Supporting Information

High-affinity anti-hapten single-domain antibodies are not favored in the llama immune response, but can be isolated by competitive selection of VHH libraries

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RUNNING TITLE: High affinity anti-hapten antibodies from VHH libraries

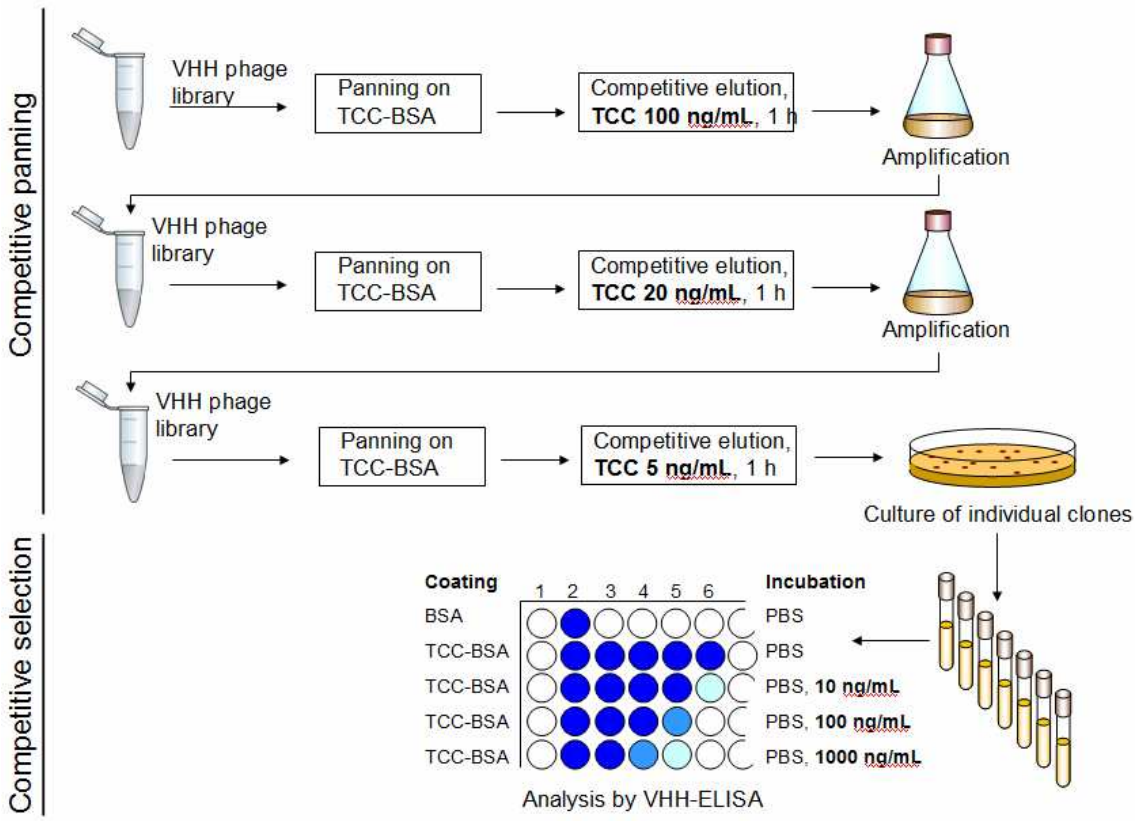
S-1. MATERIAL AND METHOS

Amplification of VHH genes. The following forward primers were use for amplification of the VHH genes: VH1: CAT GCC ATG ACT CGC GGC CCA GGC GGC C ATG GCC CAG GTG CAG CTG GTG CAG TCT GG, VH3: CAT GCC ATG ACT CGC GGCCCAGGCGGCC ATG GCC GAG GTG CAG CTG GTG GAG TCT GG, VH4: CAT GCC ATG ACT CGC GGCCCAGGCGGCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GG, VHArgento: G CTG GAT TGT TAT TACT CGC GGC CCA GGC GGC C ATG GCC CAG GTS MAR CTG CAG SAG TCW GG, and VH4Arg: CG TGG ATT GTT ATT ATC TGC GGC CCA GGC GGC C ATG GCC GAT GTG CAG CTG CAG GCG TCT GGR GGA GG. The reverse primer JH: CCA CGA TTC TGG CCG GCC TGG CCT GAG GAG ACR GTG ACC TGG GTC C was used for amplification of VHH genes [1, 2]. The forward and reverse primers contain SfiI sites (underlined) that were used to clone the amplified fragments into the pCom3X vector.

***Surface Plasmon Resonance (SPR) analysis*.** The affinity of the T4, T7 and T9 clones was determined on a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). TCC–BSA was diluted in 10 mM sodium acetate, pH 4.5 and covalently immobilized on the flow cell of a carboxymethyl dextran CM5 sensor chip (Biacore AB) by using the amine coupling method according to the manufacturer's instructions. The immobilization level was 200 resonance units (RU). A second intact flow cell (untreated) was used as reference. Different concentrations of VHHs ranging from 0.5 to 276 nM in PBS containing 0.005% surfactant P20 were injected for 120 s over the two flow cells, at a constant flow rate of 20 µL/min at 25 °C. After a dissociation step of 600 s, sensor surfaces were regenerated with 10 mM glycine pH 1.5 during 60 seconds at a constant flow rate of 60 µL/min. Experiments at different flow rates were also performed in order to discard mass transport limitation. All experiments were repeated twice. No binding of VHH to BSA carrier protein was detected when a 15-fold excess of BSA was added as competitor.

To check the affinity against free TCC, VHH T9 was diluted in 10 mM sodium acetate, pH 5.5 and immobilized at 1500 RU on a CM5 sensor chip. Different concentrations of TCC ranging from 50 to 800 nM in PBS supplemented with 0.005% P20 and 0.01% DMSO were injected at constant flow rate of 20 µL/min. All sensograms were double referenced by subtracting the signal from the reference flow cell and that of a buffer injection, and analyzed by using the BIAevaluation software, version 4.1 (Biacore AB)

S-2 FIGURES



Scheme S-1. Schematic representation of the competitive panning strategy and selection process. The VHH-phage library was panned on ELISA wells coated with TCC-BSA. After 2 h incubation at 4℃ and extensive washing, the wells were loaded with 100 µL of elution buffer (PBS-BSA containing 100 ng/mL of TCC), incubated for 1 h. The competitive eluted phage was then amplified in *E. coli* cultures and used in successive rounds of panning. Along the panning the amount of TCC used for competitive elution was decrease as shown, to promote the competitive elution of phage bearing VHHs with high affinity for TCC. After the final round of panning, *E. coli* cells were infected with the eluted phage and grown in agar plates. Individual clones were randomly picked and culture in LB. After induction with IPTG to promote the production of soluble VHH bearing the HA tag, the supernatants were diluted two-fold with 5% skimmed milk-PBS and tested by ELISA on wells coated with BSA or TCC-BSA (the anti HA-HRP conjugated antibody was used for detection). During the incubation, varying amounts of TCC were used to check for inhibition. To exemplified the competitive selection process, the schematic results of 6 clones are shown: 1, no binding; 2, unspecific binding to the plate; 3, binding to TCC-BSA but not inhibition; clones 4, 5 and 6, poor, medium and strong inhibition.

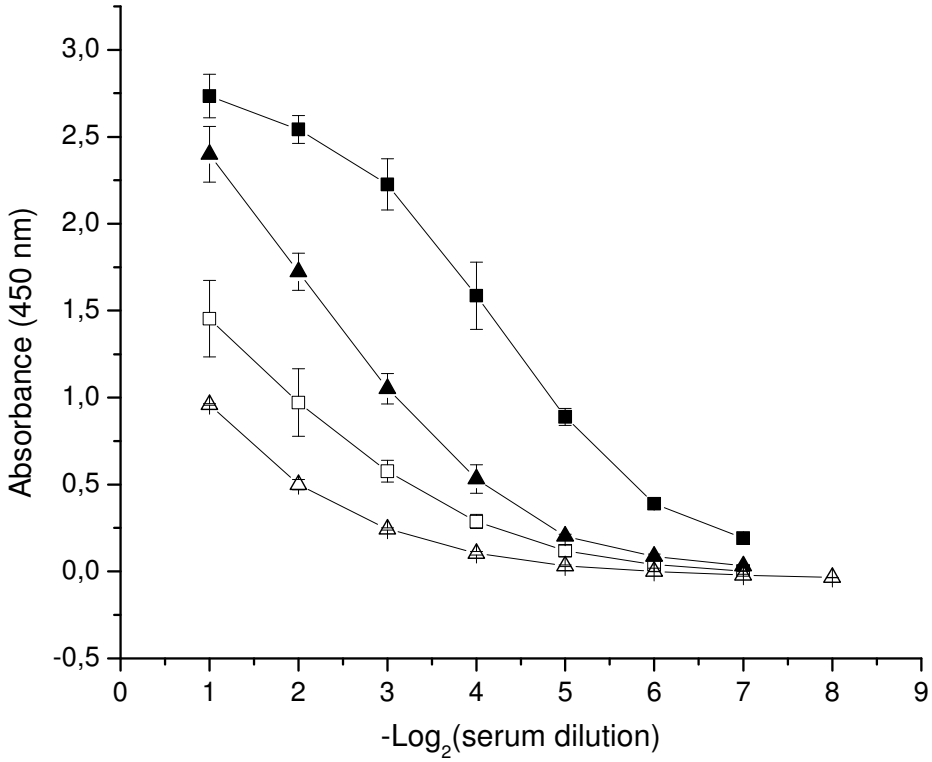


Figure S-1. Antibody response against TCC and Thy. All sera were initially diluted 1/200 and then two-fold serial dilutions were analyzed. Black and white symbols are used to denote the response to TCC or Thy, respectively. Squares and triangles are used to represent the antibody response of llama 807 and llama 856, respectively.

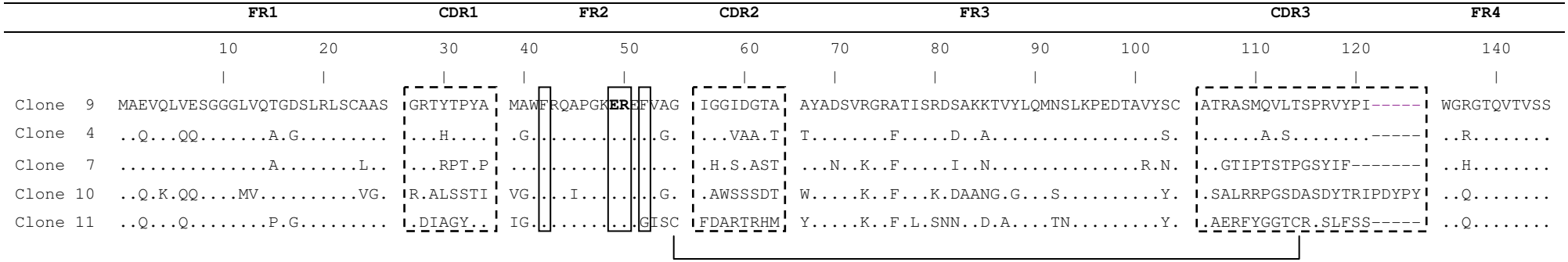


Figure S-2. Amino acid sequence alignment of the isolated clones. The deduced amino of the five clones isolated from the anti-TCC VHH library are given in the single-letter code. Residues are numbered according to the IMGT numbering system [21]. Dots are used to denote residues identical to those of clone 9 that is arbitrarily taken as a reference sequence. Gaps were introduced to improve the alignment and are marked with dashes. Dotted boxes frame the CDRs and solid-line boxes outline the characteristic amino acid substitutions of VHH FR2 [22]. The putative disulfide bond between cysteine residues in FR2 and the CDR3 of clone 11 is also drawn.

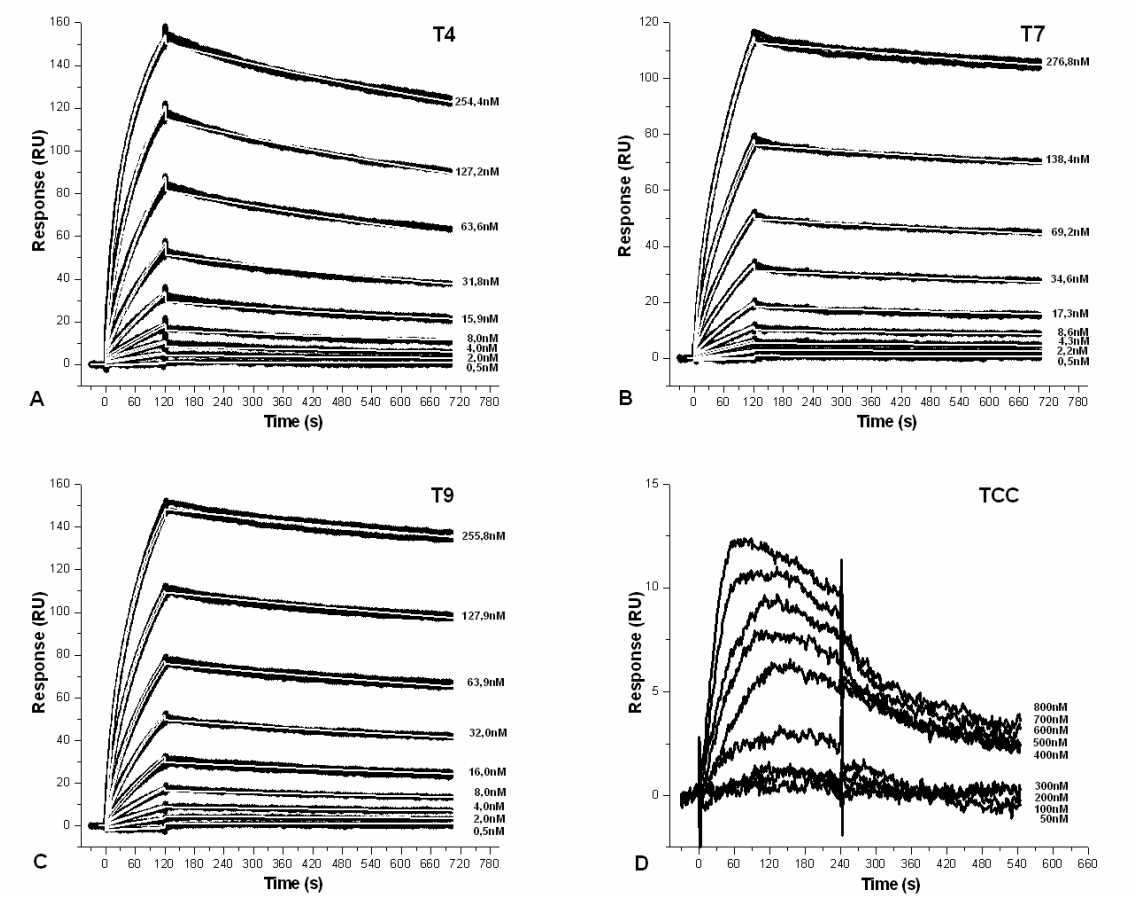
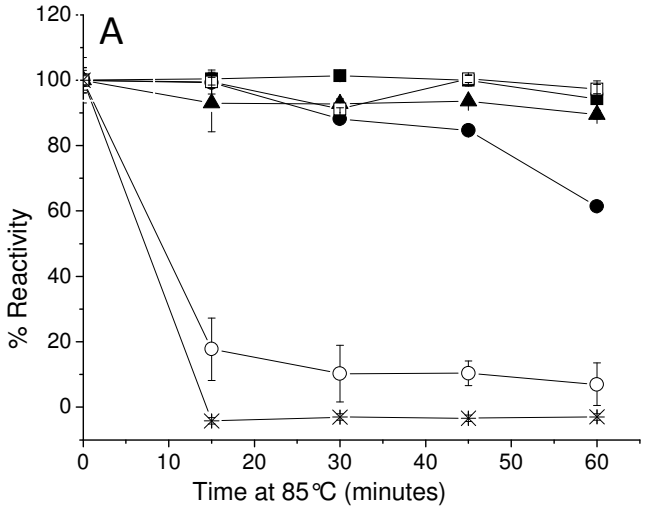
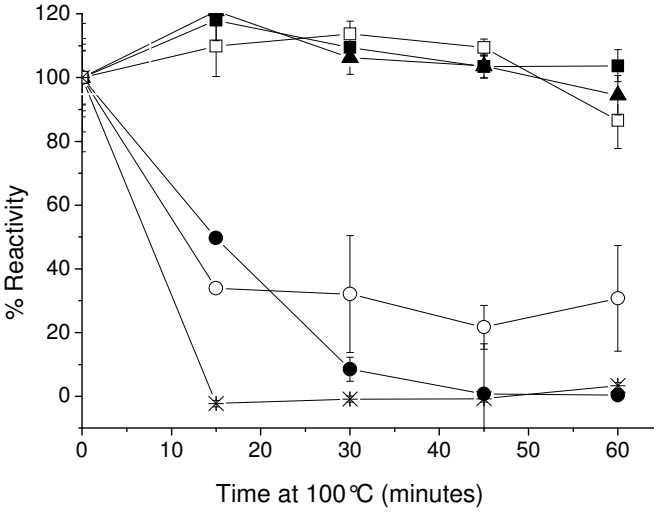


Figure S-3. Binding kinetics of the anti-TCC VHH by SPR. Sensorgrams showing the binding of T4, T7 and T9 VHH clones to TCC–BSA immobilized on a CM5 flow cell, injected at the concentrations indicated in the figure (black lines). Injections were repeated twice and best fitted with a heterogeneous ligand parallel reaction kinetic model (white lines). Panel D, sensorgram showing the binding of free TCC at the concentrations indicated in the figure to the T9 VHH clone immobilized on a CM5 flow cell. All Sensorgrams were obtained at 25°C, at a constant 20 µL/min flow rate.


A
% Reactivity
Time at 85℃ (minutes)


% Reactivity
Time at 100℃ (minutes)

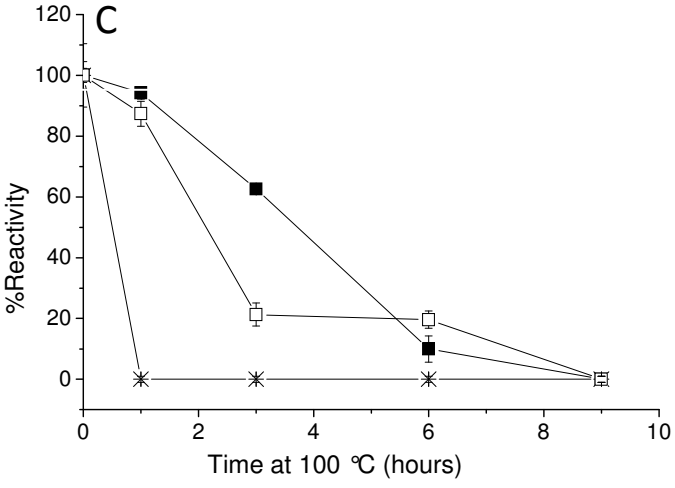

C
%Reactivity
Time at 100 ℃ (hours)

Figure S-4. **Thermal stability of anti-TCC VHH clones.** VHHs and conventional antibody fraction (IgG1) were diluted to 1 mg/mL in PBS and were heated at 85℃ or 100℃ during 1 h, graphs A and B, or at 100℃ for 9 hours (graph C). Samples at different time intervals were cooled down and the reactivity with TCC-BSA tested by ELISA. T4 (black squares), T7 (black circles), T9 (black triangles), T10 (open circles) and T11 (open squares), IgG1 (asterisk).

S-3 REFERENCES

1. Alvarez-Rueda, N.; Behar, G.; Ferre, V.; Pugniere, M.; Roquet, F.; Gastinel, L.; Jacquot, C.; Aubry, J.; Baty, D.; Barbet, J.; Birkle, S., Generation of llama single-domain antibodies against methotrexate, a prototypical hapten. *Mol Immunol* **2007,** *44* (7), 1680-90

2. Zarebski, L. M.; Urrutia, M.; Goldbaum, F. A., Llama single domain antibodies as a tool for molecular mimicry. *J Mol Biol* **2005,** *349* (4), 814-24.