Human TNF cytokine neutralization with a vNAR from *Heterodontus francisci* shark

A potential therapeutic use

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**Abbreviations:** CDR3, complementarity determining region; IgNAR, New antigen receptor; LD50, median lethal dose; ND50, median neutralizing dose; rhTNFα, recombinant human tumor necrosis factor alpha; sdAbs, single domain antibodies

The therapeutic use of single domain antibodies (sdAbs) is a promising new approach because these small antibodies maintain antigen recognition and neutralization capacity, have thermal and chemical stability and have good solubility. In this study, using phage display technology, we isolated a variable domain of a IgNAR (vNAR) from a *Heterodontus francisci* shark immunized against the recombinant human cytokine TNFα (rhTNFα). One clone T43, which expresses the vNAR protein in the periplasmic space, was isolated from the fourth round of panning. T43 had the capacity to recognize rhTNF and neutralize it in vitro, indicating that T43 has potential as a therapeutic that can be used for diseases in which this pro-inflammatory cytokine needs to be controlled.

**Introduction**

Conventional antibodies have many important features, including high affinity and selectivity for a specific target and low inherent toxicity, that make them suitable as therapeutic agents. In addition to these benefits, single domain antibodies, such as the V_{HH} (obtained from camels) and the vNAR (obtained from sharks), have other technological and biophysical advantages.4,7 Single domain antibodies are the smallest naturally occurring antibodies domain with antigen recognition capacity and, in contrast to single chain Fv (scFv), these domains do not undergo spontaneous dimerization. Furthermore, they retain binding capacity under conditions of extreme pH and temperature and also in the presence of proteases and chaotrophic agents.4

The vNAR domains display two complementarity-determining region (CDR) loops compared with conventional antibodies, which have a VH and VL domain format where the binding capacity depends on the interaction of six CDR loops.5 Differences in the length, amino acid composition and the distribution of non-canonical cysteine residues of CDR3 were observed in these molecules.6,7 Antigen binding is primarily mediated by an extended CDR3 loop, with stabilizing disulfide bounds connecting to either the less variable CDR1 region or to adjacent framework residues.4

Using phage display technology, a range of clinically important target molecules, such as cytokines, can be selected against a large repertoire, and selection of high-affinity recombinant antibodies is also possible. The IgNAR response, which has previously been analyzed in nurse (*Ginglymostoma cirratum*) and wobbegong (*Orectolobus maculatus*) sharks, is antigen driven, and both immune and naïve molecular libraries of IgNAR variable domains have been constructed and successfully screened for molecules with antigen specificity.4,7,10

Our group has analyzed the IgNAR antibodies from the horn shark *Heterodontus francisci*. The small size and the sedimentary behavior of this shark make it a good model system for raising vNAR domains.11 TNFα, a pro-inflammatory cytokine, was selected as the target because it is implicated in diseases such as rheumatoid arthritis and psoriasis. In addition, numerous other formats of monoclonal antibodies targeting TNF have been developed.12 In this report, we isolated one vNAR domain from an immunized horn shark using phage display technology. This vNAR can neutralize recombinant human TNFα in vitro. Several pharmaceutical companies are developing variable domains to neutralize TNFα, e.g., scFv (DLX105; Delenex), minibodies with a VL fused with Fc region,13 single domain VHH fragments14 and nanobodies (ATN-103, ATN-192; Ablynx). The shark vNAR platform, however, has the advantage that sharks can recognize epitopes that the immune systems of mammalians...
cannot recognize, thereby offering opportunities to isolate novel antibodies, potentially with high affinities, that could not otherwise be produced.

**Results**

**Isolation of vNAR fragments.** Total RNA was purified from the spleen of one immunized shark and 1.5 mg of total RNA was isolated. PCR reactions were performed to obtain the quantity of vNAR gene required for digestion and ligation of the amplified isolated. PCR reactions were performed to obtain the quantity of spleen of one immunized shark and 1.5 mg of total RNA was purified from the Total RNA was purified from the immunized shark, and 1.5 mg of total RNA was obtained.

**Expression and recognition ELISA assays.** After the analysis of the four different clones, we selected clone T43 based on expression and recognition ELISAs. The sequence of T43 clone is show in Figure 2. The vNAR protein was expressed in the periplasmic space of TOP10F' E. coli cells in low yield (700 μg/L). After expression and periplasmic extraction, metal affinity chromatography was performed and protein expression was detected by ELISA (Fig. 3A). Recognition of the vNAR against BSA was detected, but recognition of the specific antigen was higher (p < 0.05%); Figure 3B. The purified vNAR T43 protein (Fig. 4) had a molecular weight of ~14 kDa and could be detected with anti-HA-HRP antibody in a western blot assay. Most of the protein is eluted in fraction 2 during the purification procedure. vNAR T43 was used for thermostability assays, which demonstrate conservation of the ability of boiled vNAR to recognize antigen. An ELISA assay with the purified and boiled vNAR showed only a 15% decrease in recognition of rhTNFα compared with the non-boiled vNAR at different time points (Fig. 5).

**In vitro neutralization assays.** The cytotoxicity assay in L929 cells is a rapid and accurate bioassay with high sensitivity and reproducibility. The LD₅₀ detected in this assay for rhTNFα was 0.250 ng/mL. Accordingly, we used 5LD₅₀ (1.25 ng/mL or 0.07 nM) of rhTNFα for the inhibition assays. The ND₀ detected for the vNAR T43 was 9.78 μg/mL (650 nM). However, the ND₀ for the F(ab’)₂ was 1.62 μg/mL (16 nM) (Fig. 6).

**Discussion**

Several immunotherapies based in conventional, i.e., full-length, formats have been developed as treatments for Crohn disease, rheumatoid arthritis, spondylitis and other diseases where the TNF cytokine needs to be controlled. Nevertheless, secondary effects were observed, e.g., anti-drug antibody production, renal and liver damage and susceptibility to opportunistic infection. Many of these side effects are caused by the size of the immuno-therapeutic and its immunogenicity, which can be due to non-human sequence source and the presence of the Fc region. Single domain antibodies (sdAbs) were proposed as an attractive alternative for therapeutics and diagnostics applications because of the small size and tissue penetration; high solubility; capacity to block the enzyme active site and the nanomolar affinity of the molecules.

In this report, vNAR T43, obtained using a phage display technique with an immune library from the horn shark *Heterodontus francisci*, shows a capacity for recognition and in vitro neutralization of the human TNFα cytokine. In addition, the vNAR T43 preserved 85% of its capacity of recognition in a thermostability assay after being heated over one hour. This feature can represent a pharmaceutical advantage over commercially available immuno-therapeutic because freezing may not be necessary to conserve its integrity and functionality.

Because the expression level of the vNAR T43 protein using an *E. coli* TOP10F' system was low (700 μg/L), analysis and experimentation with diverse variables that can affect the protein production, e.g., the preferential codon use, the high GC content of the vNAR DNA sequence, the stability of the mRNA, the protein extraction process and the host expression cells will be necessary.

In the TNFα neutralization assay, we demonstrated the effectiveness of this single domain antibody in comparison with the Fab’ at the same doses. It is important to consider that the F(ab’)₂ fragment has two antigen recognition domains that can be translated in a double capture of the TNFα cytokine per molecule in comparison with the vNAR T43.

In this work, we made evident the efficacy of vNAR antibodies obtained using an immune library from a horn shark to recognize and neutralize the human TNFα cytokine in vitro assays. The vNAR T43 protein also demonstrated a high thermostability, which can make it easier to handle and preserve compared with full-length of Fab antibody therapeutics. Furthermore, the vNAR T43 showed neutralization capacity of the human TNFα cytokine in comparison with a conventional antibody fragment.
which has two binding sites per molecule. If we analyze the molar ratio between our T43 and the TNFα, it is clear that we need more than 9,000 molecules of T43 to neutralize 1 molecule of TNFα in an in vitro assay. For drug development, however, the molecule with the highest binding capacity is not always the best candidate, especially when the target is a cytokine with pleiotropic effects. To demonstrate the utility of vNAR T43 as a therapeutic, it will be necessary to understand and define the pharmacokinetics and pharmacodynamics, toxicity and immunogenicity of the molecule. The preliminary results reported here encourage us to test the vNAR T43 protein in different in vivo TNFα models that can demonstrate the potential use as a treatment in autoimmune, allergic and infectious diseases in which TNFα overproduction is mainly involved, e.g., septic shock.

Materials and Methods

Selection of vNAR fragments from an immune library. A male Heterodontus francisci shark was immunized with an emulsion of 200 μL of a 1:1 mixture of complete Freund adjuvant (CFA) and of rhTNFα 1 μg/mL (PEPROTECH, 300–01A) by intramuscular injection. The entire immunization protocol was performed over 20 weeks. Only the first immunization was performed with CFA. The boosts were administrated every 15 d using incomplete Freund adjuvant (IFA). Seven days after the final immunization, the spleen was dissected, and total RNA was isolated using TRI reagent (SIGMA, T9424) and BCP reagent (SIGMA, B9673). The RNA concentration and purity were measured by optical density at 260 nm and 280 nm, respectively, and total RNA was stored at -80°C. Reverse transcription reactions were performed using Access RT-PCR System kit (PROMEGA, A1250) with 1 μg of total RNA and a specific antisense primer (under IP protection). Hot start polymerase was used for PCR amplification of the vNAR fragments (Go Taq Hot Start Polymerase, PROMEGA, M5001). All primers used for cloning had the SfiI restriction site. vNAR fragments were cleaned, and quantified. The enzymatic digestion was performed with 5U of SfiI enzyme (PROMEGA, R4394) per μg of DNA. Target DNA was incubated for 5 h at 50°C for digestion, the enzyme was heat inactivated at 65°C for 15 min, and DNA was stored at -80°C. vNAR fragments were cloned into the pCOMb3X vector (previously digested with SfiI), using the T4 DNA ligase (10 U/mL, PROMEGA, M1804) at 1:1 molar equivalent (vector:insert). Positive and negative controls were digested vector with and without insert, respectively. The library was purified and transformed into E. coli ER2537 cells.

Immune library generation. Three hundred μL of E. coli ER2537 cells was transformed and then washed with 1 mL of SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 20 mM glucose). This method was performed two more times with 2 mL of SOC medium. The 5 mL culture was incubated at 37°C and shaken at 250 rpm for 1 h. Then, 10 mL of fresh SB medium and 3 μL of 100 mg/mL carbenicillin were added. For calculation of the transformed bacteria, 1 μL and 100 μL of cultured cells were plated on LB-Agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.2% agar) with carbenicillin at final concentration of 100 μg/mL and incubated overnight at 37°C. The 15 mL culture was incubated for 1 h at 250 rpm. Then, 4.5
rpm. The following day, the culture was centrifuged at 3,000 g for 15 min at 4°C. The re-amplified phages that were present in the supernatant were precipitated by adding 8 g of PEG-8000 and 6 g of NaCl and were then incubated on ice for 30 min. The phages were pelleted at 15,000 g for 15 min at 4°C. The supernatant was discarded, and the pellet containing the phages was resuspended in 2 mL of 1% BSA-1XPBS and filtered through a 0.2 μm membrane.

Panning was performed according to Barbas. Four panning rounds against 50 ng/well of rhTNFα were performed. In the first round, 5 washing steps were performed. Ten washing steps were performed in the second round, 15 washing steps were performed in the third, and 20 washing steps were performed in the fourth panning round.

vNAR expression and purification. Several isolated clones from the fourth panning round were analyzed by PCR reactions using the Ompseq (5'-AAG ACA GCT ATC GCG ATT GCAG-3') and Gback (5'-GCC CCC TTA TTA GCG TTT GCC ATC-3') primers at Tm 56°C. The plasmids of PCR positive clones were isolated using commercial plasmid extraction kits (QIAGEN, 27104). The recombinant plasmids were electroporated into TOP10F’ cells. For vNAR protein induction, a single colony was placed in 3 mL of SB medium with 100 μg/mL carbenicillin. The culture was shaken at 37°C and 250 rpm overnight. The 3 mL of culture was then added to 47 mL of fresh medium with the same concentration of antibiotic and under the same culture conditions. After 4 h, the entire culture volume (50 mL) was added to 450 mL of fresh medium SB enriched with antibiotic. Once the 500 ml culture reached an optical density of 0.7 at 600 nm, 1 mL of 0.5 M IPTG (SIGMA, I5502) was added. The culture was induced for 12 h at 37°C and 300 rpm. This procedure was performed for each positive clone.

The cultures were centrifuged, and a periplasmic extraction was performed by osmotic shock according to Minsky. Supernatant was filtered with a 0.2 μm cutoff membrane and purified with metal affinity chromatography using HisTrap columns (ROCHE, 17–5247–01). The column was equilibrated with wash buffer (50 mM NaPO₄, 40 mM imidazole, 0.3 M NaCl, pH 8.0), and the periplasmic extract was loaded using a syringe at 1 mL/min constant flux. After sample loading was complete, 10 mL of wash buffer was added. The sample was eluted by adding 5 mL of elution buffer (50 mM NaPO₄, 500 mM imidazole, 0.3 M NaCl, pH 8.0), which was collected in 1 mL fractions. The second fraction, which contained the vNAR protein, was extensively dialyzed against 1X PBS before proceeding with the in vitro assay. This fraction was analyzed by ELISA with anti-HA-HRP antibody (ROCHE, 12013819001) and quantified using the Micro BCA kit (THERMO SCIENTIFIC, 23235).

ELISA assays. Three wells of an ELISA plate were coated with 50 μL of the second eluted fraction and incubated for 2 h at 37°C. After decanting and washing twice with 1X PBS-0.05% Tween (PBST), the wells were blocked with 150 μL of 3% BSA-1X PBS for 1 h at 37°C. The wells were decanted and washed 3 times with PBST, and 50 μL of anti-HA-HRP (ROCHE) diluted 1:1000 in 1% BSA-1X PBS was added and
incubated for 1 h at 37°C. The plate was washed 3 times, and 50 μL of peroxidase substrate (20 mg/mL ABTS, 1X citrate buffer, 0.3% H₂O₂) was added to each well. The plate was incubated in the dark, and optical density was measured at 405 nm after 15 min. To determine whether the vNAR recognizes the antigen, a recognition ELISA was performed. For this assay, 250 ng/well of rhTNFα was added to an ELISA plate and incubated overnight at 4°C. After blocking with BSA and 3 washing steps with PBST, 100 μL of the second fraction of each clone was added and incubated for 2 h at 37°C. Three washing steps were performed, and 50 μL of anti-HA-HRP diluted 1:1,000 in 1% BSA-1X PBS was added and incubated for 1 h at 37°C. The antibody solution was discarded, and 3 final washing steps were performed. Then, 50 μL of ABTS substrate was added, and the plate was incubated in the dark until the color developed. The change of color was read at 405 nm in a Benchmark Microplate Reader (BIORAD, 170–6850).

**SDS-PAGE and western blot.** For precipitation, 1 mL of the purified vNAR was mixed with 250 μL of 100% TCA (SIGMA, T6399) and the tube was incubated overnight at 4°C. The sample was then centrifuged and washed with 1 mL of cold acetone. The pellet was resuspended in 30 μL of 2X loading buffer, boiled for 10 min at 95°C and then placed on ice. A 12% tricine SDS-PAGE was run at 50 mA for 2 h and stained with Coomassie brilliant blue, the gel was dehydrated using Gel Dryer model 583 (BIORAD, 1651745). Broad range protein molecular marker was used (BIORAD, V8491). For the western blot, the 12% tricine gel was transferred to a nitrocellulose membrane (BIORAD, 162–0115) for 1 h at 100 mA using a Trans Blot Semi-dry Electrophoretic Transfer Cell (BIORAD, 170–3,940). The membrane was blocked with 3% BSA-PBST for 1 h at room temperature with constant agitation. The blocking solution was discarded, and anti-HA-HRP diluted 1:1,000 in 1% BSA-PBST was added (ROCHE, 12013819001). The reaction was incubated for 1 h at 37°C with agitation. The antibody solution was discarded, and the membrane was washed three times with PBST for 2 min each. A page ruler plus prestained was used (Fermentas, SM1811). The membrane was incubated in a DAB solution (PIERCE, 34001) enhanced with NiSO₄ (1 mg/mL DAB dissolved in 50 mM Tris pH 7.2, 3% H₂O₂, 0.08% NiSO₄) until color developed.

**Thermostability of the vNAR.** To demonstrate the thermostability of the vNAR, we added 50 μL per well of 1 μg/mL rhTNFα to an ELISA plate in triplicate. The plate was incubated for 1 h at 37°C. Then, 150 μL per well of blocking solution was added and incubated for 1 h at 37°C. The blocking solution was decanted, and the plate was washed 3 times with PBST. Previously, the purified vNAR clone T43 was heated at 95°C for 5, 10, 20, 30, 40, 50 or 60 min. After heating, tubes were placed on ice. Fifty μL of heated protein was added per well and incubated for 1 h at 37°C. To determine whether the vNAR retained its antigen recognition capacity, we used 1% BSA as a negative control and a non-heated vNAR T43 was used as a positive control.

![Figure 5. Thermostability ELISA assays.](image)

In order to establish the neutralizing dose (ND₅₀) for each positive clone, the mouse L929 (ATCC, CCL-1) fibrosarcoma cell line was grown in Dulbecco modified eagle medium (GIBCO) supplemented with 10% horse serum and 1 μg/mL actinomycin D. For each vNAR clone 24,000 cells were added to each well and incubated for 1 h at 37°C. Then, 150 μL per well of blocking solution was added and incubated for 1 h at 37°C. The blocking solution was decanted, and the plate was washed 3 times with PBST. Fifty μL of anti-HA-HRP antibody diluted 1:1,000 in 1% BSA-1X PBS was added to each well. The plate was incubated for 1 h at 37°C. The solution was removed, and the wells were washed 3 times with PBST. Fifty μL of ABTS substrate was added, and the plate was incubated in the dark until the color developed. The change of color was read at 405 nm in a Benchmark Microplate Reader (BIORAD, 170–6850).

**In vitro neutralization assays.** To establish the neutralizing dose (ND₅₀) for each positive clone, the mouse L929 (ATCC, CCL-1) fibrosarcoma cell line was grown in Dulbecco modified eagle medium (GIBCO) supplemented with 10% horse serum and 1 μg/mL actinomycin D. For each vNAR clone 24,000 cells were added to each well and incubated for 1 h at 37°C. Then, 150 μL per well of blocking solution was added and incubated for 1 h at 37°C. The blocking solution was decanted, and the plate was washed 3 times with PBST. Fifty μL of anti-HA-HRP antibody diluted 1:1,000 in 1% BSA-1X PBS was added to each well. The plate was incubated for 1 h at 37°C. The solution was removed, and the wells were washed 3 times with PBST. Fifty μL of ABTS substrate per well was then added and incubated for 30 min at 37°C. The result was analyzed at 405 nm. Analysis was done with the GraphPad Prism v 4.02 program (GraphPad Inc.).
cells per well were incubated in 96-well plate in triplicate for 6 h with 5% CO₂, and humidity. SLD₅₀ (1.25 ng/mL) of rhTNFα was added to each well, combined with serial dilutions of purified vNAR protein were used as follow: 100, 33.3, 11.1, 3.7, 1.2, 0.41, 0.13, 0.04, 0.015 µg/mL. The assay was incubated for 18 h with 5% CO₂ at 37°C. To measure cell survival, 20 µL/well of Cell Titer One (Promega, G3582) was added and incubated at 30°C for 4 h, and the absorbance was read at 490 nm. An F(ab')₂ anti-TNFα (BIOCLON) previously proved was used to construct a reference curve for neutralization using the same concentration as the vNAR protein. The negative control was cells without vNAR antibodies. GraphPad Prism version 4.02 was used (GraphPad Inc.) for data analysis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
1. Deffas K, Shi H, Li L, Wang X, Zhu X. Nanobodies: the new concept in antibody engineering. Afr J Biotechnol 2009; 8:2645-52.
2. Wesolowski J, Alzogaray V, Reyelt J, Unger M, Juarez K, Urrutia M, et al. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. Med Microbiol Immunol 2009; 198:157-74; PMID:19529959; http://dx.doi.org/10.1007/s00430-009-0116-7.
3. Hudson PJ, Soutiaux C. Engineered antibodies. Nat Med 2003; 9:129-34; PMID:12514726; http://dx.doi.org/10.1038/nm103-129.
4. Dooley H, Flajnik MF, Porter AJ. Selection and characterization of naturally occurring single-domain (IgNAR) antibody fragments from immunized sharks by phage display. Mol Immunol 2003; 40:25-33; PMID:12990128; http://dx.doi.org/10.1016/S0161-5890(03)00084-1.
5. Roux KH, Greenberg AS, Greene L, Strelea L, Avila D, McKinney EC, et al. Structural analysis of the nurse shark (new) antigen receptor (NAR): molecular convergence of NAR and unusual mammalian immunoglobulins. Proc Natl Acad Sci USA 1998; 95:11804-9; PMID:9751746; http://dx.doi.org/10.1073/pnas.95.20.11804.
6. Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, Flajnik MF. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. Nature 1995; 374:168-73; PMID:7877689; http://dx.doi.org/10.1038/374168a0.
7. Nurtall SD, Krishnan UV, Hartrick M, De Gori R, Irving RA, Hudson PJ. Isolation of the new antigen receptor from webbogang sharks, and use as a scaffold for the display of protein loop libraries. Mol Immunol 2001; 38:313-26; PMID:11566324; http://dx.doi.org/10.1016/S0161-5890(01)00057-8.
8. Stanfield RL, Dooley H, Flajnik MF, Wilson IA. Crystal structure of a shark single-domain antibody V region in complex with lysisومة. Science 2004; 305:1770-3; PMID:15319492; http://dx.doi.org/10.1126/science.1101148.