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Research paper

## Parallel selection of multiple anti-infectome Nanobodies without access to purified antigens

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### Abstract

A strategy was developed to isolate Nanobodies, camelid-derived single-domain antibody fragments, against the parasite infectome without *a priori* knowledge of the antigens nor having access to the purified antigens. From a dromedary, infected with *T. evansi*, we cloned a pool of Nanobodies and selected after phage display 16 different Nanobodies specific for a single antigen, *i.e.* variant surface glycoprotein of *T. evansi*. Moreover 14 Nanobodies were isolated by panning on different total parasite lysates. Thus, this anti-infectome experiment generated Nanobodies, monospecific for one *Trypanosoma* species, whereas others were pan-reactive to various *Trypanosoma* species. Several Nanobodies could label specifically the coat of a set of *Trypanozoon* species. The recognized target(s) are present in GPI-linked membrane fractions of bloodstream- and fly-form parasites. Due to the omnipresence of these targets on different parasite species and forms, these antibody fragments are a valuable source for validation of novel, not yet identified targets to design new diagnostics and therapeutics.

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### 1. Introduction

The functional analysis of proteomes necessitates highly specific markers that recognize the native proteins (Bertone and Snyder, 2005; Silacci et al.,

2005). Antibodies are considered to be the first choice for such molecular recognition units (De Masi et al., 2005). To keep up with the vast number of targets envisaged for functional analysis, high-throughput techniques should be introduced to arrive at a representative set of reporter molecules against a (subset) of the proteome (Huang et al., 2002). However, (i) the availability of the target proteins and (ii) possessing over diverse libraries from which to retrieve binders are even more important. Phage- or ribosome display of large non-immune, *i.e.* synthetic or naïve, antibody libraries allows the screening of up to 10<sup>12</sup>

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different clones with very low consumption of the selected target to retrieve binders (Smith and Petrenko, 1997; Schaffitzel et al., 1999; Azzazy and Highsmith, 2002). In this approach the target should be available in a purified form either from a recombinant source or extracted from the proteome. It is clear that a direct screening of antibody libraries against an antigen mixture or a complete proteome to identify a maximum number of antigen-specific binders without an absolute need for antigen purification would constitute a serious improvement (Wingren et al., 2005). A second bottleneck comes from the weak affinity/specificity of the selected reporter molecules. Although the combination of large libraries and display techniques should theoretically yield antibodies of high affinity (in the nanomolar range) and high specificity towards the selected target, these are not routinely retrieved (Nakayama et al., 2001; Azzazy and Highsmith, 2002). Improving the antigen-binding property of a primary selected antibody is a very laborious task and the outcome remains unpredictable. Therefore, other approaches to directly isolate antibodies of high specificity and affinity, against multiple targets would be very welcome in the field of functional proteomics.

A possible way to improve on the low affinity of retrieved antibodies would consist in the employment of immune libraries. The immunization with a proteome or a complex mixture of antigens is expected to generate antibodies in the (sub-) nanomolar range against multiple antigens (Sakakibara et al., 2005). The combination of immunization and subtractive panning procedures were successfully employed to obtain antibodies towards specific targets of the proteome (Zampieri et al., 2003; Zijlstra et al., 2003; Hof et al., 2005). Different antibodies in a scFv or Fab format could be isolated from patients with auto-immune diseases (Farnaes and Ditzel, 2003; Zampieri et al., 2003), cancers (Rothe et al., 2004), or infections (Reiche et al., 2002; Kausmally et al., 2004; Ludwig et al., 2004). Although the method seems to work (Tur et al., 2003; Kramer et al., 2005), the scFv or Fab library construction is rather complex as the original VH–VL combined domains, matured as a pair during the immunization, are scrambled during the cloning procedure. In this respect, the retrieval of single-domain antibody fragments (VHH or Nanobody) from camelids immunized with a total proteome from a particular cell population or from a cell or tissue extract should facilitate the protocol. Cloning the repertoire of antigen-binding fragments from camelids in a phage display vector has an important advantage, since the binding repertoire of the unique Heavy-chain antibodies in these

animals is encoded by only a single exon in contrast to two exons for antibodies of other mammals. Consequently, for an immune VHH library, a size of  $10^6$  is already sufficient for the identification of antigen-specific single-domain binders (Muyldermans, 2001). Moreover, the camelid immune system routinely generates *in vivo* matured antibodies of (sub-) nanomolar affinity (Lauwereys et al., 1998; Conrath et al., 2001; Saerens et al., 2004). The superior intrinsic properties, *e.g.* higher *in vitro* stability (Dumoulin et al., 2002), higher solubility (Muyldermans, 2001), and their ability to target unique epitopes that differ from epitopes recognized by VH–VL pairs (Lauwereys et al., 1998; Ledebuer et al., 2002; De Genst et al., 2006), make Nanobodies potent entities in functional proteomics. Especially the latter feature is advantageous *e.g.* when differentiation between isoforms (Saerens et al., 2004) or recognition of cryptic epitopes (Stijlemans et al., 2004) of the native protein is required.

In this work, we envisaged the identification of single-domain antibodies against a *Trypanosoma* infectome in dromedary. The *Trypanosoma evansi* parasites cause trypanosomiasis or Surra, a major health and economic problem in developing countries (Wernery and Kaaden, 2002). An infection with a species within the *Trypanozoon* subgenus comprising *T. brucei*, *T. evansi* and *T. equiperdum* was chosen as model since there is a definite need for better diagnostic and therapeutic tools to combat these infectious agents (Hutchinson et al., 2004; Chretien and Smoak, 2005). The major problem consists of the antigenic variation strategy adopted by the parasite (Donelson, 2003; Pays et al., 2004; Dubois et al., 2005). This immune evasion strategy allows for the prolonged survival of the parasite in the host and is mediated by the variant surface glycoprotein (VSG) on the outer membrane of the parasite (Pays et al., 2004; Dubois et al., 2005). Although the antibody immune response mounted by the host is not protective against the infection, functional information about the interplay between parasite and host might be gained from its analysis (Mitchell and Pearson, 1986). Since the infectious agent or pathogen triggers the antibody generation, these antibodies might react with clinically significant parasite antigens, *e.g.* for identification of antigenic epitopes involved in the humoral response (Azzazy and Highsmith, 2002). For diagnostic and therapeutic purposes, it is essential to possess over both *Trypanosoma* species-specific antibodies (*e.g.* VSG-specific) and antibodies pan-reactive to multiple *Trypanosoma* species. Both these types of antibodies were isolated in a single-domain format from lymphocytes of a dromedary infected with *T. evansi*.

## 2. Materials and methods

### 2.1. Infection of a dromedary

One dromedary (*Camelus dromedarius*) kept at the Central Veterinary Research Laboratory (Dubai, U.A.E.) was infected with *T. evansi* (*T. CVRL*, approximately  $3 \times 10^6$  live parasites), isolated from a camel in the Emirate of Dubai and passed three times in white laboratory mice. Three months after infection of the dromedary, 50 ml of anti-coagulated blood was collected, from which plasma and peripheral blood lymphocytes were isolated (WAK Chemie).

### 2.2. Purification of *Trypanosoma antigens*

Male C57B1/6 mice between six to eight weeks old were injected with different *Trypanosoma* species (Table 1). Blood was collected five to seven days post-injection and the parasites were isolated from the blood on DEAE-52 resin (Lanham and Godfrey, 1970). Different kinds of antigen fractions were extracted from the parasites: (a) the total parasite lysate, (b) the glycosyl-phosphatidyl-inositol (GPI)-anchored membrane protein fraction, and finally (c) the soluble variant surface glycoprotein (sVSG).

(a) For the total parasite lysate,  $10^9$  isolated parasites were resuspended in 1 ml PBS and sonicated 3 times for 30 s (Stijlemans et al., 2004). The destruction of parasites was confirmed microscopically. The lysed parasites were centrifuged at 14,000 RPM, 4 °C for 30 min and the soluble parasite lysate was obtained from the supernatant.

(b) For the GPI-anchored membrane protein fraction, a combination of previously described protocols (Cross, 1984; Schell and Overath, 1990) was followed and described in full detail by Stijlemans et al. (2007). Briefly,  $3 \times 10^9$  parasites were resuspended in (8 ml) ice cold PBS and protease inhibitor solution (2 mM TLCK, 1 tablet complete-protease inhibitor from Roche supplemented with 20 mM *p*-chloro-mercurybenzenesulphonic acid (PCMBS, Sigma) as inhibitor of the PLC), and incubated for 30 min on ice followed by a 3 times incubation exchange from liquid nitrogen to 37 °C. Parasites were pelleted and resuspended in solution A (100 mM Hepes, NaOH pH 6.9, 10 mM PCMBS (Sigma), 1 mM TLCK, 0.1 mM PMSF). The soluble part was removed by ultracentrifugation (Centrikon T-2070 rotor, BRS, Belgium) (100,000 g, 30 min, 4 °C) and the pellet was resuspended in solution A containing 2% *n*-octylglucopyranoside (Roche) for 45 min on ice. Following a final ultracentrifugation step (100,000 g, 60 min, 4 °C) the GPI-linked membrane fraction was stored at –20 °C.

(c) For the sVSG preparation, an additional fractionation of the GPI-linked membrane protein fraction was performed by anion exchange chromatography in 100 mM NaCl solution (Cross, 1975). The purity of the sVSG containing fractions was checked by SDS-PAGE and Coomassie staining or on Western blot with a rabbit anti-VSG polyclonal antiserum. All antigens were stored at –20 °C until further use.

### 2.3. Fractionation of IgG subclasses

Separation of the different plasma dromedary IgG subclasses was performed by differential adsorption on Hitrap-protein A and Hitrap-protein G columns (Amersham Biosciences) as described previously (Hamers-Casterman et al., 1993).

### 2.4. Library construction

The mRNA was extracted from the peripheral blood lymphocytes with Quickprep™ micro mRNA purification kit (Amersham Biosciences). In a subsequent step the cDNA was prepared using Ready-to-Go You-prime-first-strand-beads (Amersham Biosciences). The gene fragments encoding the variable domain until the CH2 domain were amplified, with the specific primers CALL001 (5'-GTC CTG GCT CTC TTC TAC AAG G-3') and CALL002 (5'-GGT ACG TGC TGT TGA ACT GTT CC-3'), annealing at the leader sequence and within the CH2 exon of the H-chains of all dromedary

Table 1  
Origin of *Trypanosoma*'s

<i>Trypanosoma</i>	Origin
<i>T. evansi</i> ( <i>T. CVRL</i> )	Isolated from a camel and used for infection of a dromedary (Dubai, UAE)
<i>T. evansi</i> Kenya	Kenya
<i>T. evansi</i> ITMAS	Camel from Kenya
<i>T. evansi</i> KETRI	Kenya
<i>T. congolense</i> Tc13	Provided by Dr. H. Tabel, Canada
<i>T. b. brucei</i> AnTat 1.1	Provided by Institute of Tropical Medicine Antwerp (Belgium)
<i>T. b. brucei</i> MiTat 1.2	Variant of 427 <i>T. b. brucei</i>
<i>T. b. brucei</i> MiTat 1.5	Variant of 427 <i>T. b. brucei</i>
<i>T. b. rhodesiense</i> ETat 1.2	Uganda
<i>T. b. gambiense</i> FEO	Provided by Prof. Vincendeau, France
<i>T. b. gambiense</i> ABBA	Provided by Prof. E. Pays, Belgium
<i>T. vivax</i> ILRAD-700	Provided by Institute of Tropical Medicine Antwerp (Belgium)

IgGs, respectively. The 600 bp fragment (VHH–CH2 without CH1 exon representative for all H-genes of Heavy-chain antibodies) was eluted from a 1% agarose gel after separation from the 900 bp fragment (VH–CH1–CH2 exons, representative for the H-genes of classical antibodies). Since all VHHs belong to the same gene family (the homologue of family III in human), they are amplified with one additional PCR with nested primers A4short (5'-CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGC-3') and 38 (5'-GGA CTA GTG CGG CCG CTG GAG ACG GTG ACC TGG GT-3') annealing at the framework-1 and framework-4 regions, respectively. The final PCR fragments were ligated into the phagemid vector pHEN4 (Arbabi Ghahroudi et al., 1997), after cutting with the restriction enzymes *NcoI* and *NotI*. Ligated material was transformed in freshly prepared *E. coli* TG1 cells and plated on LB plates with ampicillin. The colonies were scraped from the plates, washed and stored at  $-80^{\circ}\text{C}$  in LB-medium supplemented with glycerol (50% final concentration).

### 2.5. Selection of specific antibody fragments

The Nanobody library was expressed on phage after super-infection with M13K07 helper phages. Specific Nanobodies (attached on virions) were enriched by several consecutive rounds of *in vitro* selection on different antigen preparations coated either on wells of microtiter plates or on immunotubes (Nunc). Bound phage particles were eluted with 100 mM Triethylamine (pH 11.0). The eluted particles were immediately neutralized with 1 M Tris–HCl (pH 7.4) and used to infect exponentially growing *E. coli* TG1 cells. The enrichment of phage particles carrying the antigen-specific Nanobodies was assessed by comparing the number of phages eluted from wells with captured versus non-captured antigen (Parnley and Smith, 1988). Individual colonies were picked and expression of recombinant phage particles or soluble periplasmic Nanobody was performed by super-infection with M13K07 or addition of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), respectively. The supernatant or periplasmic extract was tested in ELISA for antigen recognition by the Nanobody attached to phage particles or soluble Nanobody, respectively.

### 2.6. Expression and purification of antibody fragments

The VHH genes of the clones that scored positive in ELISA were recloned into the expression vector pHEN6 (Conrath et al., 2001), using the restriction enzymes *NcoI* and *BstEII*. The plasmid constructs were

transformed into *E. coli* WK6 (su $-$ ) cells. Expression in the periplasm and purification of Nanobody was performed as described previously (Conrath et al., 2001).

### 2.7. Solid-phase ELISA

Maxisorb 96-well plates (Nunc) were coated overnight at  $4^{\circ}\text{C}$  with different antigen preparations at  $1\text{--}10\ \mu\text{g ml}^{-1}$  in PBS. Residual protein binding sites in the wells were blocked for two hours at room temperature with 1% casein or 1% milk in PBS. Serial dilutions of purified IgG subclasses, virions from different rounds of panning or Nanobodies were added to the wells. Detection of antigen-bound IgG, M13 virions, or Nanobody was performed with a rabbit anti-dromedary-IgG antiserum, a horse-radish peroxidase-anti-M13 conjugate (Amersham Biosciences), a mouse anti-haemagglutinin-decapeptide-tag (BAbCO) or a mouse anti-histidine-tag (Serotec), as appropriate. Subsequent detection of the rabbit antiserum or the mouse anti-tag antibodies was done with an alkaline phosphatase anti-rabbit-IgG or anti-mouse-IgG conjugate (Sigma), respectively. The absorption at 405 nm was measured 15 min after adding the enzyme substrate *p*-nitrophenyl phosphate or 2, 2'-azino-bis (3-ethylbenzathiazoline-6-sulfonic acid) for phosphatase or peroxidase conjugates, respectively.

### 2.8. Flow cytometry analysis

Purified Nanobodies were labeled with Alexa Fluor 488 according to the manufacturer's instructions (Molecular Probes). The bloodstream form of the *Trypanosoma* was used in FACS. Either the parasites were fixed with a PBS solution (containing 3% formaldehyde and 1% glutaraldehyde) before (fixed parasites) or after (living parasites) addition of labeled Nanobodies. Aliquots of  $2 \times 10^6$  purified parasites were incubated with  $5\ \mu\text{g}$  labeled Nanobody. After 30 min parasites were pelleted, the pellet was resuspended in  $500\ \mu\text{l}$  PBS and pelleted again to remove unbound Nanobody. After five of these washes the parasites were subsequently analyzed in FACSvantage Fluorescence-Activated Cell Sorter (Becton Dickinson, San Jose, CA).

## 3. Results

### 3.1. Antibody response towards soluble VSG (sVSG)

A dromedary was infected with *T. evansi*, isolated from a camel and passed three times in white laboratory

mice (*T. CVRL*). About three months after infection blood was taken from the infected dromedary and the conventional (IgG1) and Heavy-chain (IgG2, IgG3) antibodies were fractionated from the plasma by differential absorption on protein A and G (Hamers-Casterman et al., 1993). Since the host is primarily confronted with a huge amount of VSG molecules exposed on –, or released from the parasites' surface (Dubois et al., 2005), these fractionated IgGs were tested on several sVSG proteins from different *Trypanosomas*. The sVSG proteins, prepared from either *T. evansi* Kenya or *T. b. brucei* AnTat 1.1, were recognized in solid-phase ELISA by Heavy-chain and conventional antibodies (Fig. 1). This indicates that an immune response to the sVSG antigens is raised in the dromedary classical — and Heavy-chain antibodies. Furthermore, for all three IgG fractions, the signal to sVSG from *T. evansi* Kenya was higher than that to the sVSG from *T. b. brucei* AnTat 1.1.

### 3.2. Nanobody library construction

A Nanobody library starting from  $1.5 \times 10^7$  lymphocytes was cloned in a phage display vector according to Saerens et al. (2004). Based on test ligations of vector to PCR fragment, a molar ratio of one to three appeared optimal for construction of the library. Approximately 3  $\mu$ g of the ligated vector were transformed in *E. coli* cells (TG1) to obtain a library of  $10^7$  individual transformants. As determined by colony PCR, 75% of the clones in this library contained an insert with a size of a VHH gene.

### 3.3. Selection and characterization of sVSG-specific Nanobodies

The isolation of sVSG-specific Nanobodies was performed by four consecutive rounds of *in vitro* selection on solid-phase coated *T. evansi* Kenya sVSG. Specific enrichment to *T. evansi* Kenya sVSG was observed starting from the third round of selection (results not shown). Twenty-four individual clones (randomly selected) from the second, third and fourth round of panning were used for expression of soluble Nanobodies. The capacity of these Nanobodies to bind sVSG from *T. evansi* Kenya was assessed by ELISA. After *HinfI* RFLP and sequence analysis on the VHH genes amplified from the clones positive in ELISA, 16 different Nanobodies against *T. evansi* Kenya sVSG were identified (Fig. 2A, NbTev clones).

The four NbTev (*i.e.* E25, E28, E60 and E62) giving the highest signals in ELISA, were chosen for further analysis. Both NbTev-E25 and E28 have a homologous CDR1 and large similarities in their CDR3 of 15 amino acids, whereas NbTev-E60 and E62 harbor longer CDR3 loops of 17 and 21 amino acids, respectively. These Nanobodies were expressed in *E. coli* periplasm and purified by IMAC and size-exclusion chromatography. Purified Nanobody (>95% pure) could be obtained in yields between 0.5 to 3 mg Nanobody per liter of *E. coli* culture.

The alloreactivity towards sVSG proteins from *T. b. brucei* AnTat 1.1, *T. b. brucei* MiTat 1.5, *T. b. rhodesiense* ETat 1.2 and *T. congolense* Tc13 was tested. Apparently, the four NbTev were specific for the sVSG from *T. evansi*

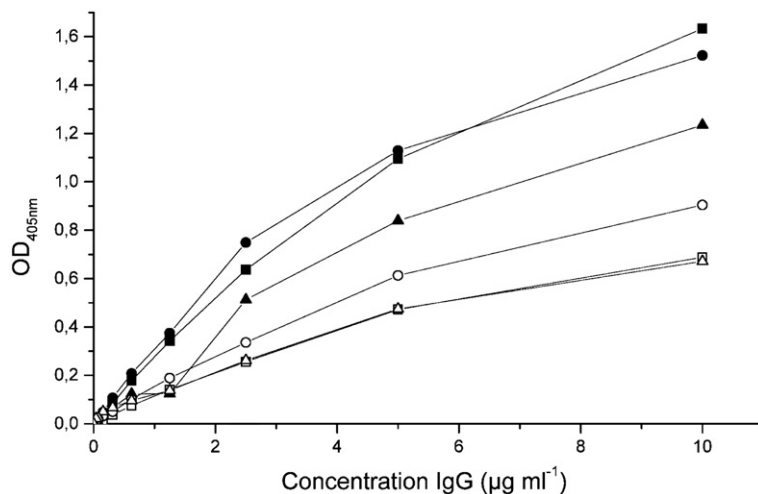


Fig. 1. The IgG response of infected dromedary measured by solid-phase ELISA. After coating soluble VSG from *T. evansi* Kenya (filled symbols) and *T. b. brucei* AnTat 1.1 (open symbols), bound IgG1 (■), IgG2 (●) and IgG3 (▲) was detected with rabbit anti-dromedary IgG antiserum. The OD at 405 nm was measured 15 min after addition of substrate.



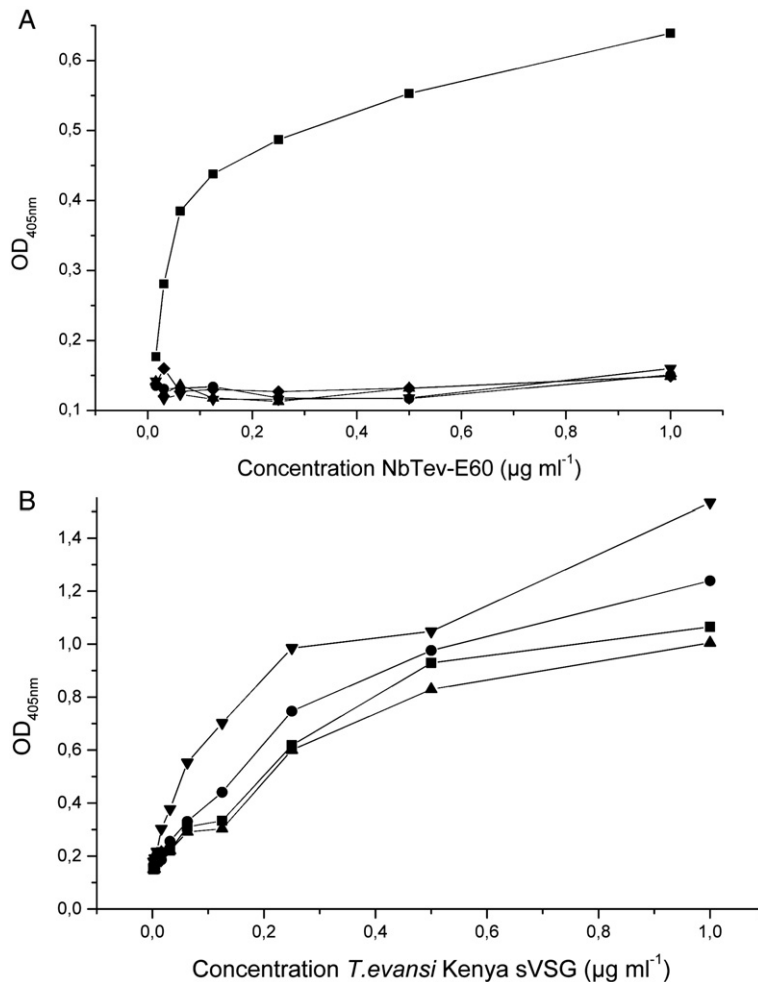


Fig. 3. (A) Solid-phase ELISA coating sVSG from *T. evansi* Kenya (■), *T. b. brucei* AnTat 1.1 (●), *T. b. brucei* MiTat 1.5 (▲), *T. b. rhodesiense* ETat 1.2 (▼) and *T. congolense* Tc13 (◆), and subsequent recognition by NbTev-E60. (B) The NbTev-E25 (■), E28 (●), E60 (▲) and E62 (▼) were coated at  $10 \mu\text{g ml}^{-1}$  in PBS. Subsequently, the *T. evansi* Kenya sVSG was loaded in serial dilution from  $1 \mu\text{g ml}^{-1}$  onwards. Captured *T. evansi* Kenya sVSG was detected with polyclonal rabbit anti-VSG antiserum. The OD at 405 nm was measured 15 min after addition of substrate.

Kenya and failed to recognize other sVSG proteins (Fig. 3A, shows the representative data for NbTev-E60). The potential of Nanobodies to capture sVSG from solution was tested in a sandwich ELISA (Fig. 3B). The four selected NbTev could easily capture sVSG from solution at a concentration of  $50 \text{ ng ml}^{-1}$  which corresponds to a detection sensitivity of  $4.6 \times 10^4$  parasites per ml of blood. This detection sensitivity is similar to that of other parasite detection techniques, such as HCT or Ag-ELISA (Kashiwazaki et al., 2000).

#### 3.4. Antibody response against different *Trypanosoma* lysates

In contrast to an immunization with purified proteins that raises a monospecific immune response, the parasite

infection of a dromedary should have elicited antibodies to multiple *Trypanosoma* proteins in parallel. The antibody response of this infected dromedary was therefore assessed in an ELISA by using various total parasite lysates as antigen. High or intermediate to low signals largely depending on the parasite species were noticed for the fractionated IgGs on these lysates. The *T. b. brucei* AnTat 1.1 and *T. evansi* ITMAS lysate as antigen gave only low signals for both, the conventional IgG1 and the Heavy-chain IgG2 fractions. In contrast, high signals were obtained for the Heavy-chain IgG3 subclass against *T. b. brucei* AnTat 1.1 (Fig. 4A). In fact a similar picture was obtained previously when a dromedary was immunized with sVSG from *T. b. brucei* AnTat 1.1 (Stijlemans et al., 2004). In that experiment, only the polyclonal Heavy-chain IgG3 antibodies (not

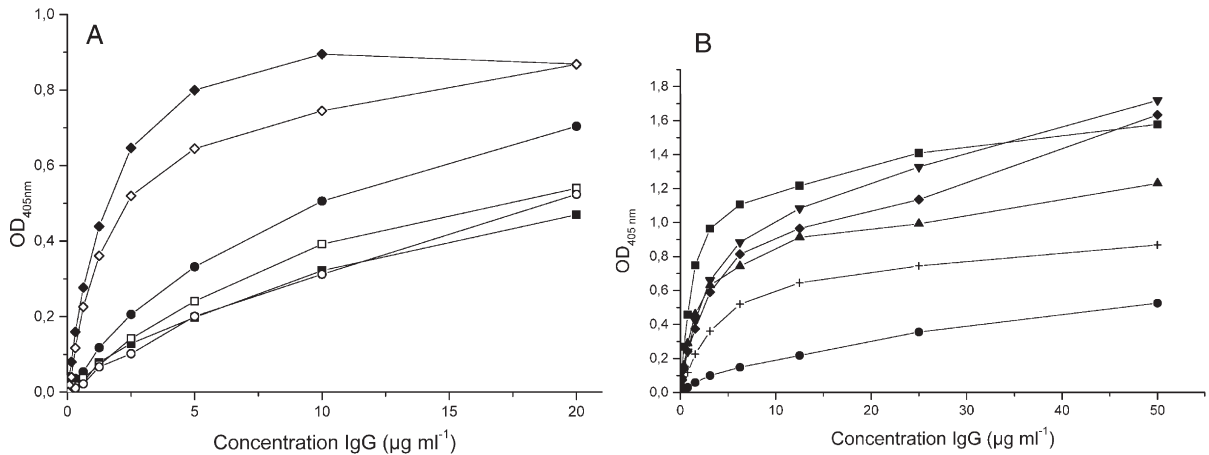


Fig. 4. The IgG response of infected dromedary to parasite lysates was measured by solid-phase ELISA. (A) After coating lysate from *T. b. brucei* AnTat 1.1 (filled symbols) and *T. evansi* ITMAS (open symbols), bound IgG1 (■), IgG2 (●) and IgG3 (◆) was detected with rabbit anti-dromedary IgG antiserum. The OD at 405 nm was measured 15 min after addition of substrate. (B) Total parasite lysate IgG3 immune response of the *Trypanosoma* infected dromedary. The IgG response of infected dromedary to different parasite lysates was measured by solid-phase ELISA: *T. b. brucei* AnTat 1.1 (■), *T. b. rhodesiense* ETat 1.2 (▼), *T. b. gambiense* FEO (◆), *T. vivax* (▲), *T. evansi* ITMAS (+) and *T. congolense* Tc13 (●). After coating lysate from different *Trypanosoma* lysates, bound IgG3 was detected with rabbit anti-dromedary IgG antiserum. The OD at 405 nm was measured 15 min after addition of substrate.

the IgG1 and IgG2) recognized sVSG from distinct classes (AnTat1.1, MiTat 1.1 and MiTat 1.5) in a dot blot. Therefore, we investigated the cross-reactivity of the IgG3 fraction to lysates of different trypanosome species (Fig. 4B). In line with the previous experiment (Stijlemans et al., 2004), it is clear that the IgG3 fractions contain antibodies that reacted strongly with all lysates (*T. b. brucei* AnTat 1.1, *T. b. rhodesiense* ETat 1.2, *T. b. gambiense* FEO, *T. vivax*, *T. evansi* ITMAS) with the possible exception of *T. congolense* Tc13.

### 3.5. Panning on total parasite lysates and selection of specific Nanobodies

Two panning procedures were followed for the isolation of Nanobodies to the antigens within a total parasite lysate. A first screening was performed using a single *T. b. brucei* AnTat 1.1 lysate during all selection rounds (Table 2, selection “A”). It was expected that all Nanobodies retrieved from that panning should recognize epitopes that are common in *T. b. brucei* AnTat 1.1

and *T. evansi* that originally infected the dromedary. For the second set of screenings alternating coatings of lysates of different *Trypanosoma* were used to retrieve Nanobodies that are reactive to epitopes shared by all these parasites (Table 2, selections “B–D”).

In selection “A”, three rounds of panning were performed on *T. b. brucei* AnTat 1.1 lysate (Table 2) with in each round an increasing number of washings to effectively remove phages carrying nonspecific binders. Enrichment of phages was observed from the second round onwards. After round two and three, 48 individual colonies were randomly picked for screening in phage ELISA. All colonies that gave a high signal in phage ELISA were further screened by PCR amplification of their VHH insert, a *HinfI* RFLP on the PCR fragment and sequence analysis. Seven different Nanobodies, *i.e.* NbTRYP01, 02, 03, 04, 06, 07 and 08 were identified (Fig. 2B). In addition to binding to antigens from the *T. b. brucei* AnTat 1.1 lysate, these Nanobodies recognize an epitope present in the *T. evansi* ITMAS and *T. evansi* Kenya lysate as well. However, no retention on

Table 2  
Adapted panning protocols

Selection	Round 1	Round 2	Round 3
“A”	<i>T. b. brucei</i> AnTat 1.1	<i>T. b. brucei</i> AnTat 1.1	<i>T. b. brucei</i> AnTat 1.1
“B”	<i>T. b. brucei</i> AnTat 1.1	<i>T. vivax</i>	<i>T. evansi</i> ITMAS
“C”	<i>T. b. rhodesiense</i> ETat 1.2	<i>T. evansi</i> ITMAS	<i>T. vivax</i>
“D”	<i>T. b. gambiense</i> FEO	<i>T. evansi</i> ITMAS	<i>T. b. rhodesiense</i> ETat 1.2

Phages from the library were selected each time on same *T. b. brucei* AnTat 1.1 lysate (“A”) or different total parasite lysate (“B”, “C”, “D”).



Table 3  
Binding properties of Nanobodies isolated by panning on different *Trypanosoma* lysates

NbTRYP	sVSG <i>T. b. brucei</i> AnTat 1.1	Lysate <i>T. b. brucei</i> AnTat 1.1	Lysate <i>T. evansi</i> ITMAS	Lysate <i>T. evansi</i> Kenya	Lysate <i>T. vivax</i>	Lysate <i>T. b. rhodesiense</i> ETat 1.2	Lysate <i>T. b. gambiense</i> FEO	Lysate <i>T. congolense</i> Tc13	Lysate <i>Leishmania</i>
01	-	+	+	+	ND	ND	ND	ND	ND
02	-	++	+	+	ND	ND	ND	ND	ND
03	-	+++	+	+	ND	ND	ND	ND	ND
04	-	+++	+	+	ND	ND	ND	ND	ND
06	-	+	+	+	ND	ND	ND	ND	ND
07	-	+++	+	+	ND	ND	ND	ND	ND
08	-	++	+	+	ND	ND	ND	ND	ND
11	-	+++	+++	ND	+++	+++	++	-	-
12	-	+++	+++	ND	+++	++	++	-	-
13	-	++	+	ND	-	++	+	-	-
14	-	++	+++	ND	+++	++	++	-	-
15	-	+++	+++	ND	+++	+++	+++	-	-
16	-	-	+++	ND	+	+	-	-	-
17	-	+	++	ND	+	++	+	-	-

Purified sVSG from *T. b. brucei* AnTat 1.1 or total parasite lysate was coated at  $10 \mu\text{g ml}^{-1}$  in PBS. Bound recombinant phage particles presenting a NbTRYP on their tip were detected by an anti-M13 horse-radish peroxidase conjugate. The OD at 405 nm was measured 15 min after addition of substrate (ND = not determined,  $- < 2$  times nonspecific signal,  $+ > 3$  times nonspecific signal,  $++ > 5$  times nonspecific signal,  $+++ > 7$  times nonspecific signal). The *Leishmania* lysate was used as a negative control in the experiment.

the *T. b. brucei* AnTat 1.1 sVSG was observed for these binders in ELISA (Table 3).

Application of the second set of panning procedures resulted in isolation of novel Nanobodies, pan-reactive across the various trypanosomes. Four rounds of selections were carried out in immunotubes as compared to microtiter plates for the first screening method, with in each round a different lysate as coating (Table 2, selections “B”, “C” and “D”). Specific enrichment of phages starting from the second round was observed on

*T. b. brucei* AnTat 1.1 lysate, even for pannings “C” and “D” that had never been selected on the *T. b. brucei* AnTat 1.1 lysate. A similar enrichment for pan-reactive Nanobodies in the phage pool was seen on the other *Trypanosoma* lysates. After the second and third round of selection, 120 randomly picked colonies were analyzed for producing virions that were binding to each lysate. The *HinfI* RFLP and sequence analysis of the VHH genes from these colonies revealed seven additional, distinct Nanobodies (NbTRYP11-17) interacting

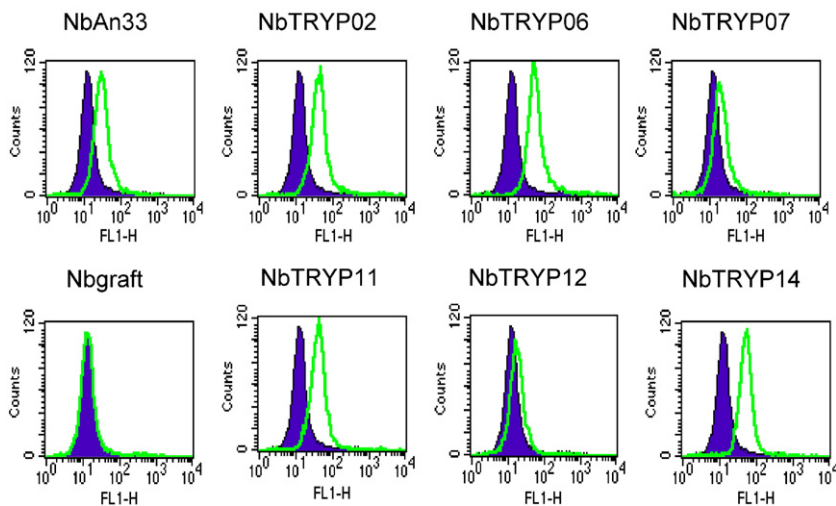


Fig. 5. The fluorescently labeled NbTRYP were incubated with  $2 \times 10^6$  fixed *T. b. brucei* AnTat 1.1 parasites. Positive control (NbAn33 binding only *T. b. brucei* AnTat1.1) and negative control (Nbgraft) were also included.

Table 4

Binding of different NbTRYPs on *T. b. brucei* AnTat 1.1 and *T. b. brucei* AnTar lysates, GPI-anchored membrane fraction (MF) of *T. b. brucei* AnTat 1.1, or the solubilized VSG from *T. b. brucei* AnTat 1.1 (sVSG)

NbTRYP	AnTat	AnTar	MF	sVSG
02	++	++	+	–
06	++	++	+	–
07	++	++	++	–
11	++	++	++	–
12	++	++	+	–
14	++	++	++	–
15	+	+	+	–

The OD at 405 nm was measured 15 min after addition of substrate ( $< 2$  times nonspecific signal,  $> 2$  times nonspecific signal,  $++ > 4$  times nonspecific signal).

with total parasite lysates. While some of the Nanobodies only recognized a few *Trypanosoma* lysates, others showed a broader specificity (Table 3).

All these Nanobodies apparently recognizing an antigen occurring in several lysates (*i.e.*, the NbTRYP clones in Fig. 2B) were cloned into an expression vector, produced and purified. Only the Nanobodies expressing at levels above 1 mg protein per liter *E. coli* culture were used for further analysis, *i.e.* NbTRYPO2, 03, 06, 07, 11, 12, 13, 14, 15, and 17.

### 3.6. Characterization of the pan-reactive Nanobodies by FACS

The pan-reactive Nanobodies were first assessed for their ability to interact with accessible epitopes on the surface of the parasite. Binding of fluorescently labeled Nanobodies to parasites was analyzed by FACS (Fig. 5). The *T. b. brucei* AnTat 1.1 parasites were stained upon addition of NbTRYPO2, 06, 11 and 14, indicating that these Nanobodies recognize epitopes at the surface of the parasite. Specific binding of these Nanobodies was also observed on fixed *T. b. gambiense* ABBA, *T. b. rhodesiense* ETat 1.2, *T. evansi* KETRI and *T. congolense* Tc13 confirming their pan-reactive potential. Remarkably those Nanobodies bound *T. congolense* Tc13 in FACS, whereas they failed to recognize their cognate epitope within the lysate when screened by ELISA (Table 3). This might be explained by the difference in epitope presentation during FACS and ELISA experiments. In addition, only low binding signals were observed for NbTRYPO7 and 12, suggesting a low affinity reaction and/or low density or restricted access of the antigen on the parasite coat. Since NbTRYPO2 and NbTRYPO12 have a nearly identical amino acid sequence, including that of their antigen-binding loops,

it is highly likely that they recognize the same epitope. Therefore, the different staining pattern of the parasites by this latter pair of binders probably reflects a net difference among these Nanobodies in their equilibrium dissociation constant for the epitope. For NbTRYPO3, 13, 15 and 17 there was no detectable staining of the parasites when analyzed by FACS. This inaccessibility suggests that their cognate antigens are most likely present intracellularly or embedded in the parasites' membrane.

The NbTRYP targeting was extended to include living parasites because such binding tests are more relevant for diagnostic or therapeutic validation of Nanobodies. All NbTRYPs positive for fixed parasites targeted equally well the living *T. b. brucei* AnTat 1.1.

### 3.7. Characterization of the pan-reactive Nanobodies by ELISA

Normally, the sVSG from different trypanosome lysates are expected to possess little sequence overlap, hence the pan-reactive Nanobodies are unlikely to target the VSG. To formally exclude the possibility that the pan-reactive Nanobodies bind to sVSG, they were tested for recognizing the sVSG from *T. b. brucei* MiTat 1.5, *T. b. brucei* AnTat 1.1, *T. b. rhodesiense* ETat 1.2, *T. congolense* Tc13, *T. b. gambiense* FEO and *T. evansi* ITMAS. None of the Nanobodies scored positive in ELISA, confirming that NbTRYPO2, 06, 11 and 14 are targeting a non-VSG type antigen(s) on the parasite surface. Neither are these Nanobodies interacting with the surface exposed antigens SRA (Radwanska et al., 2002), ESAG6 (Pays et al., 2001) and ISG75 (Tran et al., 2006) as none of these recombinant proteins were recognized by the NbTRYPs.

Furthermore, binding to *T. b. brucei* AnTar (procyclic form) lysate and the GPI-anchored membrane fraction of *T. b. brucei* AnTat 1.1 lysate was evaluated (Table 4). Binding to the procyclic form of *T. b. brucei* was observed for the NbTRYPO2, 06, 07, 11, 12, 14, and 15, which indicates that these Nanobodies recognize a protein that is also expressed during the insect cycle of the parasite. Moreover, a GPI-anchored component is targeted by these Nanobodies as seen from positive signal on the GPI-anchored membrane protein fraction. Surprisingly, the latter fraction was targeted by NbTRYPO15, a Nanobody of which the fluorescently labeled format failed to stain the whole parasites. It is surmised that this Nanobody binds an antigen that is too deeply buried in the coat to be visualized in FACS after staining with a Nanobody although the antigen is present on the membrane of the parasite.

#### 4. Discussion

This study demonstrates the feasibility to isolate a panel of *Trypanosoma* infectome-specific binders from an infected dromedary without having *a priori* knowledge of the proteins involved. Obviously, camelids also succeed in raising an immune response against several components of a proteome or infectome. It was shown that the camelid Heavy-chain antibodies were responding to various epitopes of the *Trypanosoma* infectome, and that the antigen-binding fragments, the Nanobodies, of these Heavy-chain antibodies could be efficiently cloned and selected.

The repertoire cloning of conventional antibodies, *e.g.* in the form of scFv, involves several steps. First, two independent PCRs are needed to amplify the VH and VL. Secondly, these fragments have to be joined randomly into one construct by a linker segment, either by a splicing of overlap extension or by a two-step cloning strategy. This scrambling of the individual VH and VL from a pool and the presence of artificial VH–VL combinations with decreased affinity for the original antigen implies that large libraries need to be screened in order to isolate the parental VH–VL combination, and that many binders with sub-optimal affinity and stability will be obtained. Therefore, it seems rather counter-productive to first immunize an animal in order to optimize and mature the antibodies for the target antigen, and then to split the antigen-binding partners and to scramble them in the following step. This VH–VL scrambling is avoided by cloning the repertoire of the camelid HCAbs the entire antigen-binding site of each HCAb is encoded by one single exon, the VHH.

It is well established that the VSG is the most abundant protein present on the parasite's surface (Dubois et al., 2005) and, for classical antibodies, it is an immuno-dominant antigen. Here we confirmed that *T. evansi* elicited a strong VSG-specific Heavy-chain antibody response in the infected dromedary as well. Admittedly, the immune response, raised during immunization with a parasite lysate or after a natural infection with the living parasite, might have a different outcome due to an active living parasite to host interplay. Therefore, the Nanobodies retrieved from an infected dromedary or after immunizing a dromedary with a total proteome extract from the parasite could react to a widely varying panel of antigens.

As expected, screening the Nanobody library cloned from the infected dromedary on the purified sVSG from *T. evansi* Kenya as bait yielded highly species-specific binders. Interestingly, the panning of the same Nanobody library with the total lysate of *T. b. brucei*

AnTat1.1 resulted in the selection of several binders that associated with the lysate of *T. evansi* and at least as strong, or stronger, with antigens in the *T. b. brucei* lysate. Following an adapted panning protocol whereby the bait was switched between proteome lysates of different *Trypanosoma* species during the consecutive rounds of selection allowed the identification of different Nanobodies that recognize non-VSG antigens present in multiple *Trypanosoma* species. Some of these Nanobodies (*e.g.* NbTRYP02, -06, -07, -11, -12, -14) clearly associated (to various extents) with an antigen exposed on the surface of living parasites (as revealed by FACS analysis). One particular Nanobody (NbTRYP15) failed to react with fixed or living parasites when analyzed by FACS, although it reacted well with the antigens within the GPI-anchored membrane fraction (MF, Table 4) in an ELISA. It seems that this Nanobody binds to a surface antigen, cryptic in intact parasites. In contrast, the NbTRYP03, -13, -15 and -17, apparently failed to interact with the intact parasites, or GPI-attached membrane extracts (Table 4), however, their cognate antigen was present in the total lysate from the same parasite (Table 3). Therefore, the most obvious explanation would be that these Nanobodies are directed against intracellular antigens (or transmembrane antigens).

In any case, by changing the antigen fraction during the selections we have access to a wide variety of Nanobodies, some of which are strain-specific, whereas others are pan-reactive Nanobodies, *i.e.* Nanobodies that recognize an antigen common to various *Trypanosoma* species. With sets of VSG-specific and pan-reactive Nanobodies at hand, it becomes feasible to design a protocol for a precise species typing within an infected individual. For example, antibody micro-arrays could be applied for the specific and sensitive detection of those components from an infected individual (Wingren et al., 2005).

Apart from the employment of these Nanobodies for a diagnostic test, a therapeutic utilization might be envisaged as well. To arrive at a therapeutic anti-trypanosome drug, one has to link a pan-reactive Nanobody with a potent trypanocidal compound, such as truncated human ApoLI. A potent trypanocidal drug was generated by combining a truncated human ApoLI fragment with an anti-*T. b. rhodesiense* Nanobody (Baral et al., 2006). This Nanobody, *i.e.* NbAn33, recognizing specifically multiple trypanosome species, was obtained by screening a Nanobody library from a dromedary immunized with soluble VSG of *T. b. brucei* AnTat 1.1 (Stijlemans et al., 2004). The NbAn33 targeted the high-mannose carbohydrate epitope present on various VSG proteins. As indicated above, here we

retrieved Nanobodies with a similar broad trypanosome-specific range without having access to purified antigens, neither for raising the immune response, nor for the Nanobody selections.

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