Generation of tumour-necrosis-factor-α-specific affibody[1] molecules capable of blocking receptor binding *in vitro*

Andreas Jonsson*†, Helena Wållberg*, Nina Herne‡, Stefan Ståhl* and Fredrik Y. Frejd†§[2]

**Division of Molecular Biotechnology, Royal Institute of Technology (KTH), AlbaNova University Center, SE-106 91 Stockholm, Sweden, †Affibody AB, Box 20137, SE-161 02 Bromma, Sweden, ‡NeuroNova AB, Fiskartorpsvägen 15, SE-114 33 Stockholm, Sweden, and §Unit of Biomedical Radiations Sciences, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden*

Affibody molecules specific for human TNF-α (tumour necrosis factor-α) were selected by phage-display technology from a library based on the 58-residue Protein A-derived Z domain. TNF-α is a proinflammatory cytokine involved in several inflammatory diseases and, to this day, four TNF-α-blocking protein pharmaceuticals have been approved for clinical use. The phage selection generated 18 unique cysteine-free affibody sequences of which 12 were chosen, after sequence cluster analysis, for characterization as proteins. Biosensor binding studies of the 12 *Escherichia coli*-produced and IMAC (immobilized-metal-ion affinity chromatography)-purified affibody molecules revealed three variants that demonstrated the strongest binding to human TNF-α. These three affibody molecules were subjected to kinetic binding analysis and also tested for their binding to mouse, rat and pig TNF-α. For $Z_{TNF-\alpha:185}$, subnanomolar affinity ($K_D = 0.1–0.5$ nM) for human TNF-α was demonstrated, as well as significant binding to TNF-α from the other species. Furthermore, the binding site was found to overlap with the binding site for the TNF-α receptor, since this interaction could be efficiently blocked by the $Z_{TNF-\alpha:185}$ affibody. When investigating six dimeric affibody constructs with different linker lengths, and one trimeric construct, it was found that the inhibition of the TNF-α binding to its receptor could be further improved by using dimers with extended linkers and/or a trimeric affibody construct. The potential implication of the results for the future design of affibody-based reagents for the diagnosis of inflammation is discussed.

Introduction

Therapeutic targeting and imaging of inflammatory mediators represent important recent strategies in medicine. New classes of biomolecules, engineered to selectively bind to, and block, cytokines, interleukins and similar proteins, opens up new possibilities for diagnosing and treating diseases related to inflammatory processes.

TNF-α (tumour necrosis factor-α) is a homotrimeric proinflammatory cytokine produced by monocytes and macrophages. It mediates the immune response by recruiting white blood cells to sites of inflammation and is involved in acute responses to injury and infection. TNF-α is also a central component in autoimmune and chronic inflammatory diseases such as rheumatoid arthritis, spondylarthropathies, psoriasis and inflammatory bowel disease [1–4]. There are currently four approved TNF-α-blocking protein drugs available for clinical use, namely infliximab, adalimumab, certolizumab pegol and etanercept [1,5–7]. These drugs are considered to be highly effective, but they are costly and there are patients that discontinue these treatments because of toxicity, infection, or, for unknown reasons, lack of efficacy [8]. It is therefore important to improve methods to correctly characterize and diagnose pathological conditions where TNF-α is a key molecule.

Several imaging techniques have been explored for diagnosis of inflammatory processes, including X-ray, computer tomography and radionuclide-based imaging [9–12]. Radionuclide-based imaging has the advantage of offering detection of any desired target molecule, e.g. TNF-α, if there

Key words: affibody molecule, albumin-binding-domain-based affibody-screening ELISA (ABAS ELISA), biospecific interaction analysis (BIA), immobilized-metal-ion affinity chromatography (IMAC), phage display, tumour necrosis factor-α (TNF-α).

Abbreviations used: ABD, albumin binding domain; ABAS ELISA, ABD-based affibody-screening ELISA; cfu, colony-forming unit; HBS-EP buffer, 10 mM Hepes, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20, pH 7.4; HER2, human epidermal-growth-factor receptor 2 (also known as ErbB-2); His_6, hexhistidine; HSA, human serum albumin; HRP, horseradish peroxidase; IMAC, immobilized-metal-ion affinity chromatography; IPTG, isopropyl β-D-thiogalactoside; LC/MS, liquid chromatography/MS; PEG, poly(ethylene glycol); SA, streptavidin; SPR, surface plasmon resonance; TNF-α, tumour necrosis factor-α; TPBS, 0.05% (v/v) Tween 20 and 0.02% (w/v) sodium azide in PBS; TSB, tryptic soy broth; TSB + YE, TSB plus yeast extract; VHH, single-domain variable heavy-chain antibody from camelids.

[1] The phrase 'affibody molecule' is used in the present paper instead of 'Affibody® molecule'. Affibody is a trademark owned by Affibody AB and is registered in Sweden, Europe, and the United States and under trademark application in Japan.

[2] To whom correspondence should be addressed (email fredrik.frejd@affibody.com).

is a tracer available that could specifically bind to the target molecule. As imaging tracers ideally should be rather small, able to withstand harsh chemical labelling conditions and yet capable of high-affinity recognition of target proteins, there are a limited number of basic molecular structures to explore as recognition scaffolds. Full-size antibodies generally have good affinity, but demonstrate too slow kinetics, and small peptides, with a few exceptions, often have too poor an affinity to be considered as a general tool for creating imaging agents. There are, however, some promising imaging examples using antibody fragments, and a formatted TNF-α-binding VHH (single-domain variable heavy-chain antibody from camelids) has been reported previously [13].

Affibody molecules are a class of small protein domains that have also been demonstrated previously to be good imaging agents in a clinical setting. The proteins are based on a 58-amino-acid three-helical-bundle scaffold Z, derived from staphylococcal Protein A [14]. By randomizing 13 solvent-exposed residues on helices 1 and 2, a combinatorial library can be created at the binding surface [15]. By displaying the library on a bacteriophage, and subjecting the bacteriophage to a biopanning procedure against any target proteins of choice, specific binders can be obtained with a typical affinity range of 0.1–100 nM. The binding strength can be improved by an affinity maturation procedure if a higher affinity should be required. Affibody molecules against a range of targets have been isolated [16], and preclinical studies have reported the imaging of both HER2 (human epidermal-growth-factor receptor 2; also known as ErbB-2) [17] and epidermal growth factor [18] (also reviewed in Nilsson and Tolmachev [19]). ^{111}In- and ^{68}Ga-labelled affibody molecules have also been used for detection of HER2-expressing metastatic lesions in breast-cancer patients [20].

In the present study we selected affibody molecules specific for TNF-α. The selected molecules were characterized in terms of their affinity and cross-species binding capacity. The binding site was investigated by a competition assay. Furthermore, a number of bivalent, as well as one tervalent, affibody constructs were generated with different linker lengths between the affibody moieties, and the blocking effect for these constructs of TNF-α's binding to its cellular receptor was evaluated. The potential use of this kind of engineered binding proteins in the imaging of inflammatory reactions is discussed.

Materials and methods

Strains, vectors and phagemid library

The phagemid vector pAffi1 and the phagemid library Zlib2002 (3.3×10^9 members) used in selection procedures have previously been described by Grönwall et al. [21]. The *Escherichia coli* strain TG1 was used as the bacterial host for phage production. Selected clones were subcloned into the expression vector pAY430, carrying a T7 promoter [22], a multiple cloning site, a kanamycin-resistance gene and DNA sequences encoding an N-terminal His_6 (hexahistidine) tag and, for site-specific labelling purposes, a codon for a C-terminal cysteine residue. The *E. coli* strain Top 10 was used as the bacterial host during further cloning procedures and the *E. coli* strain BL-21(DE3) was used for protein expression from the expression vector.

Phage selection

Recombinant TNF-α (Roche Diagnostics Inc., Stockholm, Sweden) was used as the target protein during phage-selection procedures. The TNF-α was biotinylated using EZ-link® Sulfo-NHS-LC-Biotin [sulfosuccinimidyl-6-(biotinamido)-hexanoate; Pierce, Rockford, IL, U.S.A.]. Biotin dissolved in water was added to 200 μg of TNF-α for a final 20-fold molar excess of biotin over TNF-α. The mixture was incubated at room temperature (25 °C) for 30 min. Unbound biotin was removed by separation on size-exclusion NAP-5 columns (GE Healthcare, Uppsala, Sweden) according to the manufacturer's recommendations, followed by dialysis against PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl and 8.1 mM Na_2HPO_4, pH 7.4) using a Slide-A-Lyser® (Pierce) dialysis cassette. The successful biotinylation of TNF-α was validated using an Agilent technologies (Stockholm, Sweden) LC/MS (liquid chromatography/MS) system.

Preparation of phage stocks from Zlib2002 and between selections was performed according to previously described methods [15,21] using the helper phage M13KO7 (New England Biolabs, Beverly, MA, U.S.A.). Phages were precipitated by addition of PEG [poly(ethylene glycol)]/NaCl, typically yielding phage titres of approx. 10^{13} cfu (colony-forming units)/ml.

The selection was carried out in solution, and the bound phages were captured on SA (streptavidin)-coated paramagnetic beads (Dynabeads M-280 Streptavidin; Dynal, Oslo, Norway). In order to avoid non-specific binding, all tubes and SA beads used in the selection were pre-treated with 5% (w/v) BSA in TPBS [0.05% (v/v) Tween 20 and 0.02% (w/v) sodium azide in PBS]. Furthermore, phages were pre-incubated with SA beads supplemented with 20 nM biotinylated bungarotoxin (Sigma–Aldrich, Stockholm, Sweden) for 1 h at room temperature for the purpose of excluding binders of both biotin and SA.

Four rounds of biopanning were performed with increasing selection pressure in each round, i.e. decreasing target concentration and/or increasing number of washes. In the first round, phages (2.0×10^{13} cfu/ml) were incubated with 100 or 10 nM biotinylated TNF-α in 2.4 ml of 3% BSA in TPBS. In the second, third and fourth rounds, fractions

of phages eluted in the previous round were incubated with 100, 20, 10 and 2 nM (round 2) and 100, 20, 4, 10, 2 and 0.4 nM (rounds 3 and 4) biotinylated TNF-α. The selection mix was incubated overnight at 4 °C in the first round of panning and at room temperature for 1 h in the second, third and fourth panning cycles. Bound phages were captured by incubation with SA beads for 15 min at room temperature under slight shaking. The beads were washed in 3% BSA in TPBS (2, 4, 8 and 11 times in the first, second, third and fourth panning round respectively). Each washing step lasted for approx. 30 sec, apart from in the fourth round of panning, where the final washing was allowed to continue for 1 h in order to favour the selection of affibody molecules with a slow off-rate. Phages were eluted with 500 μl of 0.05 M glycine/HCl, pH 2.2, for 10 min at room temperature, followed by immediate neutralization with 50 μl of 1 M Tris/HCl, pH 8.0, and 450 μl of PBS. Eluted phages were used to infect exponential-phase *E. coli* TG1 cells after each round of panning. After 30 min incubation with gentle agitation and 30 min with rigorous agitation at 30 °C, the infected cells were centrifuged, the pellet dissolved in a small volume and spread on TSB + YE (tryptic soy broth plus yeast extract) plates and incubated overnight at 37 °C. Colonies were resuspended in TSB (30 g/l; Merck, Darmstadt, Germany) and inoculated (approx. 100-fold excess of cells compared with the phage titre after elution) in 100 ml of TSB + YE (5 g/l) medium, supplemented with 2% (w/v) glucose and 100 μg/ml ampicillin (Amresco, Solon, OH, U.S.A.). The cells were allowed to grow to an attenuance (D_{600}) of 0.5 at 37 °C. A 10 ml portion of the culture was infected with M13K07 helper phages for 30 min, pelleted by centrifugation (4000 ***g*** for 15 min at 4 °C) and resuspended in 100 ml of TSB + YE medium supplemented with 100 μM IPTG (isopropyl β-D-1-thiogalactopyranoside; Acros Organics, Geel, Belgium), 50 μg/ml kanamycin (Amresco) and 100 μg/ml ampicillin, with subsequent incubation overnight at 25 °C. Phagemid particles were purified and concentrated by precipitation with PEG, followed by filtration through a 0.45-μm-pore-size filter (Sartorius AG, Göttingen, Germany). Isolated phages were precipitated and dissolved in 1 ml of PBS.

ELISA screening of TNF-α-binding affibody molecules

Selected anti-TNF-α affibody molecules were screened for their TNF-α-binding ability in an ABAS ELISA [ABD (albumin binding domain)-based affibody-screening ELISA]. A total of 279 clones from all six concentrations in the final biopanning were produced by inoculation of single colonies in 1 ml of TSB + YE medium supplemented with 100 μg/ml ampicillin and 1 mM IPTG in 96-deepwell plates (Nunc, Roskilde, Denmark) and grown overnight at 37 °C. Cells were centrifuged (3000 ***g*** for 15 min), resuspended in 400 μl of 0.1% Tween 20 in PBS and frozen at −80 °C. The frozen samples were thawed in a water bath, followed by centrifugation (3700 ***g*** for 40 min) to clarify the periplasmic fraction. Supernatants containing affibody–ABD fusion proteins were collected and stored at 4 °C until analysed by ELISA as follows. A 100 μl portion per well of the affibody solution was added to pre-blocked [with 0.5% casein (Sigma–Aldrich) in PBS] HSA (human serum albumin; Sigma–Aldrich)-coated [6 μg/ml in ELISA coating buffer (Merck, Darmstadt, Germany)] 96-well high-binding microtitre plates (Costar, Amsterdam, The Netherlands) and incubated for 1.5 h at room temperature. Biotinylated TNF-α (1 μg/ml in 0.5% Tween 20 in PBS) was added to the wells and the mixture was incubated for a further 1.5 h at room temperature. Binding of TNF-α was visualized using HRP (horseradish-peroxidase)-conjugated SA (Dako, Glostrup, Denmark). The reaction was allowed to continue for 1 h before the addition of 100 μl of TMB (tetramethylbenzidine) substrate solution (Immunopure TMB Substrate Kit; Pierce). After 30 min incubation in darkness, 100 μl of stop solution (2 M H_2SO_4) was added and the A_{450} was measured using a Tecan (Männedorf, Switzerland) Sunrise spectrophotometer.

DNA sequencing and sequence clustering

DNA sequencing of phagemid (pAffi1) inserts was performed after four rounds of biopanning. In total, 80 clones, positive for TNF-α binding according to the ABAS ELISA, were chosen for sequencing using biotinylated specific sequencing primers and ABI PRISM® dGTP, BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (GE Healthcare) according to the manufacturer's recommendations. The sequencing reaction mixtures were purified using magnetic SA-coated beads on a Magnatrix 8000 (Magnetic Biosolutions) and analysed on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems). Sequencer™ software was used to record and analyse the sequence data obtained. Subcloned DNA fragments were verified by the same procedure.

The sequences of the selected affibody molecules were clustered using the average-link hierarchical clustering method, previously described in detail by Orlova et al. [17].

DNA constructs

Five different TNF-α binding affibody variants, namely $Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:194}$, $Z_{TNF\text{-}\alpha:198}$, $Z_{TNF\text{-}\alpha:207}$ and $Z_{TNF\text{-}\alpha:210}$, containing internal amber stop codons, were subjected to site-directed *in vitro* mutagenesis to replace the stop codon with a glutamine (Q) codon. A three-step PCR-based strategy was employed as follows: two separate PCR reactions using a forward vector primer together with a clone-specific reverse primer and a clone-specific internal forward primer together with a vector-specific reverse primer generated overlapping PCR fragments. In an assembly reaction, the

two PCR products were annealed and filled in. A PCR reaction using external primers was performed to amplify the fragment, followed by subsequent purification of the affibody-encoding DNA fragments using the QIAquick PCR purification kit (Qiagen).

In addition to the five TNF-α-binding affibody molecules subjected to mutagenesis (as described above), DNA fragments encoding seven other variants of TNF-α-binding affibody molecules ($Z_{TNF\text{-}\alpha:183}$, $Z_{TNF\text{-}\alpha:192}$, $Z_{TNF\text{-}\alpha:200}$, $Z_{TNF\text{-}\alpha:203}$, $Z_{TNF\text{-}\alpha:205}$, $Z_{TNF\text{-}\alpha:209}$ and $Z_{TNF\text{-}\alpha:210}$) were subcloned into the expression vector pAY430 by PCR sticky-end cloning. The expression vector was digested using the restriction endonuclease AccI (New England Biolabs), followed by dephosphorylation with calf intestinal alkaline phosphatase (Fermentas) and subsequent purification of the linear plasmid DNA using the QIAquick PCR purification kit. Two overlapping PCR products for each affibody molecule were hybridized (90 °C → 25 °C during 45 min), generating approx. 25 % of correct fragments with the AccI site. The hybridized affibody-encoding gene fragments were ligated into the cleaved expression vector using T4 DNA ligase (Fermentas), generating a fusion protein comprising one particular affibody variant with an N-terminal His_6 tag and a C-terminal cysteine residue.

The TNF-α binding affibody molecule $Z_{TNF\text{-}\alpha:185}$ was, in addition, subcloned as head-to-tail multimers via multiples of a linker5 (GGGGS)- or linker20 (GGGGSGGGGLVGLGSGGGGS)-encoding gene fragment, creating six dimeric constructs with five-, 10-, 15-, 20-, 40- and 60-amino-acid linkers respectively and one trimeric construct with 10-amino-acid linkers (the one-letter amino acid code is used above). The gene fragments encoding the linker5 (containing a BamHI restriction site) and the linker20 (containing an SfiI restriction site) were introduced into the vector by amplification of the $Z_{TNF\text{-}\alpha:185}$ fragment in two separate PCR reactions using specific primers. PCRs 1 and 2 resulted in incorporation of the linker-encoding gene fragment 3′ and 5′ respectively. The $Z_{TNF\text{-}\alpha:185}$-linker5-$Z_{TNF\text{-}\alpha:185}$ was constructed by PstI/BamHI (New England Biolabs) restriction digestion of PCR product 1 and BamHI/AccI restriction digestion of PCR product 2, followed by ligation of the two restriction products into the expression vector pAY430. The same strategy was applied when creating the linker20 construct, but with PstI/SfiI (New England Biolabs) and SfiI/AccI restriction digestion of the resulting PCR products 1 and 2. Repeats of the above-described linkers (to generate the linker10, linker15, linker40 and linker60 constructs) were obtained by BamHI or SfiI restriction digestion, followed by ligation of hybridized linker-encoding repeats. The trimeric form of the $Z_{TNF\text{-}\alpha:185}$ molecule was assembled by amplification of one $Z_{TNF\text{-}\alpha:185}$ encoding fragment introducing one additional $(linker5)_2$ encoding unit, followed by BamHI restriction digestion and ligation into the dimeric form of the same molecule. The resulting expression vectors, encoding seven different $Z_{TNF\text{-}\alpha:185}$ variants with individual linker lengths, were electroporated into *E. coli* BL21(DE3) cells.

Protein expression and purification

Anti-TNF-α affibody molecules were expressed as His_6-tagged fusion proteins from the expression vector pAY430 in the *E. coli* strain BL21(DE3). Cells were inoculated in 50 ml of TSB medium supplemented with 50 μg/ml kanamycin and grown overnight at 37 °C. A 5 ml portion of each overnight culture was used to inoculate 1000 ml of TSB + YE medium containing 50 μg/ml kanamycin and one drop of Breox anti-foam agent (Bröste AB) in shake flasks and cultures were allowed to grow at 37 °C, with agitation at 170 rev./min, until the D_{600} reached approx. 1. Gene expression was induced by addition of IPTG to a final concentration of 0.5 mM. Cultivation was allowed to continue for 5 h at 37 °C, after which cultures were harvested by centrifugation (16 000 ***g*** for 20 min at 4 °C) and stored at − 20 °C until protein purification.

For the initial characterization of the 12 monomeric TNF-α-binding affibody variants, His_6-tagged fusion proteins were purified by IMAC (immobilized-metal-ion affinity chromatography) using a Biorobot 3000 system (Qiagen). The proteins were purified under denaturing conditions on Ni^{2+}-nitrilotriacetate Superflow columns with QIAsoft 4.1 software according to the manufacturer's recommendations. The multimeric $Z_{TNF\text{-}\alpha:185}$-variants were IMAC-purified on His-Gravitrap columns (GE Healthcare) as follows. Cell pellets were dissolved in binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl and 20 mM imidazole, pH 7.4), followed by the addition of 4 μl of Benzonase (Merck) and the cells were disrupted by sonication. Cell debris was removed by centrifugation (25 000 ***g*** for 15 min at 8 °C) and the supernatant was applied to binding-buffer-equilibrated His-Gravitrap columns. Bound proteins were washed with washing buffer (20 mM NaH_2PO_4, 0.5 M NaCl and 60 mM imidazole, pH 7.4) before being eluted with 3 ml of elution buffer (20 mM NaH_2PO_4, 500 mM NaCl and 500 mM imidazole). Following purification, buffer exchange to PBS was performed on all proteins, using PD-10 columns (GE Healthcare) according to the manufacturer's recommendations. The protein concentration was determined by measuring the A_{280} on a NanoDrop® ND-1000 spectrophotometer (Saveen Werner AB). Finally, the purified proteins were analysed by SDS/PAGE on 4–12%-(w/v)-polyacrylamide NuPAGE gels (Invitrogen).

Biosensor analysis

A Biacore® 2000 instrument (GE Healthcare) was used for real-time BIA (biospecific interaction analysis) between affibody molecules and target proteins.

In the initial experiment, 12 selected TNF-α-binding affibody variants were analysed with respect to their affinity and specificity to the target. The affibody molecules were immobilized on a carboxylated-dextran layer in separate flow cells of a CM-5 sensor chip (GE Healthcare) using the ligand-thiol method recommended by the manufacturer. The analytes, namely human TNF-α, HSA and polyclonal human IgG (Biovitrum) were diluted in HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20, pH 7.4) to final concentrations of 0.6, 0.9 and 0.4 μM respectively and injected at a constant flow rate of 10 μl/min for 20 min, followed by injection of HBS-EP buffer for 5 min.

On the basis of their performance in the initial Biacore® screening experiment, three affibody variants were chosen for further kinetic analysis using SPR (surface plasmon resonance). $Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:192}$ and $Z_{TNF\text{-}\alpha:210}$ were immobilized on CM-5 sensor chips as described above. TNF-α diluted in HBS-EP buffer was injected in duplicate over the surface at concentrations ranging from 125 to 4 nM at a flow rate of 60 μl/min for 2.5 min, followed by injection of HBS-EP buffer for 10 min. The apparent dissociation equilibrium constant (K_D) was calculated using BIAevaluation 3.2 software (GE Healthcare), assuming a 1:1 Langmuir binding model.

In a third Biacore® experiment, the anti-TNF-α affibody molecules were analysed for their ability to bind TNF-α of non-human origin. The affibody variants with the highest affinity to human TNF-α ($Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:192}$ and $Z_{TNF\text{-}\alpha:210}$), as determined in the previous experimental set-up, were immobilized as described above. Human, mouse, rat and pig TNF-α (Prospec) dissolved in HSB-EP buffer were injected over the surface at concentrations ranging from 2 μM to 1 nM at a flow rate of 10 μl/min for 10 min, followed by dissociation for 2 h. Calculations were performed as described above.

In order to investigate whether the selected anti-TNF-α affibody molecule ($Z_{TNF\text{-}\alpha:185}$) binding site on TNF-α overlaps with the interaction site between TNF-α and its receptor (in the form of etanercept; p75; Wyeth), a competition experiment was performed. Etanercept was immobilized on the surface of CM-5 chips using amine coupling according to the manufacturer's recommendation. Human TNF-α was dissolved in HBS-EP buffer to a final concentration of 2 μM, together with a large excess of $Z_{TNF\text{-}\alpha:185}$ or etanercept (20 000:1 molar excess over TNF-α) and injected at a constant flow rate of 20 μl/min for 5 min, followed by dissociation for 5 min.

In all of the experimental procedures described above, one flow cell on each chip was activated and de-activated and used as a reference. The response measured in the reference cell was subtracted from the response received in cells harbouring immobilized affibody molecules. Surfaces were regenerated with two injections of 10 mM HCl and 25 mM HCl for thiol-coupled and amine-coupled surfaces respectively.

Inhibition of the TNF-α:TNF-α-receptor interaction using affibody $Z_{TNF\text{-}\alpha:185}$ multimers

The multimeric forms of $Z_{TNF\text{-}\alpha:185}$ expressed in-frame with variants of a $(GGGGS)_x$-linker were evaluated for their potential ability of inhibiting the TNF-α interaction with its receptor etanercept in an ELISA experiment. Half-area 96-well high-binding microtitre plates (Costar) were coated with etanercept (1 μg/ml in ELISA coating buffer; 50 μl/well) overnight at 4°C. The seven multimeric anti-TNF-α affibody variants were diluted in 0.5% TPBS to concentrations ranging from 1 μM to 4 nM and mixed with biotinylated TNF-α for a final concentration of 2.5 nM TNF-α and incubated at room temperature with slight shaking. After 2 h incubation, 50 μl of the affibody/TNF-α dilutions were transferred to blocked (2 h with 0.5% casein) etanercept-coated plates and incubated for a further 1 h at room temperature with shaking. HRP-conjugated SA (diluted 1:5000 in TPBS; 50 μl/well; 1 h incubation) was used to detect the binding of TNF-α to etanercept. The A_{450} was measured using an ELISA reader (Viktor 3; PerkinElmer) after incubation with the substrate solution (Immunopure TMB) for 1 h. All samples were run in triplicate.

Results

Phage selections of TNF-α-binding affibody molecules

TNF-α-specific affibody molecules were isolated from a combinatory protein library using phage display *in vitro* selection technology. Four rounds of panning were performed using biotinylated TNF-α as the target protein and SA-coated paramagnetic beads for in-solution capture of phage. The degree of biotinylation was determined using LC/MS analysis, indicating approx. 1.5 biotin molecules per TNF-α subunit, which was considered suitable. The selection pressure was increased throughout the biopanning procedure by decreasing the target protein concentration and/or increasing the number of washes. Figure 1 outlines schematically how target concentrations were varied in the different selection schemes and between selection cycles. The phage library used in cycle 1 was pre-selected against SA-coated magnetic beads with a bound biotinylated mock protein with the aim of removing possible non-specifically binding phage. After the fourth round of panning, 279 resulting clones expressing affibody molecules were screened for TNF-α-binding activity using an ELISA (Figure 2A). The six panels show the ELISA results from clones corresponding to the six different selection schemes (as depicted in Figure 1). Positive clones did appear after five

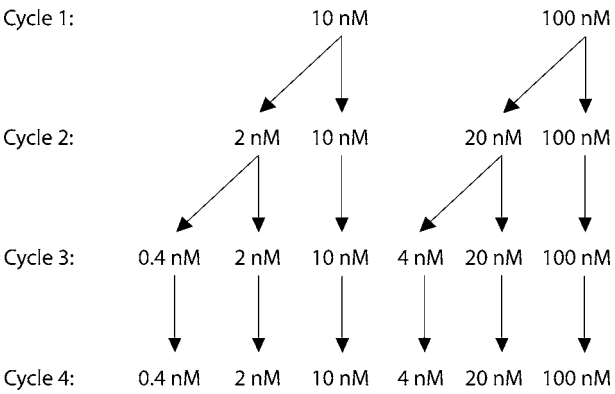

Figure 1 An overview of the scheme for the selection against TNF-α

Target protein concentrations used for each biopanning cycle (1–4) are shown.

out of six selection schemes. The most successful scheme in this analysis was when the target concentration of 10–20 nM was employed and maintained through cycles 2–4.

DNA sequencing was performed on 80 ELISA-positive clones, identifying 31 unique phagemid inserts, occurring one to ten times. The 18 sequences lacking cysteine residues are outlined in Figure 2(B). Sequence cluster analysis using so-called 'average-link hierarchical clustering' [17] revealed one dominating cluster containing the most frequently appearing clone $Z_{TNF\text{-}\alpha:185}$, but also several other smaller clusters. A total of 12 different affibody variants from the TNF-α selection, with representatives from the different groups of sequences, were chosen for further characterization.

Production of the generated affibody proteins

The twelve different affibody molecules ($Z_{TNF\text{-}\alpha:183}$, $Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:192}$, $Z_{TNF\text{-}\alpha:194}$, $Z_{TNF\text{-}\alpha:198}$, $Z_{TNF\text{-}\alpha:200}$, $Z_{TNF\text{-}\alpha:203}$, $Z_{TNF\text{-}\alpha:205}$, $Z_{TNF\text{-}\alpha:207}$, $Z_{TNF\text{-}\alpha:209}$, $Z_{TNF\text{-}\alpha:210}$ and $Z_{TNF\text{-}\alpha:211}$) from the TNF-$\alpha$ selection were chosen for further analysis on the basis of the following criteria: (1) positive results in the ELISA, (b) appearance frequency for the particular clone and (c) sequence clustering results. Five of the clones ($Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:194}$, $Z_{TNF\text{-}\alpha:198}$, $Z_{TNF\text{-}\alpha:207}$ and $Z_{TNF\text{-}\alpha:210}$), each containing an amber-stop (TAG) codon, were subjected to a three-step PCR to replace the stop codon with a glutamine (Q) codon (CAG) by site-directed *in vitro* mutagenesis. The gene fragments encoding the 12 affibody molecules were subcloned for expression in *E. coli* as monomeric affibody constructs fused to an N-terminal His_6 peptide tag and a unique C-terminal cysteine residue for directed labelling. The affibody proteins were expressed intracellularly in *E. coli* and purified on IMAC columns. The purified proteins were observed as distinct bands of the expected molecular mass when analysed by SDS/PAGE, indicating pure and stable affibody molecules (results not shown).

Biosensor binding studies and species analysis of selected affibody molecules

Binding of the 12 candidate affibody molecules to TNF-α was investigated by SPR biosensor technology using a Biacore® instrument. Taking advantage of the C-terminal cysteine residues, the monomeric ligands were thiol-coupled to the biosensor chip and TNF-α was subsequently injected over the surface. The biosensor analysis revealed that three affibody molecules, $Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:192}$ and $Z_{TNF\text{-}\alpha:210}$, displayed the strongest binding to TNF-α (results not shown). As expected, none of the affibody proteins showed any binding to the control proteins, human polyclonal IgG or HSA (results not shown), indicating specific binding to TNF-α.

Since it is likely that future studies of the TNF-α-binding affibody molecules would involve *in vivo* investigations in various animal models, it was of relevance to evaluate how the affibody variants with the highest affinity to human TNF-α ($Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:192}$ and $Z_{TNF\text{-}\alpha:210}$) bound TNF-$\alpha$ from different species. The three anti-TNF-α affibody molecules were thus immobilized on a biosensor chip as described above, and TNF-α of human, mouse, rat and pig origin respectively was injected over the surface at various concentrations. It was demonstrated that the three affibody molecules bound TNF-α from all four species, and $Z_{TNF\text{-}\alpha:185}$ was again the best binder as reflected by a significantly lower dissociation rate (Figure 3A). A comparison of the binding of $Z_{TNF\text{-}\alpha:185}$ to TNF-α from the four species is depicted as overlay sensorgrams in Figure 3(B). It can be concluded that the interaction with human TNF-α had the highest binding strength, which is not surprising since human TNF-α was used as the target protein in the phage selections. The binding to mouse and rat TNF-α seemed to be reasonably good, with a relatively low dissociation rate, in contrast with the binding to pig TNF-α, where a higher dissociation rate could be observed (Figure 3C).

$Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:192}$ and $Z_{TNF\text{-}\alpha:210}$, were further characterized by kinetic binding analyses. The monomeric affibody molecules $Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:192}$ and $Z_{TNF\text{-}\alpha:210}$ were immobilized on biosensor chips and TNF-α was injected over the surface at (monomeric) concentrations ranging from 125 to 4 nM. The apparent dissociation equilibrium constant (K_D) was calculated, assuming a 1:1 Langmuir binding model. The apparent dissociation equilibrium constants (K_D) were estimated to be 0.1–0.5 nM for $Z_{TNF\text{-}\alpha:185}$, 2–5 nM for $Z_{TNF\text{-}\alpha:210}$ and 10–20 nM for $Z_{TNF\text{-}\alpha:192}$. The affibody molecule showing the highest affinity for TNF-α was thus $Z_{TNF\text{-}\alpha:185}$, and the binding curves for the kinetic analysis of $Z_{TNF\text{-}\alpha:185}$ are shown in Figure 4(A).

Investigating the binding site on TNF-α

In order to investigate whether the binding site on TNF-α of the anti-TNF-α affibody molecule ($Z_{TNF\text{-}\alpha:185}$) overlaps

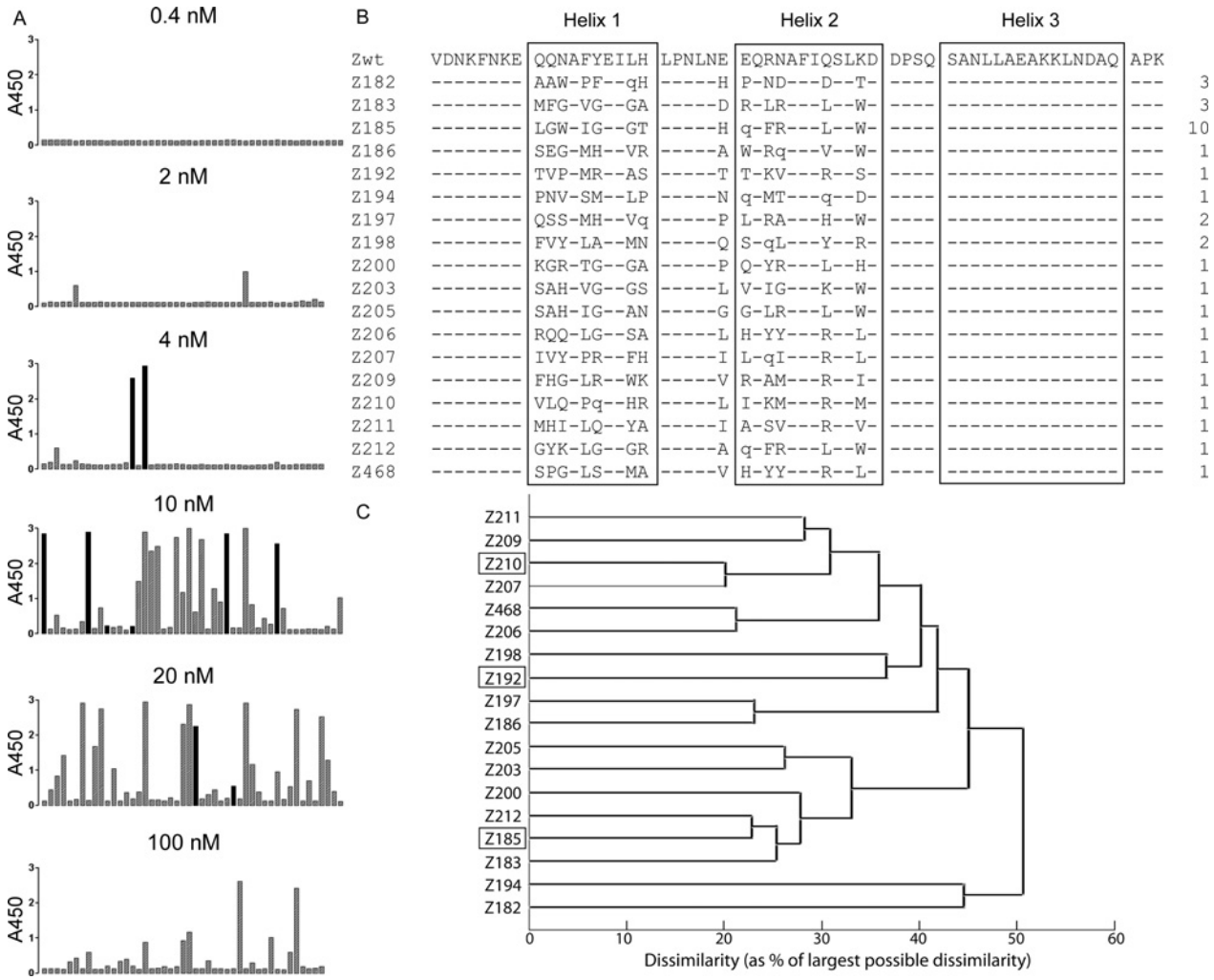

Figure 2 Screening, sequence alignment and clustering of clones after TNF-α selection

(**A**) Clones (45–48 per concentration) from selection cycle 4 were analysed in an ABAS ELISA for their TNF-α binding activity, each ELISA being marked with the final concentration used in cycle 4. Bars representing variant $Z_{TNF\text{-}\alpha:185}$ are highlighted in black. (**B**) Amino acid sequence of the wild-type Z domain aligned to amino acid sequences of 18 unique affibody variants showing binding activity in ELISA. The 13 randomized amino acid positions are presented; 'q' indicates an amber stop codon, and dashes (–) indicate amino acids identical with those in the wild-type Z domain. The values on the right indicate the number of times each variant was detected out of 279 clones. (**C**) Sequence clustering of affibody molecules using an average-link hierarchical clustering method, the result being illustrated in a dendrogram, with the three affibody variants selected for further screening boxed.

with the interaction site between TNF-α and its receptor, a competition experiment was performed. Etanercept, a recombinant TNF-α-receptor construct in clinical use under the brand name Enbrel [22a], was immobilized on the biosensor surface and human TNF-α, alone or mixed with an excess of $Z_{TNF\text{-}\alpha:185}$ or etanercept, was injected at a constant flow rate over the etanercept surface, followed by dissociation for 5 min. It was clearly demonstrated that the affibody molecule ($Z_{TNF\text{-}\alpha:185}$) and etanercept molecules recognize the same or overlapping epitopes on TNF-α, since the TNF-α-binding was efficiently blocked with both $Z_{TNF\text{-}\alpha:185}$ and etanercept (Figure 4B).

Affibody-mediated inhibition of the TNF-α–TNF-α receptor interaction

Since TNF-α exists as a homotrimer, it was of interest to investigate whether the inhibition of the interaction between TNF-α and its receptor could be further improved using dimeric affibody constructs with different linker lengths, or even trimeric constructs. Six different dimeric constructs were thus constructed, in which the two $Z_{TNF\text{-}\alpha:185}$ monomers were separated by linkers consisting of 5, 10, 15, 20, 40 or 60 amino acid residues respectively (Figure 5A). In addition, a trimeric affibody construct was generated that had three $Z_{TNF\text{-}\alpha:185}$ monomers separated by ten-amino-acid linkers (Figure 5A). The seven constructs were expressed

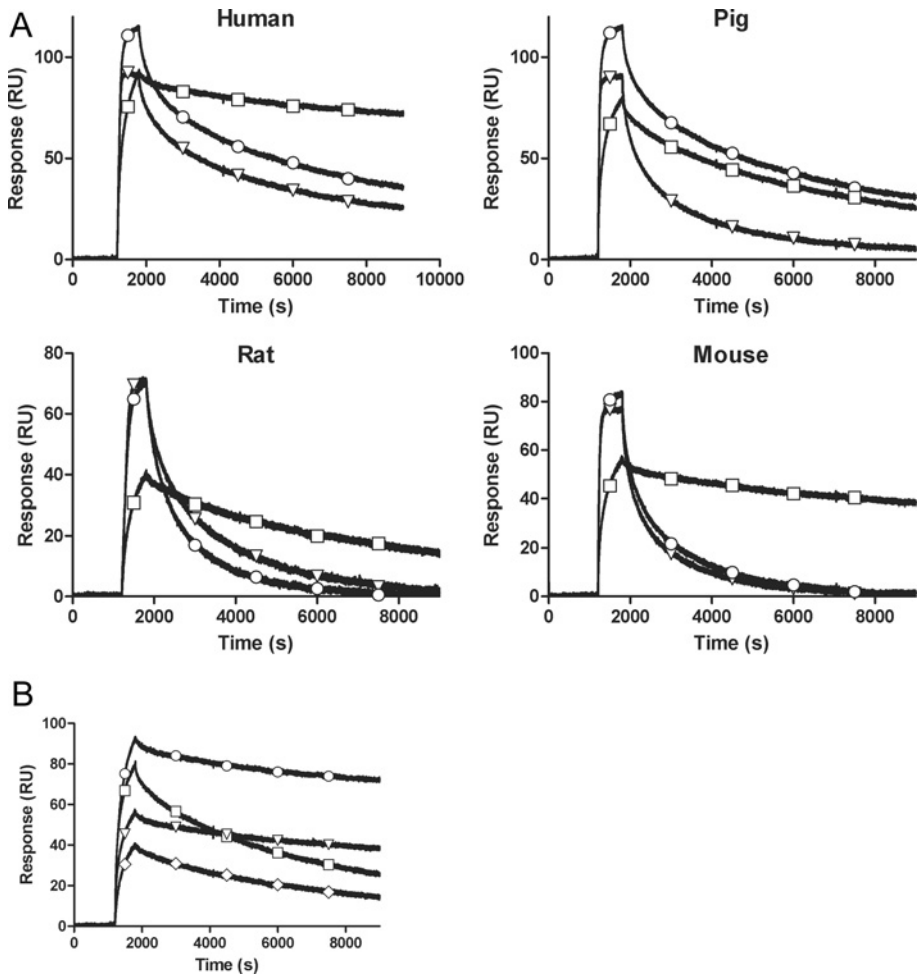

Figure 3 Biosensor analyses from a comparative study of the interactions between TNF-α from different species and affibody molecules $Z_{TNF\text{-}\alpha:185}$, $Z_{TNF\text{-}\alpha:192}$ and $Z_{TNF\text{-}\alpha:210}$

(**A**) Sensorgrams obtained after sequential injection of TNF-α molecules (400 nM) from human, pig, rat and mouse over biosensor-chip surfaces containing immobilized affibody molecules {$Z_{TNF\text{-}\alpha:185}$ (□), $Z_{TNF\text{-}\alpha:192}$ (○) and $Z_{TNF\text{-}\alpha:210}$ (Δ) via thiol coupling [~90 RU (response units) respectively]}. (**B**) A compilation from (**A**) for affibody molecule $Z_{TNF\text{-}\alpha:185}$ binding to TNF-α from different species human (○), pig (□), rat (◇) and mouse (Δ).

in *E. coli*, purified using IMAC and subsequently analysed by SDS/PAGE (Figure 5B). The purified proteins were observed as distinct bands of the expected molecular mass when analysed by SDS/PAGE, indicating pure and stable affibody fusion proteins (Figure 5B). The multimeric forms of $Z_{TNF\text{-}\alpha:185}$ were evaluated for their potential ability to inhibit the interaction of TNF-α with its receptor, etanercept, in an ELISA experiment. The seven multimeric anti-TNF-α affibody variants were diluted to concentrations ranging from 1 μM to 4 nM and mixed with biotinylated TNF-α. After 2 h incubation, the affibody/TNF-α dilutions were transferred to etanercept-coated microtitre plates. HRP-conjugated SA was used to detect binding of TNF-α to etanercept. It was demonstrated that all $Z_{TNF\text{-}\alpha:185}$ constructs could inhibit TNF-α-binding to its receptor in a concentration-dependent manner. Of the dimeric constructs, it was demonstrated that the ones having the 20- or 40-amino-acid linkers seemed to be the most potent inhibitors of TNF-α binding, and it was further found that the trimeric variant was able to inhibit TNF-α at lower concentrations than the dimeric variants (Figure 5C). Thus one more available binding site in the $Z_{TNF\text{-}\alpha:185}$ trimer resulted in a twice as potent inhibitor of TNF-α than the second-best-performing molecule.

Discussion

Inflammation is a prominent feature in many chronic disorders, but the means to diagnose inflammation are

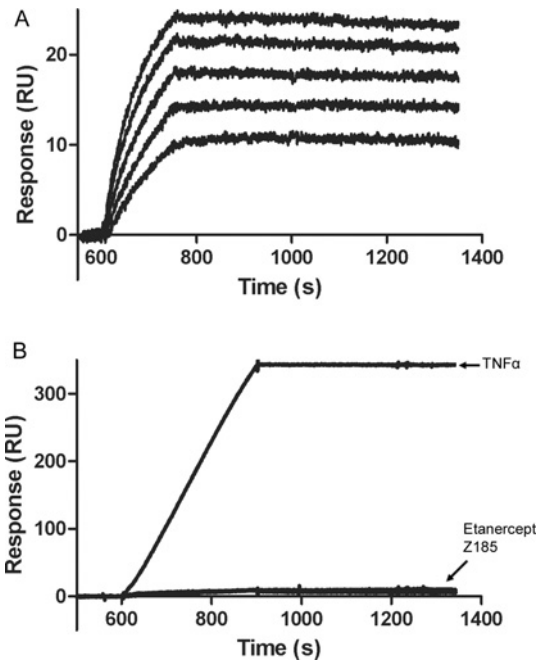

Figure 4 Affinity (K_D) determination for $Z_{TNF\text{-}\alpha:185}$ binding to human TNF-α and comparative study of inhibition between $Z_{TNF\text{-}\alpha:185}$ and etanercept

(**A**) Kinetic analysis of the interaction between $Z_{TNF\text{-}\alpha:185}$ and human TNF-α. Sensorgrams obtained after sequential double injection of TNF-α molecules at concentrations from 4 nM to 125 nM. (**B**) Sensorgrams obtained after sequential injection of TNF-α molecules (2 μM) with and without preincubation of $Z_{TNF\text{-}\alpha:185}$ or etanercept. RU, response units.

limited. A key component in the inflammatory process is TNF-α, and several TNF-α-blocking drugs have proved the clinical utility of blocking TNF-α in inflammatory disease. A TNF-α targeting agent that could aid in diagnosing and staging patients should be a useful reagent. In principle, any biomolecule could be used to target TNF-α. There are, however, differences in the application that may put certain constraints to the molecule, such as size and stability. For imaging agents, for example, small size is a key feature. There is a recent preclinical example of using the soluble TNF-α-receptor etanercept for TNF-α detection in a model of inflammation of the ear in mice [12]. Etanercept is however a relatively large molecule (150 kDa) designed for prolonged plasma residence time, which should restrict its use as an imaging agent in the clinic, since the prolonged half-life gives poor resolution in imaging. A more promising TNF-α-specific molecule was previously presented [13]. It consists of two 15 kDa TNF-α specific VHH domain antibodies, fused by a linker. The authors demonstrated imaging of TNF-α in a model of collagen-induced arthritis, but the background level for this 30 kDa protein is very high. We reasoned that it would be interesting to investigate a much smaller recognition unit for imaging applications, and hypothesized that affibody-molecule technology should be suitable, as affibody molecules have previously been shown to be good imaging agents, both in preclinical and in clinical studies [19]. We also wanted to test whether formatting the molecules into multivalent constructs could give any directions for a future therapeutic molecule. In the present study we have described the selection and *in vitro* characterization of TNF-α-specific affibody molecules. We furthermore show that their potency for blocking TNF-α from binding to its receptor *in vitro* can be enhanced by using different formats of the molecule, including dimers and a trimer with different linker lengths.

Phage display was used in a biopanning procedure, using decreasing concentrations of TNF-α as the antigen. Interestingly, the best set of binders appeared in a narrow final concentration window of 10–20 nM. Using higher antigen concentration resulted in dominating low-affinity binders. Using ELISA, a sequence-clustering algorithm and Biacore® ranking experiments, three candidates with high affinity were selected for further binding studies. It should be noted that only an 'apparent' affinity was determined, since TNF-α is a trimeric protein, which complicates the affinity determinations.

We also investigated species cross-reactivity and found acceptable binding strength for mouse and rat TNF-α, both representing relevant species for inflammation models. The *in vitro* Biacore® blocking study suggests that the monomeric affibody can block soluble TNF-α in a similar fashion to the soluble TNF-α receptor. This is in line with the results of Kronqvist et al. [23], who isolated TNF-α-binding affibody molecules using a different selection system based on staphylococcal display.

For imaging use, the small monomeric format is desirable. Given the relatively low concentration of antigen, and that much of it will be present in solution at the inflammatory site, we anticipate that affinity maturation may be warranted to increase the binding strength and therefore the detection sensitivity. For possible therapeutic applications, however, where efficient blocking is desirable, but small size is not essential, we considered the trimeric nature of the TNF-α ligand and constructed different affibody-molecule formats using bivalent molecules with different linker lengths, or even a trimeric construct. Bivalent constructs with a relatively long linker length of 20 or 40 amino acids had the best receptor-competing activity, along with the trimeric molecule, which had the best overall blocking ability. Further work with the trimeric variant, especially using different linker lengths of 20, or even 40, amino acids, could lead to a more potent molecule. It should be noted that the blocking experiments were not designed for measuring the affinity of the interaction between the affibody constructs and TNF-α in solution. Such binding experiments would need to be carefully optimized, since binding of the trimeric TNF-α molecule to the immobilized receptor

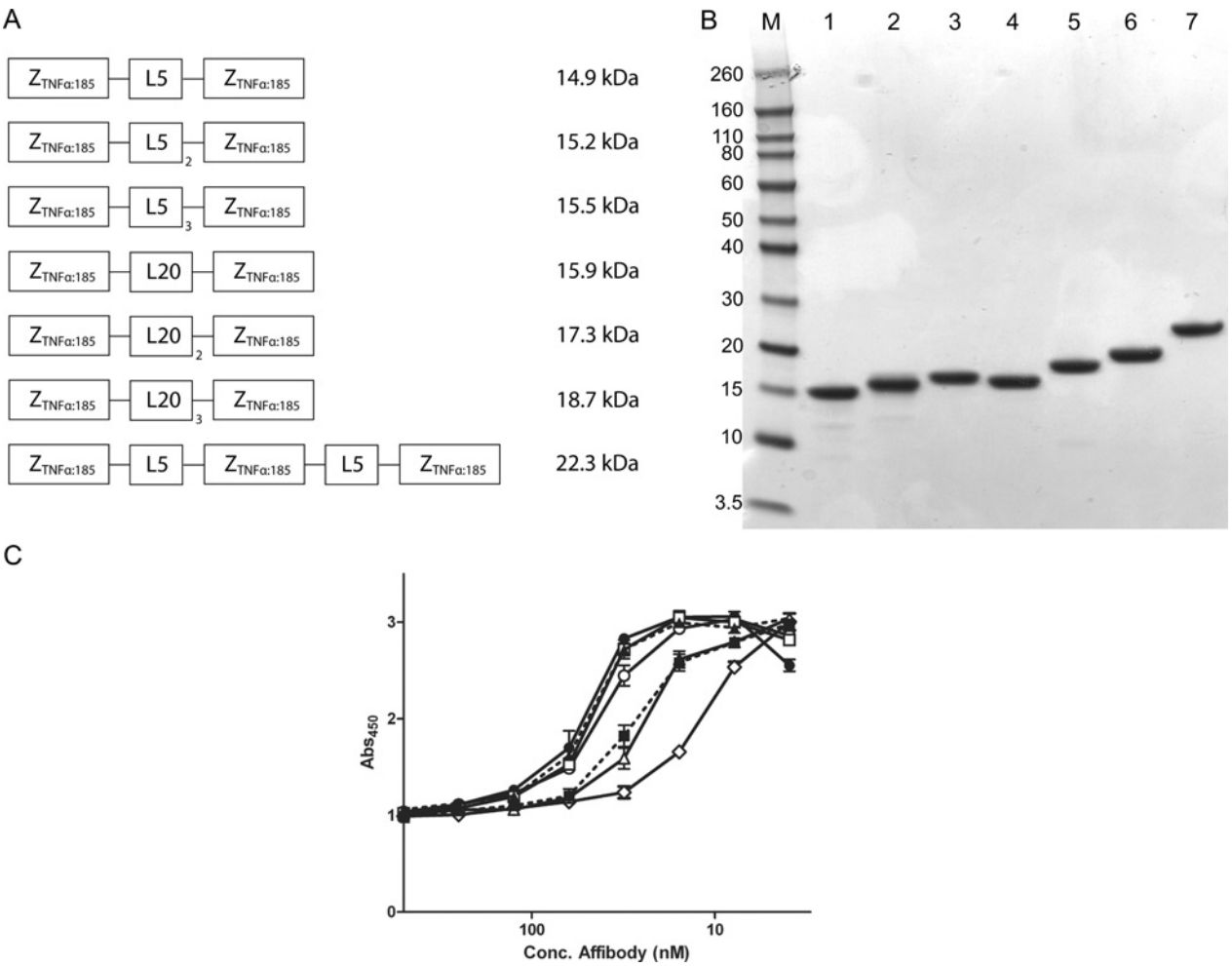

Figure 5 Schematic overview and TNF-α-blocking properties of the multimeric anti-TNF-α $Z_{TNF-\alpha:185}$ affibody variants

(**A**) A schematic overview of the di- and tri-meric affibody molecules with various lengths of connecting linker (L) between the affibody moieties. Linkers L5 and L20 comprise the amino acid sequences GGGGS and GGGGSGGGGLVGLGSGGGGS respectively. The molecular mass of the respective molecule is indicated to the right. (**B**) SDS/PAGE analysis of the IMAC-purified multimeric affibody variants $Z_{TNF-\alpha:185}$-L5-$Z_{TNF-\alpha:185}$ (lane 1), $Z_{TNF-\alpha:185}$-$(L5)_2$-$Z_{TNF-\alpha:185}$ (lane 2), $Z_{TNF-\alpha:185}$-$(L5)_3$-$Z_{TNF-\alpha:185}$ (lane 3), $Z_{TNF-\alpha:185}$-L20-$Z_{TNF-\alpha:185}$ (lane 4), $Z_{TNF-\alpha:185}$-$(L20)_2$-$Z_{TNF-\alpha:185}$ (lane 5), $Z_{TNF-\alpha:185}$-$(L20)_3$-$Z_{TNF-\alpha:185}$ (lane 6) and $Z_{TNF-\alpha:185}$-$(L5)_2$-$Z_{TNF-\alpha:185}$-$(L5)_2$-$Z_{TNF-\alpha:185}$ (lane 7). Lane M, marker proteins; molecular masses are indicated in kDa. (**C**) Inhibiting properties of the multimeric forms of $Z_{TNF-\alpha:185}$. The graph shows the detection [A_{450} (Abs_{450})] of biotinylated TNF-α after preincubation with increasing concentrations (Conc.) of $Z_{TNF-\alpha:185}$-L5-$Z_{TNF-\alpha:185}$ (○), $Z_{TNF-\alpha:185}$-$(L5)_2$-$Z_{TNF-\alpha:185}$ (●), $Z_{TNF-\alpha:185}$-$(L5)_3$-$Z_{TNF-\alpha:185}$ (□), $Z_{TNF-\alpha:185}$-L20-$Z_{TNF-\alpha:185}$ (··▲··), $Z_{TNF-\alpha:185}$-$(L20)_2$-$Z_{TNF-\alpha:185}$ (Δ), $Z_{TNF-\alpha:185}$-$(L20)_3$-$Z_{TNF-\alpha:185}$ (··■··) and $Z_{TNF-\alpha:185}$-$(L5)_2$-$Z_{TNF-\alpha:185}$-$(L5)_2$-$Z_{TNF-\alpha:185}$ (◊).

would be differently affected depending on whether one, two or three subunits would be bound by affibody molecules.

In conclusion, we show that it is possible to select affibody molecules that can bind to TNF-α from different species with reasonable affinity and that the best binder can block TNF-α from binding to its receptor *in vitro*. The blocking effect was increased by increasing valency of the binding molecule with a tuned linker length. Molecular data collected in the present study provides a strong knowledge base for affinity maturation, if increased binding affinity would be desired before undertaking *in vivo* studies for imaging and therapy.

Acknowledgements

We thank Dr Elin Gunneriusson (Affibody AB, Bromma, Sweden) for valuable technical advice and Dr Ingmarie Höidén-Guthenberg (Affibody AB, Bromma, Sweden) for critical reading of the manuscript before its submission.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

References

1 Wallis, R. S. (2008) Lancet Infect. Dis. **8**, 601–611
2 Tobin, A. M. and Kirby, B. (2005) BioDrugs **19**, 47–57
3 Stokkers, P. C. and Hommes, D. W. (2004) Cytokine **28**, 167–173
4 Van den Bosch, F., Kruithof, E., De Vos, M., De Keyser, F. and Mielants, H. (2000) Lancet **356**, 1821–1822
5 Bourne, T., Fossati, G. and Nesbitt, A. (2008) BioDrugs **22**, 331–337
6 Kaymakcalan, Z., Sakorafas, P., Bose, S., Scesney, S., Xiong, L., Hanzatian, D. K., Salfeld, J. and Sasso, E. H. (2009) Clin. Immunol. **131**, 308–316
7 Tracey, D., Klareskog, L., Sasso, E. H., Salfeld, J. G. and Tak, P. P. (2008) Pharmacol. Ther. **117**, 244–279
8 Bombardieri, S., Ruiz, A. A., Fardellone, P., Geusens, P., McKenna, F., Unnebrink, K., Oezer, U., Kary, S., Kupper, H. and Burmester, G. R. (2007) Rheumatology **46**, 1191–1199
9 Franquet, T. and Stern, E. J. (1999) Eur. Radiol. **9**, 1290–1303
10 Bleeker-Rovers, C. P., Boerman, O. C., Rennen, H. J., Corstens, F. H. and Oyen, W. J. (2004) Curr. Pharm. Des. **10**, 2935–2950
11 Tehranzadeh, J., Ashikyan, O. and Dascalos, J. (2003) Semin. Musculoskelet. Radiol. **7**, 79–94
12 Cao, Q., Cai, W., Li, Z. B., Chen, K., He, L., Li, H. C., Hui, M. and Chen, X. (2007) Eur. J. Nucl. Med. Mol. Imaging **34**, 1832–1842
13 Coppieters, K., Dreier, T., Silence, K., de Haard, H., Lauwereys, M., Casteels, P., Beirnaert, E., Jonckheere, H., Van de Wiele, C., Staelens, L. et al. (2006) Arthritis Rheum. **54**, 1856–1866
14 Nilsson, B., Moks, T., Jansson, B., Abrahmsen, L., Elmblad, A., Holmgren, E., Henrichson, C., Jones, T. A. and Uhlen, M. (1987) Protein Eng. **1**, 107–113
15 Nord, K., Gunneriusson, E., Ringdahl, J., Stahl, S., Uhlen, M. and Nygren, P. A. (1997) Nat. Biotechnol. **15**, 772–777
16 Nygren, P. A. (2008) FEBS J. **275**, 2668–2676
17 Orlova, A., Magnusson, M., Eriksson, T. L., Nilsson, M., Larsson, B., Hoiden-Guthenberg, I., Widstrom, C., Carlsson, J., Tolmachev, V., Stahl, S. and Nilsson, F. Y. (2006) Cancer Res. **66**, 4339–4348
18 Friedman, M., Orlova, A., Johansson, E., Eriksson, T. L., Hoiden-Guthenberg, I., Tolmachev, V., Nilsson, F. Y. and Stahl, S. (2008) J. Mol. Biol. **376**, 1388–1402
19 Nilsson, F. Y. and Tolmachev, V. (2007) Curr. Opin. Drug Discov. Dev. **10**, 167–175
20 Baum, R. P., Orlova, A., Tolmachev, V. and Feldwisch, J. (2006) Eur. J. Nucl. Med. Mol. Imaging **33**, S91
21 Grönwall, C., Jonsson, A., Lindstrom, S., Gunneriusson, E., Stahl, S. and Herne, N. (2007) J. Biotechnol. **128**, 162–183
22 Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) Methods Enzymol. **185**, 60–89
22a Moreland, L. W., Baumgartner, S. W., Schiff, M. H., Tindall, E. A., Fleischmann, R. M., Weaver, A. L., Ettlinger, R. E., Cohen, S., Koopman, W. J. Mohler, K. et al. (1997) N. Engl. J. Med. **337**, 141–147
23 Kronqvist, N., Lofblom, J., Jonsson, A., Wernerus, H. and Stahl, S. (2008) Protein Eng. Des. Sel. **21**, 247–255

Received 12 March 2009/9 June 2009; accepted 23 June 2009
Published as Immediate Publication 23 June 2009, doi:10.1042/BA20090085