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Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor

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High systemic drug toxicity and increasing prevalence of drug resistance hampers efficient treatment of human African trypanosomiasis (HAT). Hence, development of new highly specific trypanocidal drugs is necessary. Normal human serum (NHS) contains apolipoprotein L-I (apoL-I), which lyses African trypanosomes except resistant forms such as *Trypanosoma brucei rhodesiense*[1]. *T. b. rhodesiense* expresses the apoL-I–neutralizing serum resistance–associated (SRA) protein[2], endowing this parasite with the ability to infect humans and cause HAT. A truncated apoL-I (Tr-apoL-I) has been engineered by deleting its SRA-interacting domain, which makes it lytic for *T. b. rhodesiense*[1]. Here, we conjugated Tr-apoL-I with a single-domain antibody (nanobody) that efficiently targets conserved cryptic epitopes of the variant surface glycoprotein (VSG) of trypanosomes[3] to generate a new manmade type of immunotoxin with potential for trypanosomiasis therapy. Treatment with this engineered conjugate resulted in clear curative and alleviating effects on acute and chronic infections of mice with both NHS-resistant and NHS-sensitive trypanosomes.

T. b. rhodesiense is the causative agent of HAT in eastern Africa[4,5]. In endemic foci, large-scale infections of livestock represent a continuous threat for epidemic outbreaks[5–7]. Trypanosomes evade host immune responses by continuously changing the VSGs that cover their entire membrane, which leaves little prospect for a conventional vaccine[8,9]. Thus, the treatment of sleeping sickness relies on therapy. The disease is fatal if left untreated, and the two drugs currently available, suramin and melarsoprol[10], can cause serious adverse events resulting from drug toxicity, relapses, long duration of treatment and increasing drug resistance[11–13]. Therefore, development of new drugs is necessary.

NHS is able to lyse African trypanosomes, except those that cause HAT[14]. Recently, a human-specific serum protein, apoL-I, was identified as the trypanolytic factor of NHS[1]. When isolated from livestock, *T. b. rhodesiense* is sensitive to lysis by NHS[6]. But this parasite becomes resistant to NHS after expression of the SRA protein[2,15,16]. SRA inhibits the trypanolytic activity of apoL-I by interacting with its C-terminal domain[1]. Deleting the SRA-interacting domain of apoL-I results in Tr-apoL-I, which cannot be neutralized by SRA and thus is capable of lysing both NHS-sensitive and NHS-resistant *T. b. rhodesiense*[1].

Tr-apoL-I represents a natural trypanolytic agent to cure *T. b. rhodesiense* infections. But competition with endogenous apoL-I (10 μg/ml in NHS)[17,18], whose association with carrier high-density lipoprotein (HDL) particles allows fast uptake by the parasite[14], might interfere with its delivery to trypanosomes. Therefore, targeting Tr-apoL-I to the parasite surface is required to improve its efficiency. Nanobodies, the single-domain antigen-binding fragments derived from camel heavy-chain antibodies, represent exquisite targeting tools because of their small size (13 kDa) and strict monomeric behavior[19–21]. We identified a nanobody that binds to various VSGs (NbAn33)[3]. NbAn33 specifically recognizes oligomannose, as indicated by its binding to synthetic Man_9 and Man_7 but not Man_3, and by the strong reduction of binding to the AnTat 1.1 VSG treated with alpha-mannosidase (**Fig. 1a**). Accordingly, NbAn33 binds equally well to the MiTat 1.4, MiTat 1.2 and MiTat 1.5 VSGs that represent the three different VSG classes (I, II and III, respectively) and share the conserved N-linked $Man_{5–9}$ carbohydrate[22] (**Fig. 1a**). NbAn33 also binds to the ETat 1.2 VSG expressed in NHS-resistant *T. b. rhodesiense*. Therefore, we used NbAn33 to generate a general VSG-recognizing nanobody–apoL-I construct.

Figure 1b summarizes the design of nanobody–apoL-I constructs tested here. apoL-I contains an N-terminal signal peptide (amino acids 1–27), a C-terminal SRA-interacting domain (amino acids 343–398) and a central lytic domain (amino acids 28–342)[1,23]. In Tr-apoL-I, both signal peptide and C-terminal domain were deleted. A nanobody-encoding region was added, spaced from Tr-apoL-I by a sequence encoding the natural llama γ2c antibody hinge, allowing independent folding of the two protein subunits[24]. NbAn33–Tr-apoL-I was able to specifically recognize trypanosomes and did not bind at a detectable level to fixed blood cells or to non–fixed-cell suspensions of liver, lymph nodes and spleen of infected mice (**Fig. 1c**).

NbAn33–Tr-apoL-I exhibited a dose-dependent trypanolytic activity with a dose of 10 μg/ml (180 nM), lysing 100% of the parasites (that is, 10^6; stumpy and slender forms) within 4 h of incubation (**Fig. 2a**). Under these conditions, replacement of NbAn33–Tr-apoL-I

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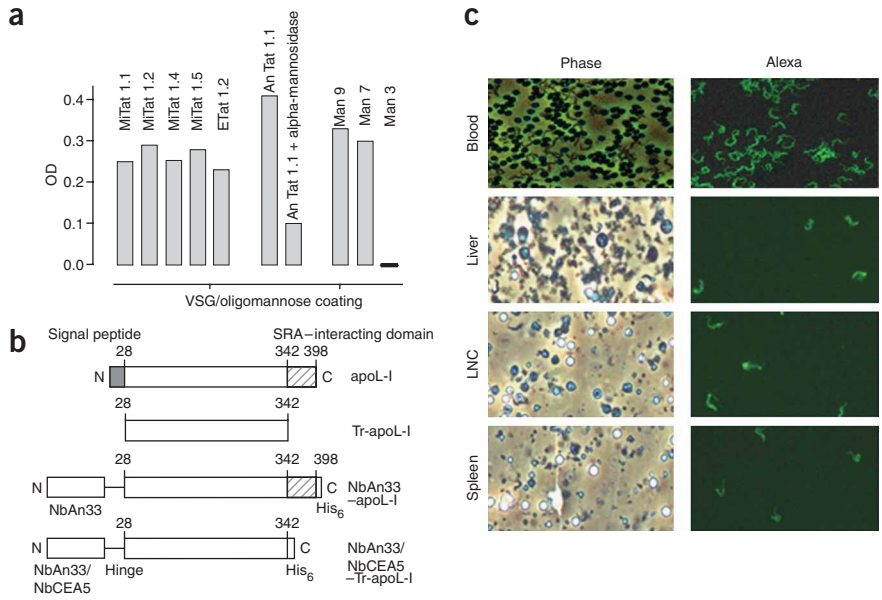

Figure 1 Targeting modules for the trypanosome surface. (**a**) Targeting specificity of NbAn33, as determined by ELISA using NbAn33. (**b**) Constructs used in this study. Chimeras contain a six-histidine (His_6) tag at the carboxy terminal for purification purposes. (**c**) Staining of *T. b. brucei* AnTat 1.1 in blood smears or various cell suspensions from an infected mouse. The samples were incubated with Alexa 488–labeled NbAn33–Tr-apoL-I and illuminated with visible (Phase) or ultraviolet (Alexa) light. LNC, lymph nodes.

with a 20-fold molar excess of NbAn33 showed no lytic activity, whereas 50% NHS (approximately 4 μg/ml apoL-I) lysed 70% of the parasites. To evaluate the contribution of the targeting module to trypanolysis, we substituted NbAn33 with NbCEA5 (ref. 24), a nanobody recognizing an irrelevant antigen. No trypanolysis was observed with 10 μg NbCEA5–Tr-apoL-I/ml (**Fig. 2b**), showing the crucial role of NbAn33. Accordingly, the lytic activity of NbAn33–Tr-apoL-I was completely inhibited after preincubation with soluble VSG (**Fig. 2b**). NbAn33–Tr-apoL-I was also highly trypanolytic for NHS-resistant *T. b. rhodesiense* ETat 1.2R (**Fig. 2b**). This required truncation of the SRA-interacting domain, because the full nanobody–apoL-I conjugate (NbAn33–apoL-I) was only lytic for ETat 1.2S (**Fig. 2b**). In accordance with the oligomannose specificity of NbAn33, all trypanosome variants used for the nanobody-VSG binding assay were lysed by NbAn33–Tr-apoL-I but not by NbCEA5–Tr-apoL-I (**Fig. 2c**). Thus, NbAn33–Tr-apoL-I efficiently lysed both NHS-sensitive and NHS-resistant trypanosomes *in vitro*.

We tested the performance of NbAn33–Tr-apoL-I in mouse models of HAT. Upon detection of virulent NHS-resistant, and as such potentially human pathogenic, *T. b. rhodesiense* ETat 1.2R parasites in blood (day 3), we treated mice with a single intraperitoneal inoculation of 10–100 μg NbAn33–Tr-apoL-I or NbCEA5–Tr-apoL-I per mouse. Treatment with a single dose of 20 μg NbAn33–Tr-apoL-I (**Fig. 3a**,**b**) or higher (data not shown) resulted in complete parasite clearance, no obvious adverse symptoms for mice and long-term survival (60 d when experiments were terminated). Mice treated with a suboptimal dose of 10 μg/mouse were partially protected, as evidenced by a delayed parasitemia and longer median survival (9 d) as compared to control NHS-treated mice (6 d). The intravenous injection of 5 μg conjugate achieved the same effects as 20 μg injected intraperitoneally. Only marginal protection was obtained after intraperitoneal injection of a high dose (100 μg/mouse) of nontargeting NbCEA5–Tr-apoL-I (**Fig. 3a**,**b**) or recombinant apoL-I, and approximately 1 mg/mouse of apoL-I was required to abrogate parasitemia (data not shown).

The NbAn33–Tr-apoL-I treatment was also assessed in a more chronic *T. b. brucei* pleomorphic AnTat 1.1 infection. Here, parasitemia was cleared completely with a single dose of 20 μg NbAn33–Tr-apoL-I injected intraperitoneally (**Fig. 3c**) and mice survived until the experiment was terminated (150 d, **Fig. 3d**). Mice treated with a subcurative dose of 10 μg showed reduced first peak of parasitemia and prolonged survival, but succumbed to late-stage parasitemia (median survival of 61 d). Control mice, treated with phosphate-buffered saline (PBS) or 100 μg nontargeting NbCEA5–Tr-apoL-I, died from parasitemia with a median survival of 30 and 36 d, respectively.

Finally, we evaluated the NbAn33–Tr-apoL-I treatment during the chronic phase of infection. Just before the onset of the second wave of parasitemia with *T. b. brucei* AnTat 1.1 (day 12), we injected mice intraperitoneally twice (2-d interval between injections) with 20 μg NbAn33–Tr-apoL-I. Parasites were promptly cleared and treated mice remained free of parasite for 50 d (**Fig. 4a**). Between days 55 and 65 of infection, however, parasites reappeared in the blood, and mice succumbed to infection with a median survival of 70 d (**Fig. 4b**). Mice treated with 20 μg nontargeted NbCEA5–Tr-apoL-I showed unaltered parasitemia and similar median survival

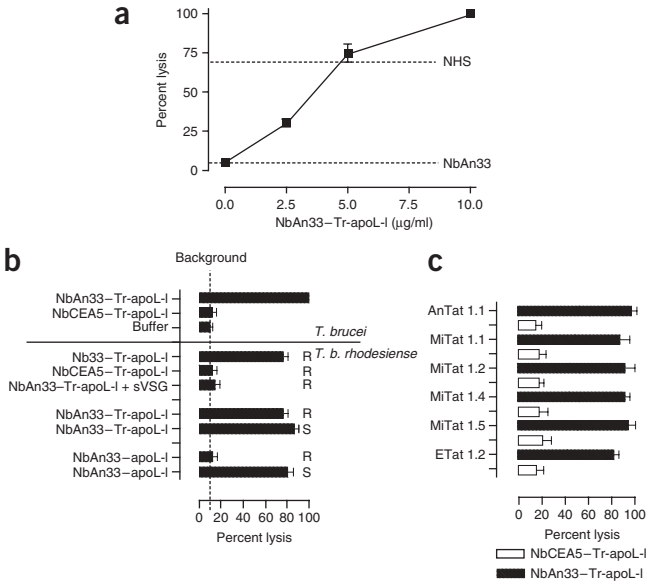

Figure 2 *In vitro* trypanolysis. (**a**) Concentration-dependent lytic activity on *T. b. brucei* AnTat 1.1 after a 4-h incubation (dotted lines, lysis by 50% NHS or 20-fold molar excess of NbAn33). (**b**) Trypanolytic activity of different chimeras (10 μg/ml) on 10^6 *T. b. brucei* AnTat 1.1 and *T. b. rhodesiense* ETat 1.2S (S) and ETat 1.2R (R; 4-h incubation). (**c**) Trypanolytic activity of NbAn33–Tr-apoL-I and a nontargeted control chimera (10 μg/ml, 4-h incubation) on trypanosomes expressing unrelated VSGs that share the conserved Man_{5-9} carbohydrate[22].

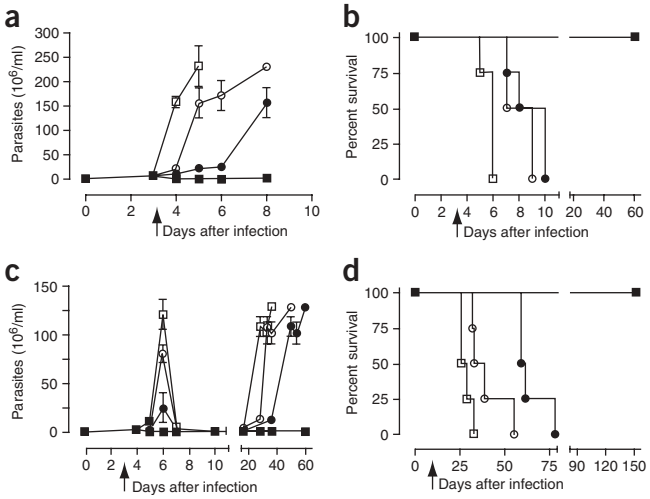

Figure 3 Therapeutic effects of NbAn33–Tr-apoL-I in acute infection. (**a**,**c**) Parasitemia and (**b**,**d**) survival of mice infected with *T. b. rhodesiense* ETat 1.2 R (**a**,**b**) or *T. b. brucei* AnTat 1.1 (**c**,**d**) and treated with 20 μg (filled squares) or 10 μg (filled circles) NbAn33–Tr-apoL-I, or 100 μg NbCEA5–Tr-apoL-I (open circles), with NHS (**a**,**b**; open squares) or PBS (**c**,**d**; open squares) at 3 d after infection (arrow).

time (30 d) as PBS-treated mice. To trace the origin of the re-emerging infection in the NbAn33–Tr-apoL-I–treated mice, the post-treatment presence of mRNA encoding VSGs was checked in lungs, liver, spleen, lymph nodes, peritoneal exudate cells (PECs), thymus, kidney, eyes, ovaries and brain. VSG-encoding RNA was detected only in liver samples taken 4 weeks after treatment (**Fig. 4c**), indicating that parasites might first re-emerge in the liver.

During chronic trypanosome infection, systemic inflammation is associated with anemia, severe weight loss, splenomegaly, reduced locomotor activity and increased level of serum aspartate transaminase (AST)[25–28]. Treatment with 20 μg NbAn33–Tr-apoL-I at the onset of the second peak of parasitemia rescued infection-associated anemia, and the level of red blood cells remained normal until parasites reappeared (**Fig. 4d**). This treatment also abolished other infection-associated pathological features (**Fig. 4e**). Hence, even though a treatment of 20 μg/mouse was insufficient to completely cure a chronic infection, it was beneficial in alleviating several infection-associated pathologies.

Administering NbAn33–Tr-apoL-I (20 μg/mouse) when parasites reappeared in the bloodstream (day 60) of mice already treated at the onset of the second parasitemia wave had no effect on parasitemia or mice survival. The parasites from the final parasitemia had undergone antigenic variation, as they were not expressing the original AnTat 1.1 VSG (**Supplementary Fig. 1** online). Although variants lacking the high-mannose NbAn33 target exist[22], it is unlikely that in this case parasites switched to such VSG expression, as trypanosomes purified from treated or infected mice were found to be equally susceptible to lysis by NbAn33–Tr-apoL-I *in vitro* and *in vivo* (**Supplementary Fig. 1**). Induction of the host humoral response is more likely to be responsible, as sera from mice treated twice with the chimera (50 μg/mouse intraperitoneally on alternate days and boosted after 10 d) reacted strongly with apoL-I (ELISA signal, $OD_{405} > 0.65$ at 1:500 dilution) and neutralized its activity. These sera did not react with NbAn33, confirming its low immunogenicity in mice[29]. An even lower immunogenicity is expected in humans, given the higher sequence identity between nanobodies and human variable immunoglobulin domains[30]. Moreover, as apoL-I is a human self-antigen, treatment of humans with NbAn33–Tr-apoL-I should not induce a humoral response against this moiety.

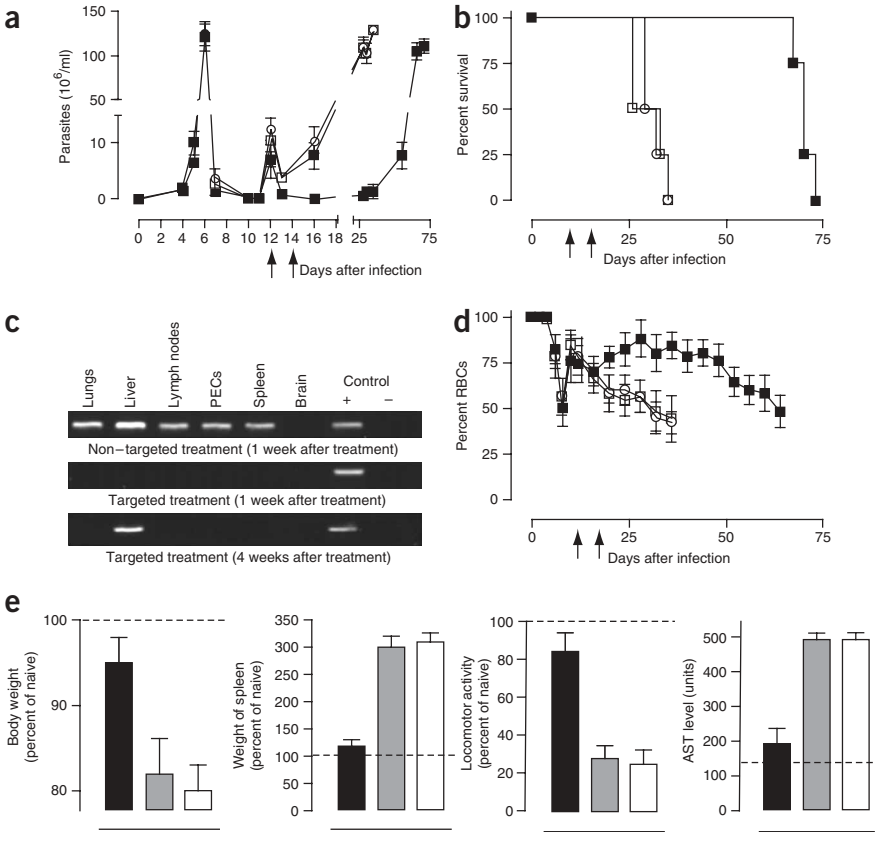

Figure 4 Therapeutic effects of NbAn33–Tr-apoL-I treatment at the second peak of parasitemia. (**a**) Parasitemia and (**b**) survival of mice infected with *T. b. brucei* AnTat 1.1. Treatment with NbAn33–Tr-apoL-I (filled squares), NbCEA5–Tr-apoL-I (open circles) or PBS (open squares). (**c**) Detection of *T. brucei* VSG RNA in various organs at different time points after treatment with NbAn33–Tr-apoL-I (targeted treatment) or NbCEA5–Tr-apoL-I (nontargeted treatment) at the second peak of parasitemia. Results from 2 weeks and 1 week after treatment were indistinguishable (data not shown). (**d**) Anemia expressed as percent of red blood cells (RBCs) in *T. b. brucei*–infected mice treated with NbAn33–Tr-apoL-I (filled squares), NbCEA5–Tr-apoL-I (open circles) or PBS (open squares) at days 12 and 14 after infection (arrows). (**e**) Pathological features associated with trypanosome infection in mice (25 d after infection). In each panel, the black, gray and white bars represent values for mice treated with NbAn33–Tr-apoL-I, NbCEA5–Tr-apoL-I and PBS, respectively. In all cases treatments were performed on days 12 and 14 after infection (dotted line, naive mice).

In conclusion, we have developed a new trypanocidal modality amenable to treatment of HAT, either alone or in combination with less-curative doses of other trypanocidal drugs. Further developments are possible, as substitution of NbAn33 with NbES31, a nanobody directed against the ESAG6 subunit of the *T. brucei* transferrin receptor, was equally potent in eliminating the parasite infection in mice (T.N.B., K.C. & E.P., unpublished data). Finally, we anticipate that this modality will be amenable to treatment of other infections, depending on the availability of specific targeting nanobodies and a host-derived natural defense molecule.

METHODS

Solid-phase binding ELISA. We coated 96-well plates with different VSGs (1 μg/ml in 0.1 M $NaHCO_3$, pH 8.2) overnight (4 °C), and performed ELISA as previously described[3]. We removed mannose from AnTat1.1 VSG by incubating 100 μg VSG for 18 h at 25 °C with 10 units of alpha-mannosidase (Sigma) followed by enzyme inactivation at 65 °C for 10 min and gel filtration. We coated synthetic oligomannoses (Oxford Glycoscience) on peptide immobilizer plates (Sanvertech), and then performed ELISA[3].

Cloning and expression of nanobody–apoL-I. The truncated *APOL1* gene was amplified by PCR from a plasmid containing the *APOL1* gene[1] as *Not*I-*Xho*I fragment with the following primer set (*Not*I-*Xho*I restriction sites are underlined): Apo42F, 5′-ATAAGAATGCGGCCGCAGAGGAAGCTGGAGCGAGGGT-3′; Apo-TrR, 5′-ATCCGCTCGAGGAAGCTTACAGGGGCCACAT-3′. The amplified truncated *APOL1* fragment was cloned in *Not*I-*Xho*I–digested pET-21d (+) (Novagen). The NbAn33–llama γ2c hinge was amplified as *Bam*HI-*Not*I fragment with the following primer set (*Bam*HI-*Not*I sites are underlined): nanobody F, 5′-ATCCGGGATCCCAGATGTGCAGCTGGTGGACTCT-3′; nanobody–MH-R, 5′-ATAAGAATGCGGCCGCGGGAGCTTTGGGAGCTTTGGAGCTGGGGTCTTCGCTGTGGTGCGCTGAGGAGACGGTGACCTGGGT-3′. The reverse primer includes the nucleotide sequence of the 15-mer llama γ2c hinge, coding for the amino acid sequence AHHSEDPSSKAPKAP. The amplified fragment was cloned in pET-21d (+) containing truncated *APOL1*. The construct was transfected into BL21 *Escherichia coli*. The NbCEA5 conjugated to truncated *APOL1* was engineered likewise. As additional control, full *APOL1* was amplified as *Not*I-*Xho*I fragment with the following primers set: Apo42F, see above; ApoR, 5′-ATCCGCTCGAGCAGTTCTTGGTCCGCCTGCA-3′. All recombinant proteins were expressed as cytosolic His-tagged products. After lysis of the transformed BL21 cells, the recombinant fusion proteins were purified using a Ni-NTA Superflow column (Qiagen). Further purification was performed by gel filtration by high-performance liquid chromatography using a Superdex 75 (10/30) column (Pharmacia, Akta Explorer 10S) equilibrated with PBS. The proteins were checked in 10% SDS-PAGE.

Parasite binding of NbAn33–Tr-apoL-I. We labeled NbAn33–Tr-apoL-I with Alexa 488 (Invitrogen). During parasitemia (*T. b. brucei* AnTat 1.1), we air-dried thin blood smears and fixed them with methanol. After washing, we flooded slides with Alexa-labeled NbAn33–Tr-apoL-I for 10 min and washed them twice. We homogenized spleen, lymph nodes and liver of infected mice and filtered cell suspensions through a cotton sieve. We incubated these cell suspensions with Alexa-labeled NbAn33–Tr-apoL-I for 30 min and washed them twice before mounting them on microscopic slides. The slides were visualized with visible as well as ultraviolet light for immunofluoroscence microscopy (Nikon ECLIPSE E600 with phase contrast).

***In vitro* trypanolysis assays.** Parasites used for this assay included *T. b. brucei* AnTat 1.1 (Institute of Tropical Medicine, Belgium), MiTat 1.1, MiTat 1.2, Mitat 1.4 and MiTat1.5 (a gift from M.A.J. Ferguson, University of Dundee) and *T. b. rhodesiense* ETat 1.2 S and ETat 1.2 R (Institute of Tropical Medicine, Belgium). We expanded and purified parasites as previously described[3]. We incubated purified parasites (10^6 parasites/ml phosphate saline-glucose buffer with 5% FCS) with different chimeric proteins at 37 °C.

***In vivo* therapy experiments.** Mouse care and experimental procedures were performed under approval from the Ethical Committee of the Vrije Universiteit Brussel. We intraperitoneally infected C57BL/6 mice with 5,000 parasites. Once we detected parasites in blood by microscopy, mice received different doses (from 10 to100 μg/mouse, four mice per group) of NbAn33–Tr-apoL-I. Control mice were either left untreated or received NbCEA5–Tr-apoL-I or NbAn33–apoL-I. After treatment, we followed parasitemia microscopically every other day and recorded the survival of mice. To ensure the NHS resistance of *T. b. rhodesiense* ETat 1.2 R, we infected mice with 500 μl NHS and subsequently infected them with 5,000 parasites in 100 μl PBS. For the treatment at the second peak of parasitemia, mice infected with *T. b. brucei* (AnTat 1.1) were left untreated on the first peak of parasitemia. When we detected the second wave of parasites microscopically, mice received intraperitoneal injections of NbAn33–Tr-apoL-I (20 μg/mouse), twice on alternate days. Control mice were either left untreated or received NbCEA5–Tr-apoL-I. We treated cohorts of four to five mice in parallel for each experimental setting and repeated these experiments at least three times.

Detection of parasites by PCR. We prepared cell suspensions of various organs at different time points after treatment on second peak of parasitemia. We extracted total RNA from about 10^7 cells using the Trizol reagent (Invitrogen). We used oligo$(dT)_{12–18}$ primers (Gibco BRL) to synthesize cDNA and performed PCR on mRNA encoding VSG with universal VSG primers (mini-exon, 5′-GCTATTATTAGAACAGTTTC-3′; conserved 3′-terminal 16-mer, 5′-GTGTTAAAATATATCA-3′) and checked the amplicon in 1.5% agarose gel.

Assessment of pathological parameters. After treatment, we counted total red blood cells every other day. We recorded the body weight, weight of spleen, locomotor activity and serum AST levels of infected mice at 25 d after infection for treated and untreated mice. We measured locomotor activity as the total time spent per hour by mice on running in their cage, eating, drinking and cleaning their fur and nest. The AST levels were measured from individual mouse serum by a commercial kit (Boehringer Mannheim).

Accession codes. GenBank accession codes: *Camelus dromedarius* anti-VSG immunoglobulin heavy chain variable domain cAbAn33 mRNA, AY263490; llama γ2c hinge, AX800153.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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