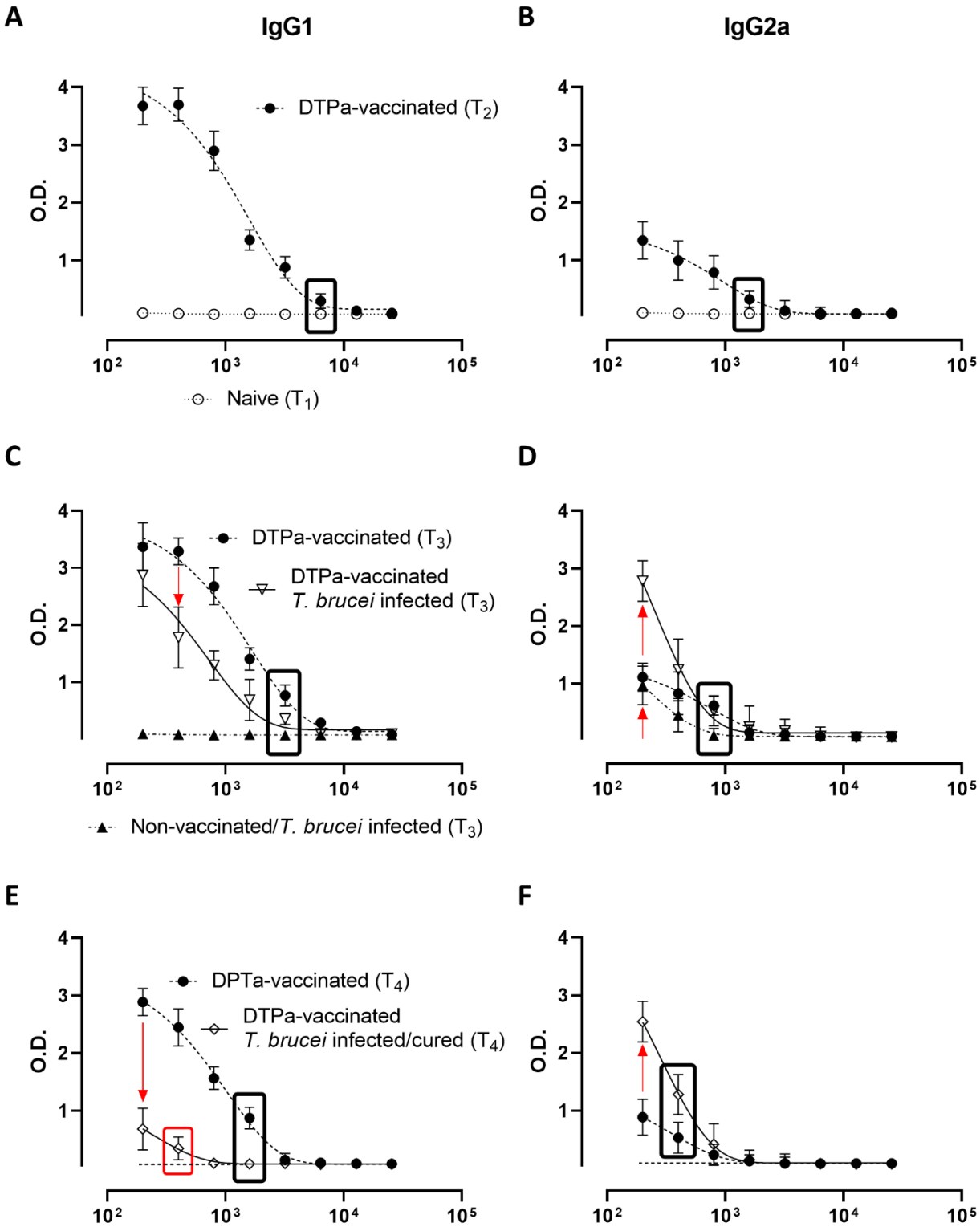

Figure 2: DTPa induced IgG1 (left panels) and IgG2a (right panels) titers are strongly affected by *T. b. brucei* infection. (a,b) Plasma samples were collected prior to the start of the DTPa vaccination (T_1), and 2 weeks after the vaccine boost (T_2). (c,d) Plasma samples were collected at experimental timepoint T_3 from both DTPa-vaccinated and non-vaccinated *T. b. brucei* AnTat 1.1 challenged mice (14 dpi) as well as control DTPa-vaccinated mice that were not infected by *T. brucei*. (e,f) Plasma samples were collected at experimental timepoint T_4 from 6-week cured DTPa-vaccinated mice that had recovered from their *T. b. brucei* infection. Control samples were obtained here from DTPa-vaccinated mice that had not been challenged with *T. b. brucei*. Plasma dilution series (1:2) were made to determine anti-pertussis antibody endpoint titers for all experimental groups indicated as black boxed data points. This value represents the last serial dilution resulting in an OD that is significantly different from the OD value obtained at the same dilution in naïve mice (represented in d, e and f as a dashed line). The red boxed value in (e) indicates the significant *T. b. brucei* induced reduction of IgG1 titers at T_4. Red arrows indicate the *T. brucei* induced trends in reduction (c,e) or increase (d,f) of anti-pertussis antibody ELISA OD readings. Values are represented as the mean ± SD of 5 individual mice per time point.

3.3. T. b. brucei infection results in the rapid destruction of the host spleen B cell compartment, while anti-trypanosome treatment results in a full cellular spleen recovery.

In the past, we reported that *T. b. brucei* AnTat 1.1 infection results in the rapid destruction of the spleen architecture and in the depletion of mature B cells from the spleen [30]. Monitoring this infection-associated pathology was used here to confirm the damage done to the immune system by the parasite, as well as to confirm the repair of the immune system 6 weeks after diminazene diaceturate treatment of infected mice. Flow cytometry analysis was implemented to track all mature spleen B cell populations, T cells, NK/NKT cells, and macrophage/monocyte as well as granulocyte populations. Measurements were done using naïve mice (T_1), DTPa-vaccinated mice (T_2), DTPa-vaccinated / trypanosome infected mice (T_3) and vaccinated cured mice, just prior to the *B. pertussis* challenge (T_4). Results are presented as flow cytometry measurements (Fig. 3) as well as total spleen cell number counts (Fig. 4). First, the data indicates that while the spleens of vaccinated mice show a similar cellular composition to those of naïve mice for most cell compartments (Fig. 3 T_1, compared to Fig. 3 T_2), a marked increase in $CD11b^+Ly6G^+Ly6C^{Int}$ granulocytes occurs after vaccination (Fig. 3 T_1f) compared to Fig. 3 T_2f). This observation is confirmed when absolute spleen cell numbers are calculated (Fig. 4, with Fig. 4j showing granulocyte counts). Two weeks into infection (T_3), trypanosomosis-associated destruction of the $B220^+CD1d^+$ Marginal Zone compartment is prominent, while destruction of the $B220^+CD1d^{Low}$ Follicular B cell compartment is measurable as well (Fig. 3 T_3a). This coincided with an increase in $CD138^+$ Plasma B cells (Fig. 3 T_3b) and $GL7^+$ Germinal Center B cells (Fig. 3 T_3c). $CD4^+$ and $CD8^+$ populations remain almost unchanged (Fig. 3 T_3d), while both $NK1.1^+$ and $NK1.1^+TcR\beta^+$ T cell populations decline (Fig. 3 T_3e). During infection the $CD11b^+Ly6G^+Ly6C^{Int}$ granulopoiesis exacerbates, accompanied by an influx of $Ly6C^+$ monocytes and macrophages (Fig. 3 T_3f). Six weeks post-treatment, all cell counts regain a status that is roughly comparable to naïve mice (Fig. 3 T_4). All numerical spleen cell data obtained by converting flow cytometer 'percentage' observations into absolute number data, confirms that 6 weeks post treatment the immune system of all cured mice was reset into a naïve mode, but as shown in Fig. 1b, this did not result in a restoration of previously acquired DTPa-induced vaccine protection against *B. pertussis*. Hence, we conclude that while curative anti-trypanosome treatment restores full naïve immune function in previously infected mice, *T. b. brucei* infection itself induces a rapid and permanent destruction of the immunological memory compartment.

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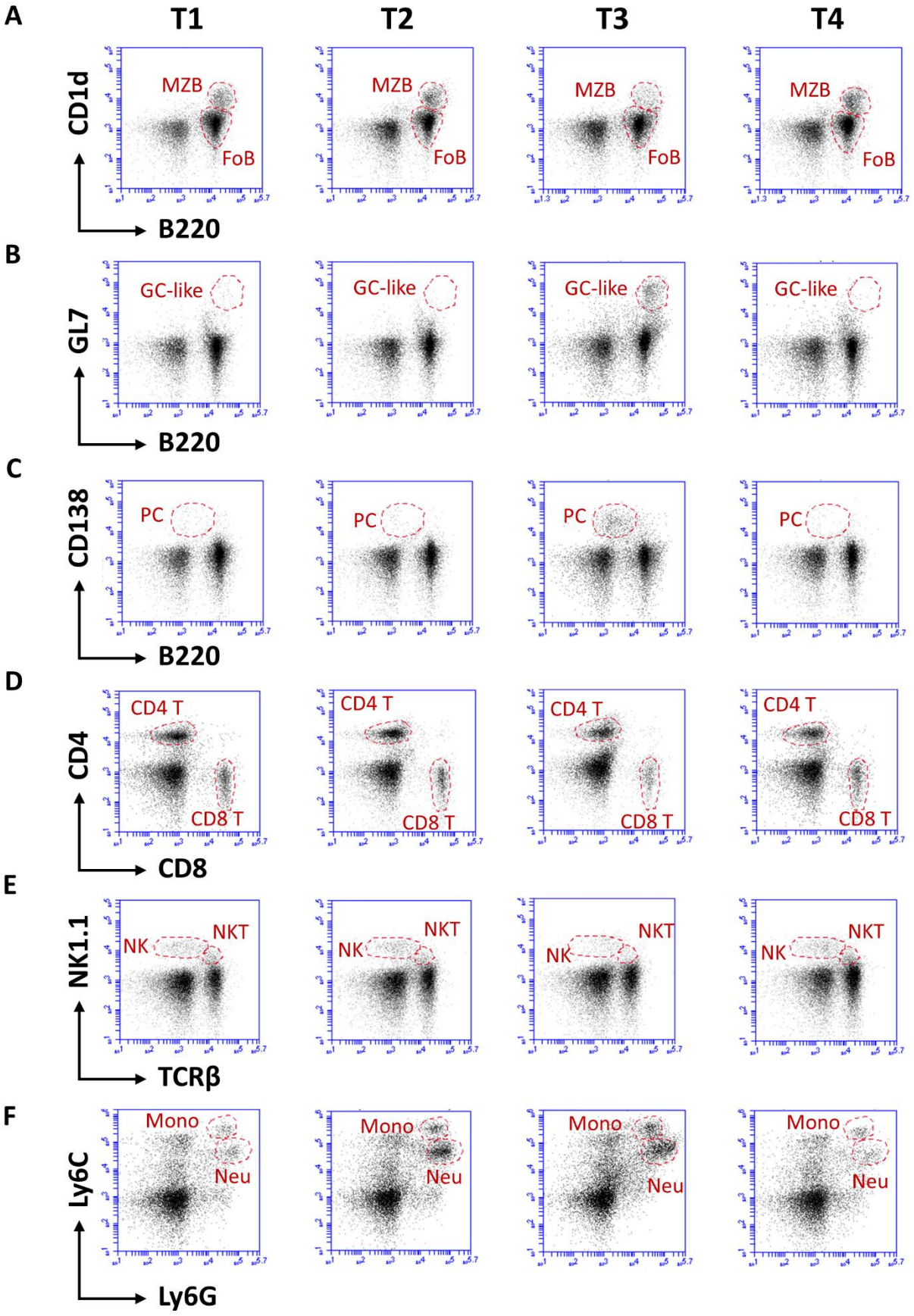

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Figure 3: Early-stage *T. b. brucei* infection has a detrimental impact on the spleen composition in DTPa-vaccinated mice. Flow cytometry analysis of spleens of naïve mice (T1), DTPa-vaccinated mice prior to parasite challenge (T2), DTPa-vaccinated *T. b. brucei* AnTat 1.1 infected mice (T3), and DTPa-vaccinated T. brucei AnTat 1.1 challenged and cured mice (T4). Analysis was performed for (a) Marginal Zone and Follicular B cells using a CD1d+/B220+ gating, (b) Germinal center B cells using GL7+B220+ gating, (c) Plasma B cells using a CD138+B220Int gating, (d) CD4+ and CD8+ T cells, (e) NK1.1+ and NK1.1+TcRβ+ spleen cells, and the (f) granulocyte/monocyte spleen compartment, using a Ly6C+/Ly6G+ gating strategy. One presentative plot from a biological triplicate experiment is shown for each population.

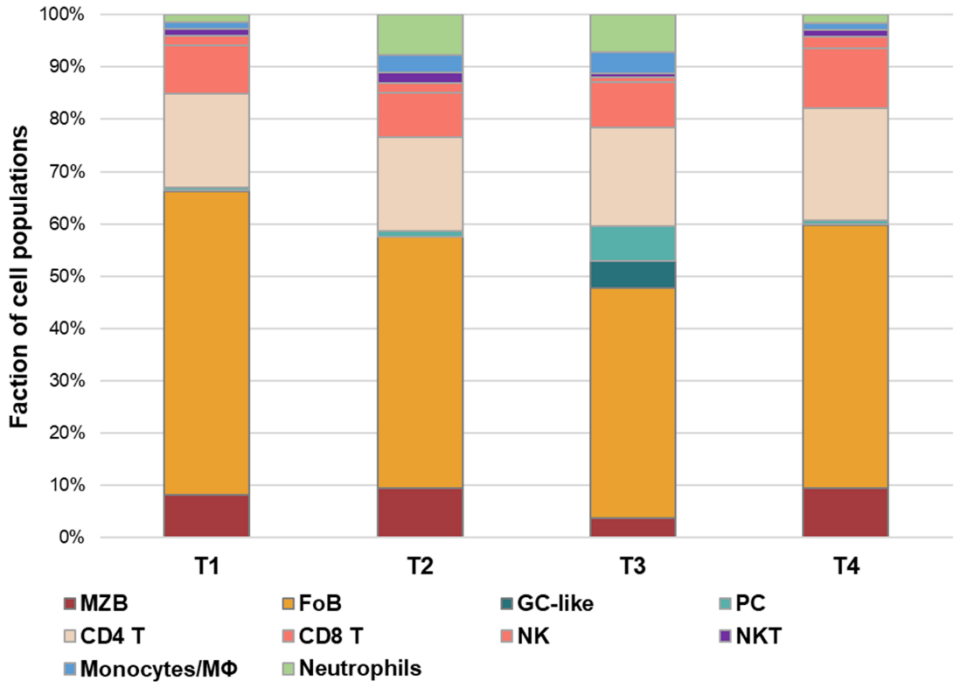

Figure 4: Early-stage *T. b. brucei* infection affects mainly the spleen B cell (reduction) and neutrophil (increase) numbers in DTPa-vaccinated mice. Numerical data, expressed as %-value of the total spleen is plotted for Marginal Zone, Follicular, Germinal and Plasma B cells, CD4+ and CD8+ T cells, NK1.1+ and NK1.1+TcRβ+ spleen cells, and the monocyte/macrophage compartment as well as the spleen neutrophil population.

3.4. DTPa vaccination results in the temporary improvement of trypanosomosis control through an improved anti-parasite IFNγ response.

During the execution of the experimental setup outlined above, parasitemia development of *T. b. brucei* AnTat 1.1, as well as the subsequent treatment success were monitored in order to validate the model. Hence, this was done in both DTPa vaccinated and non-vaccinated mice. Surprisingly, DTPa exposure itself appeared to have a parasite suppressive effect, as peak parasitemia in vaccinated mice were significantly reduced (Fig 5a). Two weeks into infection, all mice were cured by the drug regiments, and were found to be parasite-free for the remaining 7 weeks of the experiment. As there was no reason to assume that DTPa-induced antibody responses had any parasitemia altering effect, the occurrence of anti-trypanosome (VSG) antibodies were measured in DTPa-vaccinated and non-vaccinated experimental groups, both before *T. b. brucei* infection and 2 weeks into infection. Data shows that DTPa vaccination itself indeed did not induce any cross-reactive anti-VSG antibodies (Fig. 5b). Hence, an explanation for the improved parasitemia control in the DTPa-vaccinated mice had to coincide with another vaccination-associated

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immune parameter. Interestingly, when plasma IFNγ levels were measured in all experimental groups at the peak stage of infection (T_P), both non-vaccinated mice as well as DTPa-vaccinated mice exhibited increased circulating cytokine levels, with the latter being significantly higher (Fig. 5c). As IFNγ itself has been shown to be a resistance factor in *T. b. brucei* control involving innate TLR/MyD88 signaling [45-47], it appears that in this case, the non-specific immune activation by the DTPa vaccine formulation coincided with improved parasitemia control through an increased Type-1 inflammatory response.

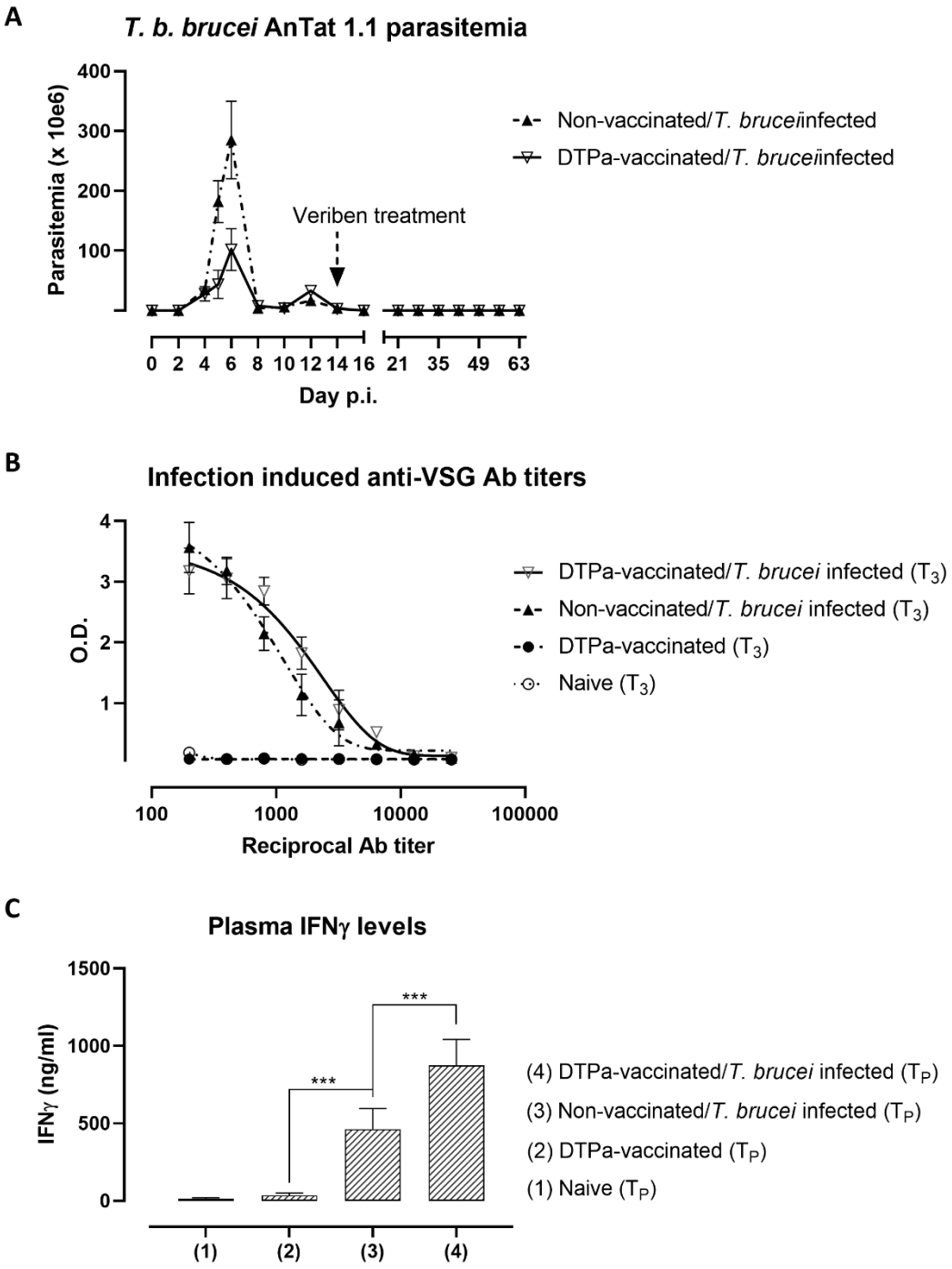

Figure 5: DTPa vaccination results in the temporary improvement of *T. b. brucei* control. (a) Parasitemia in DTPa-vaccinated and non-vaccinated mice was recorded with 2 to 3-day intervals during the first 2 weeks of infection, and one-week intervals after curative drug treatment (63 dpi). All mice were infected through i.p. injection of 5000 *T. b. brucei* AnTat 1.1 trypanosomes. (b) Anti-trypanosome antibody tires were measured in a VSG-specific ELISA format with samples derived from naïve mice, DTPa vaccinated mice, non-vaccinated *T. b. brucei* infected mice and DTPa-vaccinated *T. b. brucei* infected mice. All samples were analyzed at timepoint T_3, using 1:2 plasma dilutions. (c) Plasma INFγ concentrations were determined by ELISA in both non-vaccinated and DTPa-vaccinated mice at 6 dpi (T_P). Values are compared to control plasma INFγ levels in naïve mice and DTPa-vaccinated mice that were not exposed to a *T. b. brucei* AnTat 1.1 challenge. Values are represented as the mean ± SD of 5 individual mice per time point. *** indicates a significance level of $p \leq 0.001$.

4. Discussion

Salivarian trypanosomes cause diseases in humans, livestock, and game animals throughout most of the developing world. Human trypanosomosis is mostly confined to Africa, where it occurs as two distinct diseases being the West- and Central African sleeping sickness caused by *Trypanosome brucei gambiense* and East-African *T. b. rhodesiense* HAT. While for long there has been a call for the development of a vaccine strategy for HAT, recent successes in the control of *T. b. gambiense* infection have shown that persistent surveillance of the human population at risk, combined with dedicated drug treatment and vector control, can bring down the disease burden even without access to a vaccine intervention strategy. In fact, it is expected that by 2030 HAT should no longer be considered as a significant human threat [48]. With *T. b. gambiense* historically being responsible for over 95% of all HAT cases, this has to be considered as a great 'global-effort' success, as it involved many players in a consorted South-North strategy [49]. However, it is important to stress that *T. b. gambiense* is an anthroponotic parasite, placing it in a rather unique context in terms of disease control. In view of this, the control *T. b. rhodesiense* HAT is a completely different problem. This is a zoonotic infection in which the parasite reservoir is not found in the human population, but in livestock and game animals that roam East Africa. In such setting, infection surveillance and targeted treatment becomes a virtually impossible task and vaccination of the commercial animal reservoir is the only long-lasting sustainable solution, preventing the intermediate step in disease transmission from a wildlife reservoir to the human population. Besides this, there are other reasons why anti-trypanosome vaccine development is still on the agenda of many health organizations. First, there are a number of trypanosomes (*T. congolense*, *T. vivax* and *T. evansi*) that cause considerable livestock losses without posing a direct risk for human health [6]. These parasites obviously are the cause of economic hardship, preventing the human development of mainly smallholder farmers. For *T. vivax* and *T. evansi* this includes vast territories in South America and Asia, as these parasites have been able to move out of Africa through non-tsetse mechanical transmission [50]. Treatment of these infections are often done by herd-approach, without proper diagnosis, and with drugs that can cause serious residue issues in consumption products such as milk and meat. For example, ethidium bromide is currently still being used and advertised as anti-trypanosome drug, and is freely available for online purchase as 'injectable solution' for the treatment of animal trypanosomosis. Obviously, this results in the fact that anti-trypanosome treatment itself becomes a direct health hazard for humans. In case of *T. evansi*, there is an additional complication, as this parasite has been reported to cause atypical Human Trypanosomosis trough zoonotic transmission [7]. While human *T. evansi* susceptibility was initially reported to be associated with a mutation-linked absence of the human trypanolytic factor APOL1, subsequent studies showed that also fully APOL1 competent individuals can succumb to *T. evansi* infections [8,9]. Together, these finding show that anti-trypanosome vaccination should still be considered as the ultimate goal in the fight against both animal and human trypanosomosis,

particularly limiting a zoonotic transmissions. Unfortunately, despite many promising laboratory reports, not a single vaccine-based solution has found its way into field application so far [17].

Anti-trypanosome vaccination is hampered by a number of fundamental problems, of which some have only become clear in recent years. In order to understand the difficulties in vaccine development for trypanosomosis, one should take a holistic view at the biological niche, occupied by this parasite: salivarian trypanosomes have evolved to be extracellular free living parasites that dwell throughout the blood and lymph, in plain sight of the adaptive immune system, host antibodies and the complement system. This is where they thrive, this is not a hostile environment for these parasites! This is also a biological niche where trypanosomes encounter virtually no resource competition by other pathogens or microorganism. For decades, it has been known that antigenic variation of the Variant Surface Glycoprotein (VSG) coat plays a key role here as it (i) allows regular escape from high-affinity binding antibodies, by surface exposed epitope alteration [16], (ii) allows the rapidly clearance of surface bound antibodies trough endocytosis [19], and (iii) prevents complement mediated lysis both through its physical barrier function and antigen shedding capacity [25]. However, looking at the fundamental way the adaptive immune system works, it is clear that antigenic variation per se would not allow trypanosomes to 'eternally' outrun the mammalian defense system. Indeed, the main issue any trypanosome would face is the fact that VSG molecules do not just have B cell epitopes that trigger antibody production, but they also have T cell epitopes that are vital in the development of the T cell help needed for B cell activation, differentiation and affinity maturation. Many of these T cell epitopes are conserved [22], meaning that once T cell help has been generated by one early VSG variant, B cell against newly arising variants could be generated at a much higher rate. Secondly, while molecular biology approaches have shown that individual trypanosomes can harbor over thousand different *vsg* genes and pseudogenes [51], it has never been proven that these genes all encode for antigenically distinct antigens that are not being recognized by cross-reacting antibodies. Together this makes that while trypanosomes can most likely use VSG antigenic variation to establish a successful early-stage infection, the system would not provide an efficient defense system for long-term survival inside a given mammalian host. In order to ensure the latter, trypanosomes had to adopt a second layer of defense, i.e. the induction of B cell dysfunction, rendering these cells inefficient in producing truly detrimental antibody responses. This pathology involves the disruption of regular B cell affinity maturation pathways as well as the induction of low-affinity polyclonal antibody responses that result in an antibody 'dilution' response [52]. While most data documenting this has been obtained using experimental mouse infections models, multiple reports of *T. evansi* induced immune suppression show that B cell dysfunction is a real pathology in animal trypanosomosis [37]. For HAT, this problem has been known since long [53] but situation is less well studied, with one thorough study describing the damaging effect of *T. b. gambiense* infections on vaccine-induced anti-measles response [38], and one study describing the problem of HAT-induced non-specific polyclonal B cell activation hampering HIV diagnosis [54]. In an experimental setting, we have previously shown that the human DTPa vaccine loses efficacy during active *T. b. brucei* infection. This observation could be explained by the multiple levels of immune-suppression that is induced during active trypanosomosis [55-58]. In contrast, the study here provides data that shows however that the problem at hand is that trypanosomes permanently destroy host immune compartments. This extends to vaccine induced memory responses that do not recover after anti-trypanosome treatment. The nature of the damage has to be related to the vast destruction of both the bone marrow and peripheral B cell compartments that are induced during infection [15,27,30]. While anti-trypanosome treatment allows a full recovery of these compartments, it appears that the host B cell poesies in this case is reset to a naïve condition, while pre-existing memory that was eradicated by the trypanosome, did not recover. This trypanosomosis-associated

pathology makes perfect sense in the biology of VSG antigenic variation. Indeed, destruction or dysfunction of immunological memory would allow the parasite to escape from the buildup of cross-reactive detrimental antibodies, and even with time the re-use of previously expressed VSGs or nearly identical VSGs. The fact that non-related immune memory (such as the one induced by the DTPa vaccine) is destroyed during infection, has to be considered as collateral damage. Important to mention is that the experimental vaccine setting used here, does not determine whether final immune dysfunction was due to B cell compartment or T cell compartment damage. Both have been previously shown to be important for immunity against *B. pertussis* [59,60], and both have been reported to be affected by trypanosomosis [61]. Interestingly, previous DTPa vaccine data has shown that protective responses coincided with increased IgG1 antibody titers. In line with these findings our results show that breakdown of DTPa induced protection against *B. pertussis* coincided with a very significant drop in anti-pertussis IgG1 plasma titers, from 1/1600 to 1/400 at the timepoint where the intranasal bacterial challenge was administered (T_4 in this setup). The ablation of protection also coincided with the trypanosomosis-induced deregulation of the spleen B cell compartment, and a trypanosome-induced bias towards IgG2a antibody production. This Ig isotype switch coincides with a strong *T. brucei* associated IFNγ response, which further enhances the vaccine associated IFNγ response [62,63]. However, IgG2a pertussis-binding antibodies that are induced during the trypanosomose infection appear to be of low affinity, as binding is rapidly lost with increasing plasma dilutions. Hence, we conclude that the observed IgG2a induction has the typical characteristics of a polyclonal B cell activation response caused by the parasite, in the immunological environment of a pathological trypanosomosis-associated inflammatory Type-1 IFNγ context [64,65], and is unable to confer any detrimental biological activity against *B. pertussis*. Hence, coinciding with an increase in low-affinity anti-pertussis IgG2a antibody plasma titers, a total loss of vaccine-induced protection is observed. Finally, the trypanosomosis-associated IFNγ response is most likely also linked to the immune destructive effect observed at the level of the spleen B cell compartment [66], and could also explain the spleen macrophage/granulocyte expansion observed during trypanosomosis [61].

When considering the future of anti-trypanosome vaccination, one last immunological/infection hurdle that needs to be taken into, is the speed at which the mammalian immune system can trigger an immune memory recall response, before infection-associated B cell destruction sets in. Our data, and those of others, have shown that trypanosomes initiate the destruction of the host B cell compartment within the first week after infection [27-29]. Destruction of T cell functionality further undermines humoral immune activity [67]. This has to be the result of evolutionary pressure, as the latter is a race between parasites that try to undermine the immune system, and the immune system that tries to eliminate the parasites. With trypanosomes being very successful organisms that are able of infecting virtually all mammals, it is clear who has gained the upper hand in this race. Hence, even if a vaccine were to be developed against a non-variable conserved surface exposed trypanosome molecule, such as a nutrient receptor, it remains to be seen whether a protective recall response could be triggered fast enough to stop the emergence of a fist peak of parasitemia after an infectious challenge. Indeed, in this case a successful approach would necessitate the full activation of the antibody production capacity within hours after infection, a requirement that appears unrealistic taken the nature of the B and T cell memory recall responses. However, in a setting of regular pathogen exposure in endemic trypanosome areas, the need for a memory recall response might be replaced by the presence of a continued memory maintenance response. As such, efforts to develop an anti-trypanosome vaccine for the prophylactic protection of livestock animals should be continued, as it will not only protect agriculture economies, but also reduce the risk for zoonotic transmission that could result in the re-emergence of human trypanosomosis [67].

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