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Selection of CD4-specific Affibody® molecules to be used for human T lymphocyte separation

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Title (English)

Selection of CD4-specific Affibody® molecules to be used for human T lymphocyte separation

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Abstract

Human CD4 (hCD4)-specific ligands were selected from a phage display library containing $3.3 \cdot 10^9$ variants. The library was previously constructed with the well-characterised protein A-derived three-helix bundle domain Z as a scaffold. Selections were performed using two different strategies: Soluble selection with biotinylated hCD4 and streptavidin beads as well as solid phase selection using tosylactivated beads with immobilised hCD4. After four cycles with successively increasing selection stringency, six binding (Affibody®) molecules were observed using biosensor analysis.

Keywords

Affibody®, magnetic beads, CD4, cell separation

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Selection of CD4-specific Affibody® molecules to be used for human T lymphocyte separation

Pontus Hedberg

Sammanfattning

Inom cellseparation används ofta antikroppar som binder till cellspecifika ytproteiner. Genom att fästa antikropparna på magnetiska kulor och sedan exponera dessa för en blandning av celler så kommer avsedd(a) celltyp(er) fångas upp via de särskiljande ytproteinerna och bindas till kulorna. Med hjälp av en magnet kan uppfångade celler sedan separeras från övriga. Eftersom antikroppar är relativt bräckliga kan det under vissa förhållanden vara önskvärt att använda en tåligare molekyl med samma specifika bindningsförmåga.

Då det visat sig komplicerat att konstruera helt nya proteiner, tillämpas ofta en teknik kallad fagpresentation. Genom att mutera specifika positioner i ett sedan tidigare välkarakteriserat protein skapas ett bibliotek bestående av miljarder olika varianter. Dessa 'lagras' i arvsmassan hos, och presenteras på ytan av, bakterieinfekterande virus (fager). Följaktligen kan man fånga upp proteiner med önskade bindningsegenskaper genom att blanda biblioteket med ämnet man vill ha bindare till. De varianter som inte fastnar tvättas bort och de bindande mångfaldigas genom att fagerna tillåts infektera bakterier. Det anrikade biblioteket kan sedan utsättas för ytterligare selektionsrundor under allt strängare förhållanden för att till slut endast innehålla bindare.

I det här arbetet har fagpresentation använts för att ta fram molekyler vilka binder till CD4, ett ytprotein specifikt för T-hjälparceller. Detta har gjorts i syfte att möjliggöra senare utvärdering av bindarnas förmåga att separera T-hjälparceller jämfört med existerande antikroppar.

Examensarbete 20 p i Molekylär bioteknikprogrammet
Uppsala universitet augusti 2003

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2 LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ABD	Albumin Binding Domain
Amp	Ampicillin
CD4	Cluster of differentiation 4 (aka T4, Leu-3, L3T4)
cDNA	complementary DeoxyriboNucleic Acid
cfu	Colony-forming units
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
e-o-e	End-over-end
FACS	Fluorescence Activated Cell Sorter
Fc	Fragment, crystallisable. The non-binding 'stem' of an antibody
HSA	Human Serum Albumin
Ig	Immunoglobulin
IPTG	Isopropyl thio-β-D-galactoside
Kan	Kanamycin
K_D	Equilibrium dissociation constant (low K_D = high affinity)
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
PAA	Polyacrylamide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque-forming units
r.t.	Room temperature
RU	Response Units
ScFv	Single-Chain Variable-Fragment. The antigen-binding part of an antibody
SPR	Surface plasmon resonance
ssDNA	Single stranded DNA
TSB	Tryptic Soy Broth
TSB-YE	TSB supplemented with Yeast Extract
TST	Tris-buffered saline + Tween
w.t.	Wild-type

3 INTRODUCTION AND BACKGROUND

3.1 INTRODUCTION

The aim of this project was to generate Affibody® molecules that bind human Cluster of differentiation 4 (hCD4). In a combinatorial protein engineering approach, phage display technology was used to select putative binders from a library based on the well-characterised protein A-derived three-helix bundle, domain Z, as a scaffold.

The separation of human blood cells is a procedure routinely performed at research departments in hospitals and universities as well as in many companies. While there are numerous methods to separate different types of cells, two of the most common systems are based on magnetic beads and fluorescence-activated cell sorting (FACS). The former employs monoclonal antibodies (mAbs) immobilised on paramagnetic beads to achieve specific cell separation. All cell types have their particular telltales that can be used in order to distinguish one from the other. For example, CD4 molecules can be used to differentiate T-helper cells from cytotoxic (cell-killing) T cells, marked with CD8 and in the same fashion, other specific markers key out individual cell types. mAbs bound to paramagnetic beads can recognise and bind to these particular cell surface proteins, allowing for subsequent separation of single cell types by simply using a magnetic device.

While mAbs are highly specific, they are generally fragile under non-physiological conditions. Sometimes this negatively affects their use, e.g. by restraining elution conditions and the number of possible regeneration cycles in affinity chromatography. Furthermore, mAbs are quite expensive to develop and produce. Artificial specific binders, engineered from libraries based on molecules with desirable properties, would not only provide more stable ligands but could also reduce both developmental and production costs. In addition, the freedom to choose scaffolds with tailored characteristics matched with the binding specificities and strengths of mAbs may permit entirely new applications for affinity ligands previously unfeasible due to the large size and relative frailty of mAbs.

3.2 HIGH-THROUGHPUT PROTEIN SCREENING

3.2.1 Protein engineering vis-à-vis directed evolution

De novo design of proteins with ameliorated properties, altered specificities or even entirely new functions has proven a formidable challenge [1]. This is mainly due to the inherently complex nature of protein function *per se*, including but not limited to the intricacies of accurately predicting protein folding and resulting functionality, as well as the enormous number of possible variants that need to be evaluated in order to find promising candidates. As an illustration; if a mere five positions in a protein were to be randomised, one would end up with a daunting 20^5 different variants. Even if rational elimination of several amino acid combinations would be performed, a deterring number of variants would still remain to be investigated.

In an attempt to circumvent these obstacles, a different approach of directed evolution has proven highly successful. Libraries containing millions, if not billions, of mutants are

generated simultaneously and subsequently screened for desired properties. Naturally, the two approaches can be combined if the library is constructed by randomising specific positions in a previously engineered scaffold.

Although methods for mutagenesis and the sequencing of proteins are commonplace today, the lack of a protein-equivalent of PCR makes amplification significantly simpler to perform at gene rather than protein level. This creates a need to express the corresponding proteins and ways to tag each protein with its DNA in order to enable further amplification of promising candidates.

There are several methods to tag the protein with its corresponding DNA sequence, although all of them ultimately accomplish the same thing, i.e. linking genotype with phenotype. The methods can be categorised into following three groups: i) those based on physical linkage; ii) compartmentalisation or iii) spatial separation [1]. Compartmentalisation involves restricting each protein and its corresponding DNA sequence to a distinct compartment. This can be accomplished either by introducing plasmid DNA encoding the protein into a cell. Alternatively, water-in-oil emulsions can be used to create artificial, "cell-like", compartments. Compartmentalisation methods have proven particularly powerful as assays for enzyme catalysis. Spatial separation tags the protein to its DNA indirectly. By associating the protein with a unique spatial address, e.g. a well in a microtiter plate or a part of a solid support, proteins can be traced back to their DNA sequence. The testing of complementary DNA (cDNA) libraries to ascertain protein function is one area where spatially addressable methods have been productive [1].

The most straightforward method to establish the protein-DNA link is probably that of direct physical linkage. This method is highly compatible with assays based on binding to an affinity matrix and this tag-method/assay combination has proven to be a tremendously powerful tool for high-throughput protein screening [2]. Only the different aspects of physical linkage in general and that of phage display in particular will be discussed further in this report.

Finally, a high-throughput assay, which is compatible with the chosen genotype-phenotype link, is required to select and/or screen the library for promising candidates (Fig. 1).

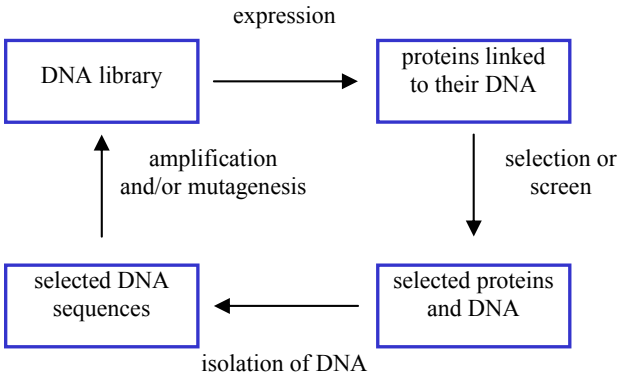

Figure 1. General strategy for large-scale analysis of protein function.

3.2.2 Methods of Physical Linkage

In cell surface display, the physical link between genotype and phenotype is established by genetically fusing a library to the gene of an anchoring membrane protein such as *E. coli* LamB or OmpA [3]. Gene fusion products are presented on the surface of the recombinant bacteria carrying the DNA coding for the displayed protein, and selection or screening can then be performed using for instance flow cytometry. Bacterial systems are

most often used [4], but yeast [5] and eukaryotic [6] systems have also been developed. Although the concept of cell surface display has been available for quite some time, its previous uses have not been focused on library screening or protein selection [7].

It has been shown that larger libraries result in both higher affinity molecules and a greater diversity of sequences with similar function [8], such as affinity for a particular target. In contrast to phage and cell-surface display, other techniques such as ribosome display and mRNA-peptide fusion allow protein selection entirely *in vitro*. This absolves potential library size from the constraints placed by transformation limits of *in vivo* systems, making libraries as large as 10^{15} both theoretically and practically possible (at least for shorter peptides) [9]. The main difference between the two approaches is that ribosome display makes use of stalled, noncovalent ribosome-mRNA-protein complexes to carry out protein selection, whereas mRNA-peptide fusion utilises puromycin to form covalent complexes between an mRNA molecule and its corresponding peptide.

3.3 PHAGE DISPLAY

3.3.1 Background

Phage display was introduced already in 1985 [10] and has since proven to be a tremendously versatile and powerful tool for selecting proteins from vast libraries. Once the hurdle of creating a sufficiently large library is surmounted, the technique is comparatively simple, rapid to set up and perhaps most important, reasonably inexpensive since it requires no special equipment.

Although several different phage display systems have been developed, they are all based on the same basic principle. By fusing the nucleotide sequence corresponding to the protein to be displayed to a gene encoding a phage coat protein, the protein will be presented at the phage surface and its DNA 'stored' inside the phage particle. Consequently, the needed link between genotype and phenotype is established.

Even though other phage systems (e.g. Lambda and T7) have been successfully used as phage display vehicles [11, 12], the original M13 filamentous phage and its close relatives belonging to the Ff family remain popular. One of the main advantages of this particular cloning vehicle is that the length of the filamentous phage is determined by the size of the genomic DNA. The packaging process is therefore not linked to any size constraint of the M13. However, since M13 is non-lytical, only proteins capable of being exported through the bacterial inner membrane may be displayed [13].

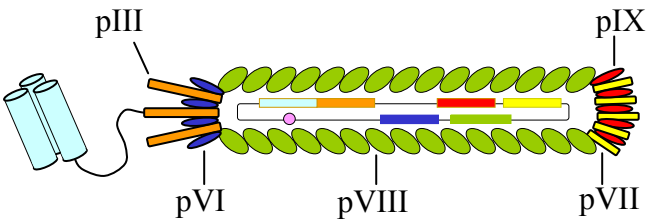

Figure 2. Schematic view of a M13 filamentous bacteriophage. The phage particle consists of a single stranded DNA molecule surrounded by several different coat proteins. pVIII is the major coat protein with pIII and pVI on one end, and pVII and pIX on the other. Foreign protein sequence fused to pIII.

The Ff phage particles are 800-2000 nm long, 6-7 nm wide and consist of single stranded DNA (ssDNA) packaged into a coat made up from approximately 2700 copies of pVIII, which constitutes the major coat protein. The particle is flanked by three to five copies each of pIII and pVI on one end and five copies of pVII and pIX,

respectively, on the other (Fig. 2). The two coat proteins pVIII and pIII each have their respective merits as fusion partners [14]. In general terms it can be said that pIII is suitable for smaller numbers of larger proteins whilst pVIII is generally chosen when larger numbers of smaller proteins are to be displayed. Henceforth only the option of using pIII will be discussed.

One of the more significant improvements to phage display is the introduction of co-infection by helper phage. This introduces a tool to control the valency of display, allowing for larger foreign proteins to be handled and avoiding the potential problems with avidity [15]. Instead of fusing the foreign DNA to gene III in a single phage chromosome, resulting in all pIII being fusion proteins, the recombinant gene III is expressed from a plasmid containing both *E. coli* and phage origins of replications (i.e. phagemid). A wild-type (w.t.) version of pIII is introduced together with the rest of the phage proteins through the separate genome of a helper phage.

Apart from a phage origin of replication, the phagemid carries antibiotic resistance. Since the packaging signal of the helper phage is less efficient than that of the phagemid, less helper phage particles are assembled. As a result, two types of virion particles are secreted, a minority carrying helper phage DNA and a majority with phagemid DNA. The coats of both will be mosaics comprised of a mixture of both recombinant and w.t. pIII molecules [16].

3.3.2 Selections with phage display

Selection of phage-borne peptides and proteins consists of culling an initially large population, typically 10^9 clones, each represented by 100 particles on average, to give a subpopulation that is only a fraction in size but with an improved fitness for the target. The subpopulation is then amplified in bacteria and used for further rounds of selection.

Among the different selection pressures applied to phage display libraries, affinity selection is by far the most common [16]. Briefly, after binding target molecules to a solid support, the phage library is allowed to pass over or be incubated with the immobilised target. The small fraction of phage displaying proteins that bind to the target will be captured while the reminder will be washed away. Bound phage are subsequently eluted and propagated by infecting fresh bacterial host cells resulting in an amplified and greatly enriched subpopulation. This subset can then be subjected to further rounds of affinity selection, often with successively raised stringency, i.e. higher selection pressure (Fig. 3). Monoclonal phage populations from the final eluate, usually after three to five rounds of selection, are propagated and analysed individually.

In order to increase the efficacy of selection, two different parameters need to be taken into consideration: i) Yield is the fraction of particles with a given fitness that survive each round of selection while ii) stringency is the degree to which high-fitness peptides are preferred over those less desirable [16]. Although the final goal of selection generally is to isolate the peptide(s) with highest fitness, it is important not to focus on stringency alone. Particularly during the first round of selection, high yield is of great importance if the risk of loosing potential candidates is to be avoided.

The previously discussed restrictions in library size caused by transformation limitations can in part be compensated for by using several independently created libraries [17] or library maturation. The latter, also known as directed evolution, involves subjecting the already selected subpopulations to mutagenesis. Since the starting point is a "fitter-than-average" library, the idea is to introduce variants with higher fitness, absent from the initial population. The great advantage of directed evolution over various approaches to rationally engineer the properties of proteins is that the need for *a priori* knowledge of the particular molecular structures involved in binding is reduced.

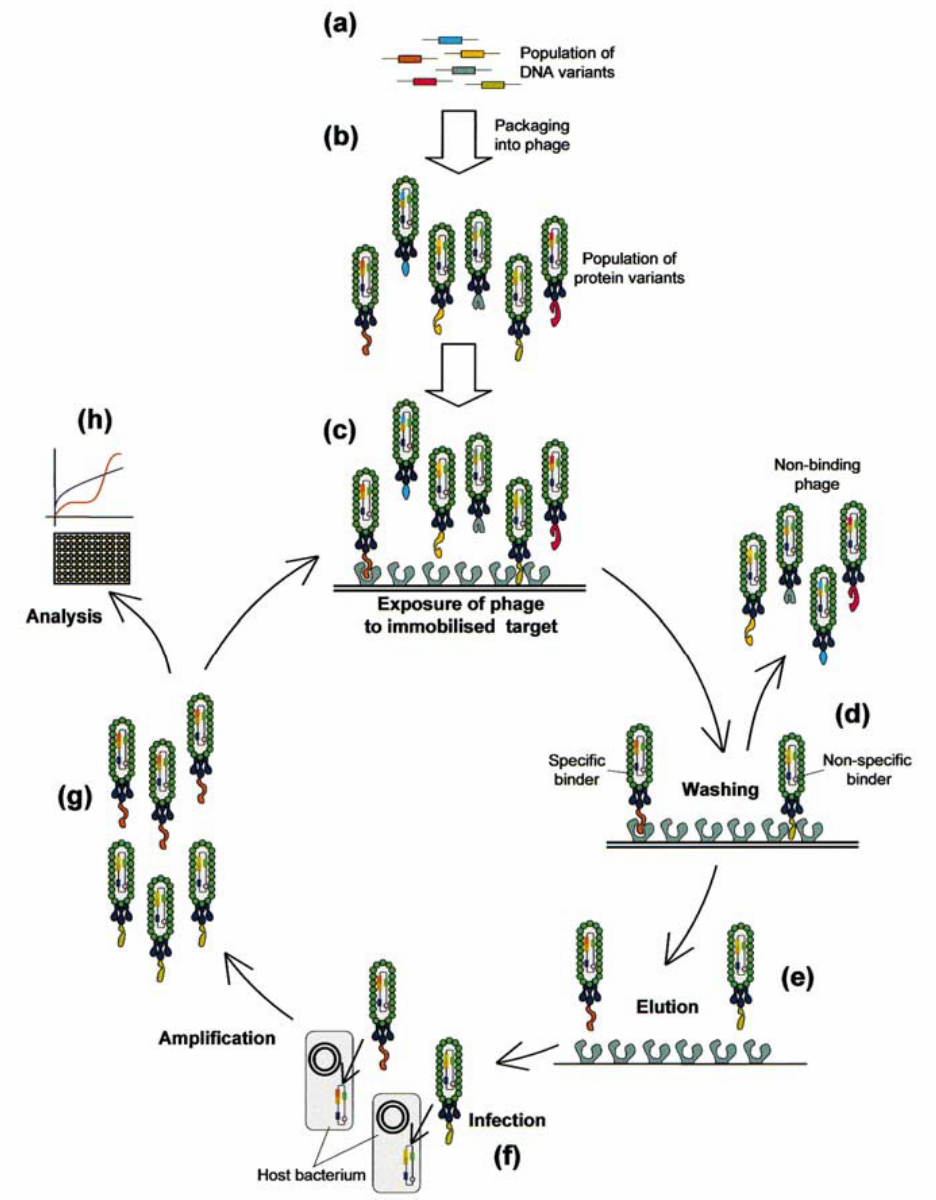

Figure 3. The phage display cycle. (a) A DNA library is created and (b) cloned into phage genome or phagemid as fusions to a coat protein gene. The phage library is exposed to immobilised target molecules (c) and phages displaying proteins with appropriate characteristics are captured while non-binding phages are washed off (d). Bound phages are eluted (e) and subsequently amplified (f) by infecting fresh host bacterial cells resulting in a sub library with an overall higher fitness (g). Further enrichment can be accomplished by repeating the steps (c) to (f). Monoclonal populations are analysed individually (h) in the end. (Illustration used with permission from Willats WG. 2002 [13])

3.4 THE AFFIBODY®

3.4.1 Origin of molecule

Affibody® molecules are ligands derived from one of the (IgG) Fc-binding domains of staphylococcal protein A. This 58-residue three-helix bundle was used as the scaffold for a phage display library constructed through the randomisation of 13 specific surface-exposed amino acid (aa) positions distributed over helices one and two (Fig. 4). The rationale for choosing this particular scaffold is that it has several attractive features. For instance: i) its folding characteristics are very fast and it can be boiled at natural pH without significant loss of activity after cooling [18]; ii) it is highly soluble in aqueous solutions [19]; iii) it is remarkably stable under alkaline conditions [20]; iv) it is secretion competent in *E. coli* [21] and v) it can successfully be displayed using M13 phage display [22]. Furthermore, in accordance with many antigen-Ab interactions [23], the surface area of helices one and two available for binding is approximately 800 $Å^2$, ensuring a sufficiently large 'starting point' for modifications although being presented by a significantly smaller scaffold.

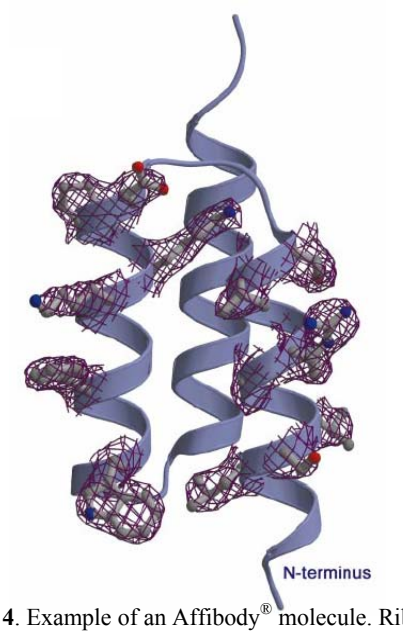

Figure 4. Example of an Affibody® molecule. Ribbon plot with backbone shown in blue and side chain electron densities of 13 randomised residues highlighted in red (Illustration used with permission from Högbom M *et al.*, 2003 [24]).

3.4.2 Affibody® phage display library

When the Affibody® based phage display library was created, essentially as described earlier [18], the core of the three-helix bundle needed to remain unaltered in order for the molecule to retain its protein structure. Since only the residues comprising the 800 $Å^2$-binding surface could be variegated, traditional random mutagenesis was abandoned for a novel solid-phase-assisted method. In short, randomised single-stranded oligonucleotides were assembled successively, enabling randomisation of appropriate positions while retaining the intermittent invariant segments crucial for scaffold integrity. The library was created using a NN(G/T) codon degeneracy and reached a size of $3.3 \cdot 10^9$ Affibody® variants (unpublished data).

Using this library, phage display selections have been successfully carried out against a wide range of different targets. Specific Affibody® variants with micromolar range K_D-values have been selected against such diverse proteins as *Taq* DNA polymerase, apolipoprotein A-1_M, insulin, respiratory syncytial virus (RSV) G protein, Immunoglobulin A (IgA) and Immunoglobulin E (IgE) [25].

3.5 TARGET

Human CD4 is a 55 kDa transmembrane protein belonging to the Immunoglobulin (Ig) superfamily and functions as a co-receptor in the cellular immune response. It is mainly expressed on class II major histocompatibility complex (MHC)-restricted T cells, thymocyte subsets, monocytes and macrophages and is commonly used as a T helper cell marker [26]. The structure of hCD4 is outlined in Figure 5.

By interacting with the T-cell receptor MHC class II complex, hCD4 increases the avidity of association between a T cell and an antigen-presenting cell [27]. hCD4 also serves as the high-affinity receptor for cellular attachment and entry of the human immunodeficiency virus (HIV) [28]. The 371 aa extracellular region consists of four Ig-like domains (D1-D4) with disulfide bonds stabilising domains 1, 2 and 4 [27]. Baculovirus-expressed CD4 is commercially available, adding to the proteins suitability as an attractive target candidate. (hCD4 sequence and domain distribution in APPENDIX A.)

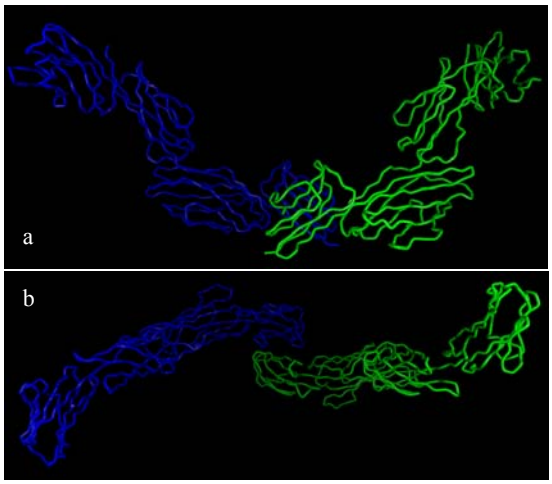

Figure 5. The hCD4 homodimer. Backbone diagram showing the extracellular domains (D1-D4) of two hCD4 molecules dimerised at D4. Side (a) and top (b) views at 3.9 Å resolution. PDB id 1wio [27].

4 MATERIALS AND METHODS

4.1 AFFIBODY® SELECTION

4.1.1 Strains and vectors

E. coli strain RRIΔM15 (*supE*) [29] does not read the amber stop codon and was used for phage particle amplification. For protein expression, non-suppressor *E. coli* strain RV308 [30] was used as well as RRIΔM15. Phagemid vector pAffi1 (Fig. 6) is derived from vectors pKN1 [18] and pUC119 [31] and encodes for the OmpA signal peptide, the Affibody®-based library, an albumin binding domain derived from streptococcal protein G and a truncated version of the M13 phage coat protein pIII. The latter is separated from the others by the amber stop codon. Phage stocks were prepared using M13K07 helper phage (New England Biolabs, Beverly, MA).

4.1.2 Target verification and biotinylation

The purity of target human soluble CD4 (Protein Sciences, Meriden, CT) was investigated on a 4-12% polyacrylamide (PAA) gradient SDS-NuPAGE gel (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The protein was investigated under reducing as well as non-reducing conditions and MultiMark LC5725 (Invitrogen) was used as molecular weight marker. The gel was subsequently silver-stained [32].

Throughout the report, CD4-buffer refers to the phosphate buffer in which hCD4 was delivered (10 mM NaH_2PO_4, 300 mM NaCl, 0.01% Tween 80, pH 7.0).

hCD4 was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (21335, Pierce, Rockford, Il). 20 µg hCD4 (2.4 µM) was incubated on ice during 2 hrs with a 10:1 molar excess of biotin reagent. Free biotin was removed through dialysis in CD4-buffer (2x500 mL, 1x1000 mL) using a 0.5-3.0 mL Slide-A-Lyzer MWCO 10,000 (Pierce). In order to assess the level of biotinylation, a HABA-Avidin assay (Pierce) was performed according to the manufacturer's recommendations.

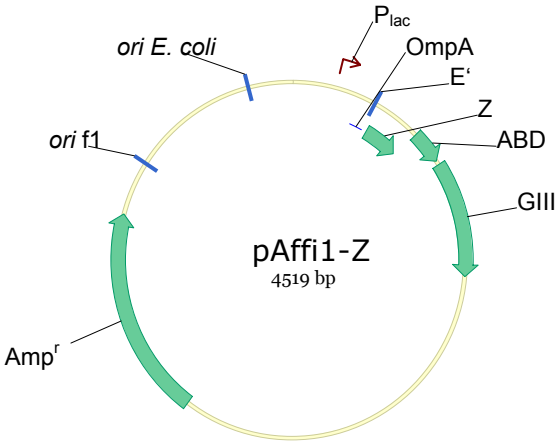

Figure 6. The phagemid vector pAffi1. Apart from the library itself (Z), the vector codes for the OmpA signal peptide and the six first aa of domain E in protein A (E'), an albumin-binding domain fused to the truncated gene of M13 phage coat protein III, phage and *E. coli* origins of replication and ampicillin resistance.

4.1.3 Binding assay

To investigate the amount of biotinylated hCD4 (b-hCD4) able to bind to streptavidin coated paramagnetic beads (Dynal, Oslo, Norway), 0.5 mg beads were washed twice with 500 µL CD4-buffer and then incubated with 5 µg b-hCD4 end-over-end (e-o-e) for 30 min at room temperature (r.t.). The supernatant containing unbound b-hCD4 was transferred to 0.5 mg new beads, washed as above, and incubated e-o-e for 30 min at r.t. This was repeated a third time after which the beads from each of the three incubations were resuspended in 5 µL reducing sample buffer (diluted from a 5x stock solution of 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 12.5% SDS, 25% β-mercaptoethanol, 0.05% bromo phenol blue). One µL of each was investigated on a 10-15% PAA gradient SDS-PAGE Phast gel (Amersham Biosciences, Uppsala, Sweden).

4.1.4 hCD4-coating of tosylactivated paramagnetic beads

Tosylactivated beads (Dynal) were used to covalently attach hCD4 target protein. 6 mg beads were washed once and resuspended in 300 µL CD4-buffert containing 30 µg hCD4 and the mixture was incubated e-o-e at 4°C during three days. The beads were washed twice with phosphate buffered saline (PBS) + 0.1% gelatine during 5 min at 4°C and incubated with 0.2 M Tris-HCl pH 8.5 + 0.1% gelatine e-o-e during 3 min at r.t. The beads were finally washed a third time with PBS + 0.1% gelatine during 5 min at 4°C and resuspended in 200 µL fresh PBS + 0.1% gelatine.

The amount of unbound hCD4 remaining in the supernatant after coating was determined on a 4-12% PAA gradient SDS-NuPAGE gel (Invitrogen) according to the manufacturer's instructions using MultiMark LC5725 (Invitrogen) as molecular weight marker. The gel was subsequently silver-stained.

4.1.5 Preparation of phage stocks

Phage stocks between selections were propagated by cultivation in 5 L E-flasks containing 500 mL Tryptic soy broth supplemented with yeast extract (TSB-YE: 30 gL^{-1} Tryptic soy broth, 5 gL^{-1} yeast extract) and 20% glucose and 100 μgmL^{-1} ampicillin (Amp) at 37°C, 100 rpm. When the cultures reached log-phase (OD_{600} = 0.5-0.8), an aliquot containing 10^{10} cells (OD_{600} = 1 approximately equals $5 \cdot 10^8$ cells) was incubated with $2 \cdot 10^{11}$ plaque forming units (pfu) M13K07 helper phages during 30 min at 37°C without shaking. The cells were centrifuged at 3,300 x *g* for 15 min and the pellet was resuspended in 250 mL TSB-YE supplemented with 100 μgmL^{-1} Amp, 25 μgmL^{-1} kanamycin (Kan) and induced with 0.1 mM isopropyl thio-β-D-galactoside (IPTG). The cultures were grown over-night at 30°C, 80 rpm.

Cells were pelleted by centrifugation at 2,100 x *g* for 30 min. Phages were recovered by poly ethylene glycol precipitation (PEG solution; 20% PEG, 2.5 M NaCl). Supernatants were mixed with PEG solution to 1/5 of the volume and incubated on ice for 1 hr. The mixtures were centrifuged at 10,700 x *g* at 4°C for 30 min and resuspended in water. The procedure was repeated once. The final phage-containing pellets were resuspended in 2.5 mL CD4-buffert supplemented with 0.1% gelatine and filtered through a 0.45 µm filter.

4.1.6 Selections from phage library

Selections against hCD4 were performed using two different strategies: Soluble selection using biotinylated hCD4 and streptavidin beads as well as solid phase selection using tosylactivated beads with immobilised hCD4. All steps were performed at r.t., tubes and streptavidin beads were preblocked with CD4-buffer + 0.1% gelatine (2 hrs on bench and 30 min e-o-e, respectively).

Soluble selection: 1 mL phage stock from a phagemid library with $3.3 \cdot 10^9$ Affibody® molecule variants (Zlib2002, Elin Gunneriusson, personal communications) was preselected against streptavidin beads by incubating e-o-e during 30 min (cycle 1: 2 mg beads; cycles 2-4: 0.5 mg). Supernatant from the preselection was incubated with decreasing concentrations of b-hCD4 e-o-e during 1 h 45 min after which streptavidin beads were added and the mixture was incubated for 15 min (cycle 1: 2 mg beads, 200 nM b-hCD4; cycle 2: 0.5 mg, 100 nM, cycle 3: 0.5 mg, 100 nM; cycle 4: 0.5 mg, 100 and 20 nM). Supernatant containing unbound phages was removed and beads were washed with PBS-T according to the following; cycle 1: 1 x 1000 µL; cycle 2: 3 x 500 µL; cycle 3: 6 x 500 µL and cycle 4: 12 x 500 µL. Bound phages were eluted with 500 µL 0.05 M glycin-HCl, pH 2.2 during 10 min and the supernatant was neutralised by addition of 50 µL 1 M Tris-HCl, pH 8.0 in 450 µL PBS.

Solid phase selection: Tosylactivated beads with immobilised hCD4 were used for solid phase selection. 1 mL Zlib2002 phage stock was directly incubated with hCD4 immobilised on beads (cycle 1: 1.5 mg beads, 73 nM hCD4; cycle 2: 0.37 mg, 36 nM; cycle 3: 0.37 mg, 36 nM; cycle 4: 0.37 mg, 36 and 18 nM) e-o-e during 2 hrs. Unbound phages were removed, the beads were washed and the selected phages were eluted as above.

Eluted phages were mixed with 50 mL RRIΔM15, grown to OD_{600} = 0.6, incubated during 20 min at 37°C and plated on large agar TYE plates. Phage concentrations were determined by allowing serial dilutions (10^2-10^{13}) of phages infect *E. coli* grown to log-phase. Phages and cells were incubated at 37°C for 20 min and plated on TYE agar plates.

4.2 SCREENING OF CLONES FOR CHARACTERISATION

4.2.1 Phage ELISA

Phages from clones obtained after four rounds of selection were produced in 96 well microtiter plates and an Enzyme Linked ImmunoSorbent Assay (ELISA) was used to screen for phages expressing hCD4-binding Affibody® molecules.

Randomly picked individual colonies were used to inoculate 250 µL TSB-YE supplemented with 2% glucose and 100 µgmL^{-1} Amp in 96-deepwell plates (Nalge Nunc, Rochester, NY) and were grown overnight at 37°C, 1000 rpm. 5 µL overnight culture was added to 500 µL TSB-YE supplemented with 0.1% glucose and 100 µgmL^{-1} Amp in new plates. After growing to log-phase (approximately 3 hrs), $7 \cdot 10^9$ pfu M13K07 helper phages in 50 µL TSB-YE were added per well and left to infect during 30 min at 37°C. Subsequently, 450 µL TSB-YE was added to a final volume of 1 mL TSB-YE (100 µgmL^{-1} Amp, 25 µgmL^{-1} Kan and 0.1 mM IPTG) and grown overnight at 30°C, 1000 rpm. Overnight cultures were pelleted by centrifugation at 3,100 x *g* during 30 min at 4°C. The phage-containing supernatants were transferred to a new plate and incubated with 1/5 volume PEG-solution on ice for 1 hr. Finally, the supernatants were centrifuged at 3,100 x *g* during 30 min and the supernatant discarded. The pelleted phages displaying Affibody® molecules were resuspended in 110 µL CD4-buffer and further used in ELISA.

Streptavidin coated 96-well plates (Nalge Nunc) were coated with 0.1 µg b-hCD4 or b-IgG in 100 µL CD4-buffer and high binding polystyrene 96-well plates (Corning inc., Corning, NY) with 0.1 µg hCD4 or IgG in 100 µL CD4-buffer over-night at 4°C. After initially blocking the wells with blocking buffer (2% dry milk in CD4-buffer) for 1 hr at r.t., 150 µL phages in blocking buffer were added to each well and incubated for 2 hrs at r.t. Primary antibodies (Abs) goat α-hCD4 (#AF-379-NA, R&D Systems, Minneapolis, MN) and rabbit α-M13 (#6188, Abcam, Cambridge, UK) were diluted in blocking buffer according to the manufacturer's ELISA recommendations, dispensed on the plate in 100 µL aliquots and incubated for 1 hr at r.t.

Secondary Abs used for streptavidin coated 96-well plates: Rabbit α-goat IgG (#305-055-003, Jackson ImmunoResearch, West Grove, PA) and goat α-rabbit IgG (#4050-04, Southern Biotech, Birmingham, AL) conjugated with alkaline phosphatase were diluted 1:10,000 and 1:2,000, respectively, in blocking buffer. 100 µL in each well was incubated for 1 hr at r.t. Developing solution was prepared by dissolving three phosphatase substrate tablets (#104, Sigma, St. Louis, MO) in 7.5 mL water and mixed with 7.5 mL 1 M dietanolamin (5 mM $MgCl_2$, pH 9.8). 100 µL substrate solution were added to each well and A_{405} was measured after 4 hrs.

Alternatively, for the polystyrene plates, secondary antibodies rabbit-α-goat IgG (#6160-05, Southern Biotech) and goat-α-rabbit IgG (#A0545, Sigma) conjugated with horseradish peroxidase were both diluted 1:8,000 in blocking buffer and 100 µL per well were incubated for 1 hr at r.t. 100 µL ImmunoPure TMP Substrate solution (#34021, Pierce) was added. The reaction was terminated with 2 M H_2SO_4 after 20 min and A_{450} was measured. Excess phages, primary- and secondary Abs were removed by washing wells 3 times with CD4-buffer, followed by 1 x PBS.

4.2.2 DNA sequence analysis

PCR fragments from individual clones were amplified using oligonucleotide primers Affi21 (5'-TGCTTCCGGCTCGTATGTTGTGTG-3') and Affi22 (5'-CGGAACCAGAGCCACCACCGG-3'). Following initial denaturation during 5 min at 94°C, the program consisted of 30 cycles with denaturation at 96°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 40 s, and a final extension step at 72°C for 3 min, holding at 4°C after completion. PCR reaction was performed on a PTC-0225 DNA Engine Tetrad (MJ Research, Waltham, MA). To verify a successful reaction, 15 samples were investigated on a 1% agarose gel stained with ethidium bromide and using EZ Load Precision Molecular Mass Ruler (Bio-Rad, Hercules, CA).

Sequencing was performed with ABI Prism dGTP Big Dye Terminator 3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the supplier's recommendations using biotinylated (forward) Affi71 (5'-b-TGCTTCCGGCTCGTATGTTGTGTG-3'). PCR-reaction was carried out on a GeneAmp PCR System 9700 (Perkin Elmer, Foster City, CA) with 25 cycles 96°C for 10 s, 50°C/5 s and 60°C/4 min, holding at 4°C after completion. Sequence reaction product was purified using paramagnetic streptavidin coated beads on a Magnatrix 8000 (Magnetic Biosolutions, Stockholm, Sweden) and subsequently sequenced in a 3100 Genetic analyzer (Applied Biosystems). Results were analyzed with Sequencher v. 4.0.5. (Gene Codes Corp., Ann Arbor, MI).

4.3 AFFIBODY® PRODUCTION

4.3.1 Plasmid preparation and transformation

Plasmids of individual clones were amplified in RRIΔM15 over-night in 5 mL TSB (100 μgmL^{-1} Amp) at 37°C. Plasmids were subsequently prepared using a QIAprep spin miniprep kit (Qiagen, Venlo, Netherlands) according to manufacturer's instructions and verified on a 1% agarose gel.

Transformation to $CaCl_2$ competent RV308 cells by heat-shock was performed by adding 400 ng plasmids to 100 µL RV308, thawed on ice. The mixture was incubated for 30 min on ice, heated at 42°C during 90 s in a heating block and finally left to recover first on ice for 2 min and then at 37°C during 10 min. All transformed cells were grown at 37°C over-night on Amp plates.

4.3.2 Protein expression and purification

Affibody® molecules were expressed fused to an albumin binding domain in *E. coli* RV308 or RRIΔM15 bacteria. Cells were grown over-night in 10 mL TSB (100 μgmL^{-1} Amp) at 37°C, 100 rpm, and 200 µL were used to inoculate 100 mL TSB (100 μgmL^{-1} Amp). At log-phase (OD_{600} = 0.5-0.8), IPTG was added to the cultures to a final concentration of 1 mM. Cultures were incubated over-night at 30°C, 100 rpm. Cells were harvested by centrifugation at 2,600 x *g* for 10 min and periplasmic proteins were released by osmotic chock treatment. Cells were resuspended in 25 mL osmotic shock buffer (20% sackarose, 0.3 M Tris-HCl, pH 8.0 and 1 mM EDTA) and incubated for 10 min in r.t. After centrifugation at 7,400 x *g* for 10 min, the pellet was resuspended in 25 mL ice-cold MQH_2O and put on ice for 10 min. Cells were removed through a final centrifugation at 8,400 x *g*, 4°C for 10 min, and the protein-containing supernatants were transferred to new tubes.

After dilution to 50 mL in 1xTST (25 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, 0.05% Tween 20, pH 8.0) and 0.45 µm filtration, putative Affibody® candidates were purified through affinity chromatography using PD-10 columns (Amersham Biosciences). The columns were packed with 2 mL human serum albumin (HSA)-sepharose. 1xTST was used as running- and washing buffer and 0.5 M HAc pH 2.8 as elution buffer. The columns were initially pulsed with HAc and subsequently equilibrated with 4 column volumes (cv) 1xTST. The samples were loaded onto the columns and washed with another 4 cv 1xTST. The buffer was changed with 1 cv NH_4Ac and samples were eluted with HAc in 8 x 1 mL fractions. Absorbance at 280 nm was measured for all fractions using a SmartSpec 3000 spectrophotometer (Bio-Rad). Protein-containing fractions were subsequently pooled and A_{280} was measured. Pools were aliquoted and freeze-dried. All samples were investigated on 20% homogenous Phast gels (Amersham Biosciences) stained with coomassie brilliant blue R-250.

4.4 BIOSENSOR ANALYSIS

Binding of the purified Affibody® proteins to hCD4 was analysed using surface plasmon resonance (SPR) on a BIAcore 2000 (Biacore AB, Uppsala, Sweden). hCD4 was immobilised on a CM-5 sensor chip by activating the carboxylated dextran layer with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide (EDC) according to the manufacturer's recommendations. 210 µL of hCD4 (10 μgmL^{-1}) in 10 mM sodium acetate pH 4.5 was injected at a flow rate of 5 $\mu Lmin^{-1}$. IgG was immobilised as above in 10 mM acetate pH 4.0 and used as a negative control. Remaining activated groups on immobilised surfaces were blocked with an injection of ethanolamine. One sensor chip surface was deactivated immediately after activation and used as a blank (reference).

Protein samples of Affibody® molecules were dissolved in HBS (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% P-20, pH 7.4) to final concentrations of either 5 or 10 µM. Duplicate samples were injected in random order at a flow rate of 5 $\mu Lmin^{-1}$. Injections were made during 5 min followed by dissociation during 15 min. After each sample injection, the surfaces were regenerated with two 30 s injections of 10 mM HCl.

5 RESULTS

5.1 SELECTIONS

Human CD4 specific Affibody® variants were selected from the combinatorial phage display library Zlib2002 in two parallel strategies; selection in solution where phages were selected against suspended biotinylated hCD4 and subsequently captured on streptavidin coated beads (soluble selection) and solid phase selection with hCD4 bound to tosylactivated beads.

Target purity was verified with SDS-PAGE analysis (>90%, data not shown). hCD4 was biotinylated with biotin reagent in a molar excess of 10:1. The level of biotinylation was determined in a HABA assay. The result showed a biotinylation molar ratio of 6:1 (Fig. 7).

A binding assay was performed to determine the maximal amount of b-hCD4 possible to bind to 1 mg streptavidin beads. The binding assay confirmed that 2 mg streptavidin beads is a sufficient amount in order to capture the full 0.2 µM of b-hCD4 used in the first cycle of soluble selection (Fig. 8).

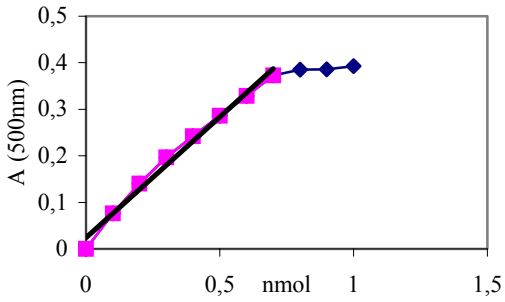

Figure 7. HABA assay. Standard curve used to determine the level of hCD4 biotinylation. Fitted curve equation (before saturation) was y = 0.5189x + 0.0237. Sample A_{500}= 0.1 indicated a biotinylation molar ratio of 6:1.

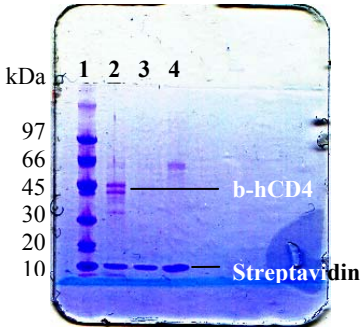

Figure 8. Binding assay. A 50 µL aliquot with 5 µg b-hCD4 was consecutively incubated e-o-e with 3 x 0.5 mg streptavidin coated beads (wells 2-4). The level of bound b-hCD4 in all three incubations was investigated on an SDS-PAGE Phast gel.

hCD4 was covalently bound to tosylactivated magnetic beads for use in solid phase selection. The level of hCD4 successfully bound to the tosylactivated beads, was deduced by investigating the amount of remaining hCD4 in the supernatant after incubation. Approximately 2 µg hCD4/mg beads was bound, implicating a binding efficiency of roughly 40% (data not shown).

Selections were performed in four cycles for both the soluble and the solid phase strategy. In order to increase selection stringency, the number of washes was increased for each additional cycle and the target concentration was successively decreased. An overview of the selection conditions used is outlined in Table 1. Starting target concentrations of 73 nM (solid phase) and 200 nM (in solution) during cycle 1 was gradually reduced to 18 and 20 nM, respectively. Enrichment factors were calculated as amount phages out

divided with input phages, in percent. Increasing figures for soluble selection confirmed that a selection was taking place (Tab. 1).

Table 1. Selection conditions and phage titers. Selection stringency was increased by reducing target concentrations and increasing number of washes for each additional selection cycle. Enrichment was calculated as amount phages out divided with input phages, in percent.

		Phages in (cfu)	Target konc (nM)	No of washes	Phages out (cfu)	Enrichment (%)
Cycle I	In solution	$4.03 \cdot 10^{12}$	200	1	$1.69 \cdot 10^{6}$	$4.19 \cdot 10^{-5}$
	Solid phase	$4.03 \cdot 10^{12}$	73	1	$9.55 \cdot 10^{6}$	$2.37 \cdot 10^{-4}$
Cycle II	In solution	$1.00 \cdot 10^{11}$	100	3	$2.93 \cdot 10^{5}$	$2.93 \cdot 10^{-4}$
	Solid phase	$1.00 \cdot 10^{11}$	36	3	$6.76 \cdot 10^{5}$	$6.76 \cdot 10^{-4}$
Cycle III	In solution	$1.94 \cdot 10^{11}$	100	6	$1.92 \cdot 10^{5}$	$9.90 \cdot 10^{-5}$
	Solid phase	$2.52 \cdot 10^{11}$	36	6	$1.38 \cdot 10^{5}$	$5.48 \cdot 10^{-5}$
Cycle IV	In solution	$2.90 \cdot 10^{11}$	100	12	$3.40 \cdot 10^{5}$	$1.17 \cdot 10^{-4}$
	In solution	$2.90 \cdot 10^{11}$	20	12	$6.80 \cdot 10^{5}$	$2.34 \cdot 10^{-4}$
	Solid phase	$3.37 \cdot 10^{11}$	36	12	$2.50 \cdot 10^{4}$	$7.42 \cdot 10^{-6}$
	Solid phase	$3.37 \cdot 10^{11}$	18	12	$2.80 \cdot 10^{4}$	$8.31 \cdot 10^{-6}$

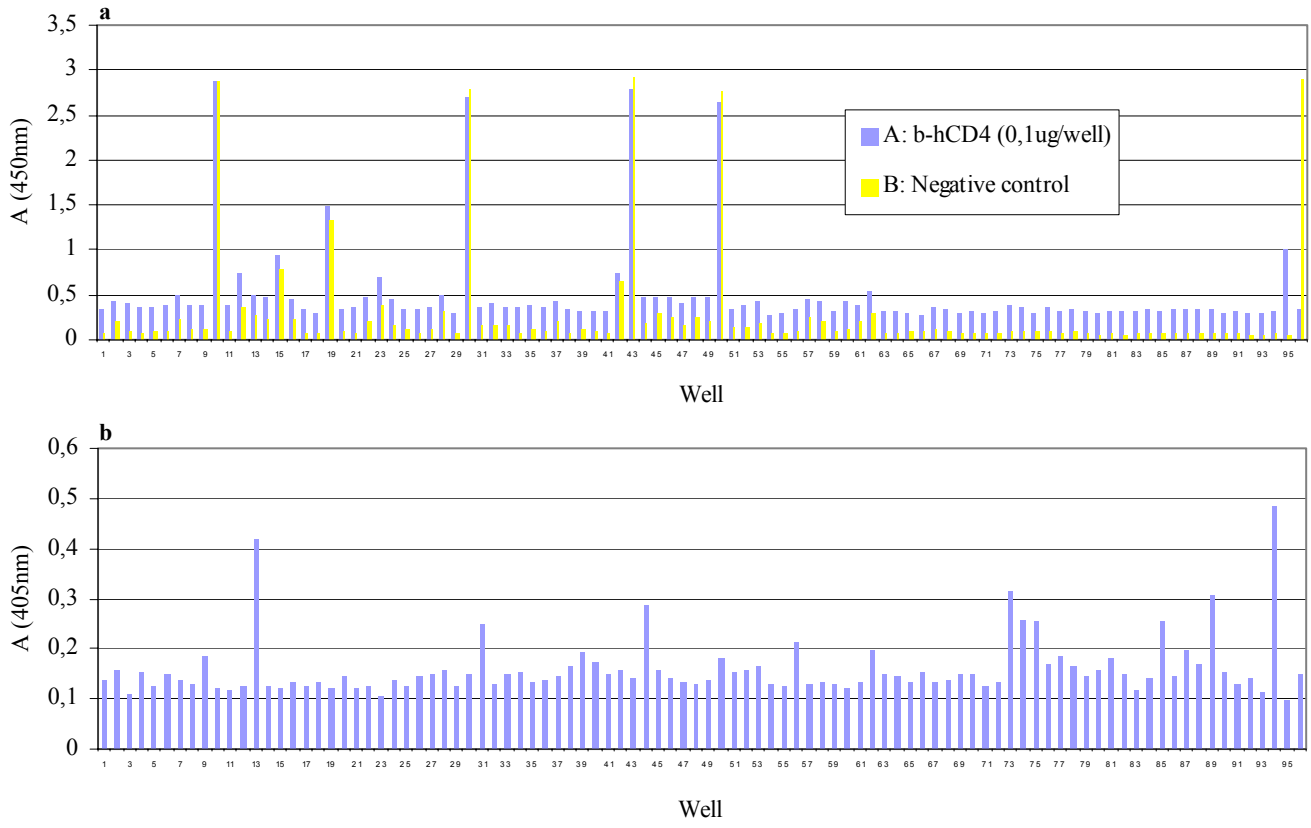

Figure 9. Phage ELISA. **(a)** ELISA using streptavidin coated plates. (A) b-hCD4 immobilised in streptavidin coated wells with (B) "empty" streptavidin coated wells as negative control. Phages exposing Affibody® variants on their surface were added to wells 1 through 61 (soluble selection) and 62 through 94 (solid phase selection). Bound phages were detected using secondary Abs conjugated with alkaline phosphatase. **(b)** ELISA where hCD4 was covalently bound directly to the plastic surface of microtiter wells. 91 Affibody® presenting phage variants were screened in wells 1 through 62 (soluble selection) and 63 through 91(solid phase selection). Bound phages were detected using secondary Abs conjugated with horseradish peroxidase.

5.2 PHAGE ELISA

After four selection cycles, 94 randomly picked clones were screened for their binding to hCD4 using Enzyme-Linked Immunosorbent Assay (ELISA). In an initial ELISA, b-hCD4 were immobilized in streptavidin-coated plates and phages exposing Affibody® variants on their surface were allowed to bind to their target. As a negative control, binding against empty streptavidin wells was also tested. The experiment indicated that many clones showed equally well (or better) binding to streptavidin than hCD4 (Fig. 9a). In a second ELISA, hCD4 was covalently bound directly to the plastic surface of a microtiter plate and 91 clones tested in this alternative format (86 clones from the first ELISA and 5 new clones. The result indicates a number of hCD4-binding clones (Fig. 9b).

Table 2. Sequenced clones. Variegated regions are highlighted and the Z w.t. showed as a reference. The 12 clones that were expressed are marked in bold and sequence homologies are boxed. Amino acids are given by their one-letter code (B = Amber TAG stop codon, expressed as Q in RRIΔM15)

		HELIX 1						HELIX 2							HELIX 3
		10					20				30				40 50
Z_{wt}:	VDNKFNKE	**QQN**	A	**FY**	EI	**LH**	LPNLN	**EE**	Q	**RN**	AFI	**Q**	SL	**K**	DDPSQSANLLAEAKKLNDAQAPK
$Z_{hCD4-Ab31}$:	--------	BEC	-	RS	--	PG	-----	RE	-	ER	---	A	--	N	-----------------------
$Z_{hCD4-Ba16}$:	--------	AMC	-	TQ	--	LS	-----	TR	-	RG	---	E	--	G	-----------------------
$Z_{hCD4-Aa20}$:	--------	CVV	-	VL	--	CH	-----	GT	-	GL	---	P	--	P	-----------------------
$Z_{hCD4-Ab9}$:	--------	CVV	-	VL	--	CH	-----	GT	-	GL	---	P	--	P	-----------------------
$Z_{hCD4-Aa31}$:	--------	DKS	-	GM	--	SS	-----	EA	-	PD	---	P	--	G	-----------------------
$Z_{hCD4-Ba12}$:	--------	ERA	-	ST	--	DC	-----	QT	-	TR	---	F	--	A	-----------------------
$Z_{hCD4-Ba8}$:	--------	GES	-	GA	--	EP	-----	QQ	-	ED	---	S	--	E	-----------------------
$Z_{hCD4-Ba9}$:	--------	GES	-	GA	--	EP	-----	QQ	-	ED	---	S	--	E	-----------------------
$Z_{hCD4-Aa9}$:	--------	GIK	-	FH	--	ES	-----	PY	-	CG	---	I	--	S	-----------------------
$Z_{hCD4-Ab41}$:	--------	HAS	-	GS	--	QY	-----	GI	-	LQ	---	C	--	A	-----------------------
$Z_{hCD4-Ab8}$:	--------	ILF	-	VC	--	AP	-----	DR	-	SL	---	I	--	S	-----------------------
$Z_{hCD4-Ab38}$:	--------	KRK	-	EG	--	NT	-----	WT	-	LS	---	H	--	I	-----------------------
$Z_{hCD4-Bb60}$:	--------	NWH	-	IA	--	WK	-----	VS	-	IE	---	L	--	P	-----------------------
$Z_{hCD4-Bb64}$:	--------	PHS	-	CL	--	FR	-----	PW	-	EF	---	B	--	I	-----------------------
$Z_{hCD4-Aa8}$:	--------	QMK	-	RV	--	QA	-----	GY	-	CI	---	F	--	G	-----------------------
$Z_{hCD4-Bb56}$:	--------	RPG	-	VR	--	TY	-----	TR	-	BE	---	H	--	G	-----------------------
$Z_{hCD4-Aa13}$:	--------	RRV	-	WK	--	VL	-----	GQ	-	YK	---	R	--	L	-----------------------
$Z_{hCD4-Aa39}$:	--------	RVF	-	CF	--	SI	-----	AD	-	DE	---	G	--	G	-----------------------
$Z_{hCD4-Ab22}$:	--------	RVF	-	CF	--	SI	-----	AD	-	DE	---	G	--	G	-----------------------
$Z_{hCD4-Ba13}$:	--------	TRL	-	SD	--	QN	-----	ID	-	DB	---	Q	--	E	-----------------------
$Z_{hCD4-Ba14}$:	--------	TRL	-	SD	--	QN	-----	ID	-	DB	---	Q	--	E	-----------------------
$Z_{hCD4-Ab14}$:	--------	TVW	-	CI	--	TI	-----	GE	-	NM	---	N	--	S	-----------------------
$Z_{hCD4-Bb65}$:	--------	VCT	-	AR	--	EA	-----	GE	-	SQ	---	B	--	Y	-----------------------
$Z_{hCD4-Ba11}$:	--------	WLM	-	LP	--	AP	-----	VS	-	FT	---	Q	--	Q	-----------------------
$Z_{hCD4-Aa44}$:	--------	VYR	-	MW	--	GF	-----	LP	-	IR	---	R	--	S	-----------------------

5.3 DNA SEQUENCING ANALYSIS

40 clones were selected for DNA sequence analysis. Affibody® inserts were PCR amplified and the fragment length was confirmed on agarose gel (~580 bps; 25 fragments

had an estimated correct length, 15 clones had a deviating band size). All clones were sequenced and 15 clones showed large substitutions/deletions, as expected from the length analysis. Of the remaining 25, four sequences appeared in duplicate and nine belonged to those scoring higher than average in the ELISA screening (Tab. 2). Six of the clones had the amber (TAG) stop-codon within the variegated region translated to a Q (glutamine) in a *E. coli* suppressor cell strain. Clones were selected for protein expression based on ELISA results and sequence homologies.

5.4 PROTEIN EXPRESSION AND PURIFICATION

Plasmids from selected Affibody® clones were propagated and transformed to RV308 bacterial cells. Protein expression from the phagemid vector in the non-suppressor *E. coli* strain RV308 generates the fusion protein E'-Z_{hCD4}-ABD (Fig. 10). Clones carrying the amber stop codon were produced directly in the suppressor strain to yield full-length proteins. Individual clones were grown and periplasmic proteins were collected by osmotic shock treatment. The fusion proteins were purified with affinity chromatography taking advantage of the HSA-binding domain ABD.

Figure 10. E'-Z_{hCD4}-ABD fusion protein. When expressed in RV308, Affibody® clones are fused with the first six residues of domain E in staphylococcal protein A (E') as well as an albumin binding domain derived from streptococcal protein G (ABD), omitting the M13 pIII part of the gene.

Total amounts of purified proteins from 100 ml culture of the hCD4 specific Affibody® clones were determined spectrophotometrically at A_{280} and calculated using the absorbance for 1 mgmL^{-1} of each respective protein (Table 3). The purity of the proteins was analysed with SDS-PAGE under reducing conditions (data not shown). The proteins appeared pure. However, since several clones yielded only minute concentrations, it was difficult to determine if any impurities were present or not.

Table 3. Protein concentrations after expression.

Clone	A_{280}	A_{280} of 1 mgmL^{-1}	c (µgmL^{-1})	Strain
$Z_{hCD4\text{-}Aa13}$	0.008	1	8.0	RV308
$Z_{hCD4\text{-}Aa31}$	0.002	0.49	4.1	RRIΔM15
$Z_{hCD4\text{-}Ab09}$	0.002	0.51	4.0	RV308
$Z_{hCD4\text{-}Ab22}$	0.833	0.5	1670	RV308
$Z_{hCD4\text{-}Ab31}$	0.023	0.49	47.0	RV308
$Z_{hCD4\text{-}Ab38}$	0.016	0.91	18.0	RV308
$Z_{hCD4\text{-}Ba08}$	0.117	0.49	240	RV308
$Z_{hCD4\text{-}Ba11}$	0.012	0.91	13.0	RV308
$Z_{hCD4\text{-}Ba12}$	0.023	0.49	47.0	RV308
$Z_{hCD4\text{-}Ba13}$	0.011	0.48	23.0	RRIΔM15
$Z_{hCD4\text{-}Bb60}$	0.04	1.34	30.0	RV308
$Z_{hCD4\text{-}Bb64}$	0	0.91	0	RRIΔM15

5.5 BIOSENSOR ANALYSIS

Binding analysis of the purified proteins against hCD4 was performed on a BIAcore 2000 using surface plasmon resonance. Immobilization of hCD4, and hIgG as a negative control, on a CM-5 chip resulted in 3810 resonance units (RU) and 3460 RU, respectively. Injection of all 12 Affibody® variants revealed six binding candidates: $Z_{hCD4\text{-}Aa13}$, $Z_{hCD4\text{-}Ab38}$, $Z_{hCD4\text{-}Bb60}$, $Z_{hCD4\text{-}Aa31}$, $Z_{hCD4\text{-}Ba11}$ and $Z_{hCD4\text{-}Ab22}$ (Fig. 11, a-f). The two former had responses above 100 RU whereas the remaining four were significantly lower.

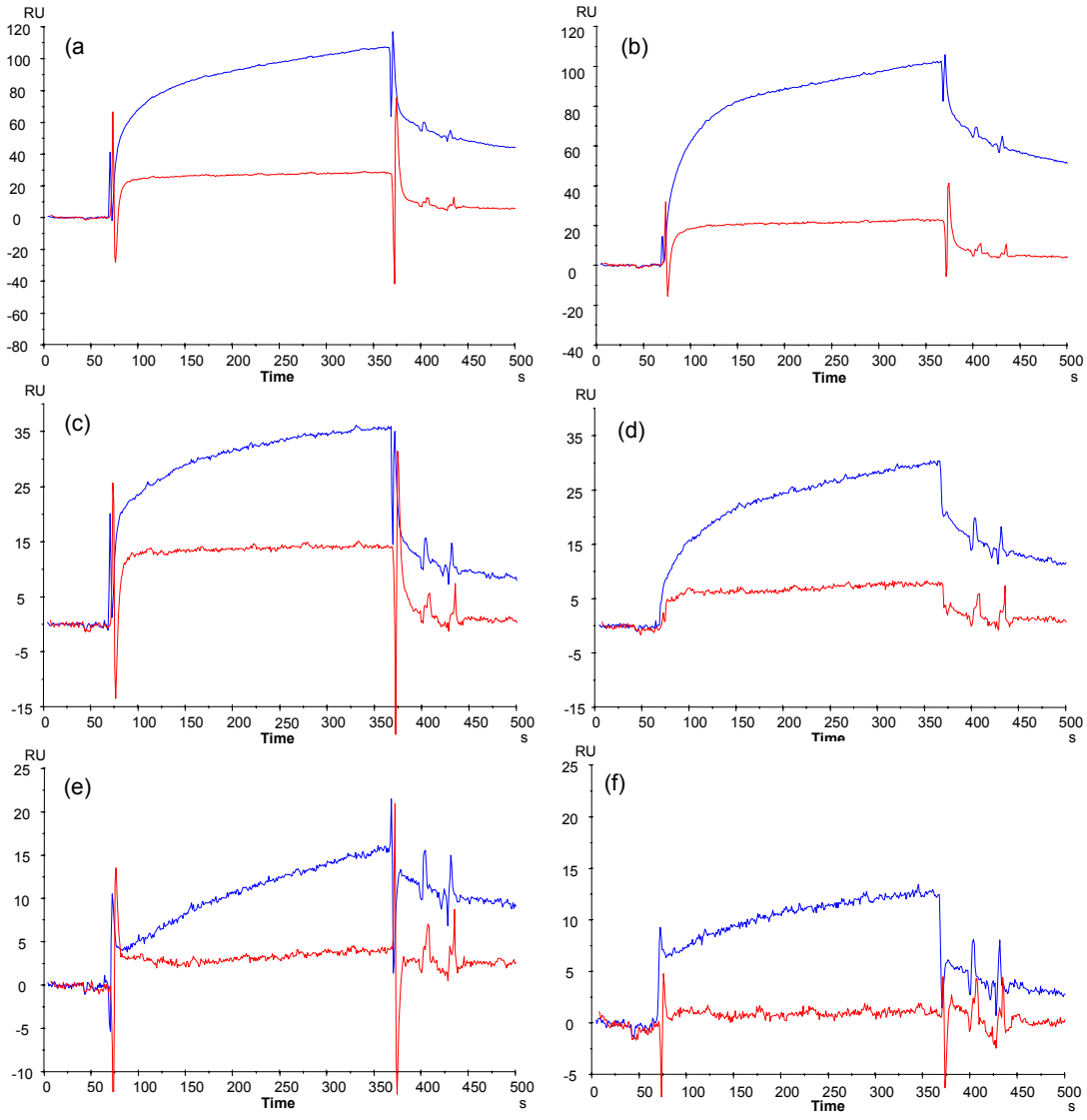

Figure 11. Sensorgrams. Subtractive sensorgrams of putative hCD4-binding Affibody® clones injected over CM5 sensor chip surfaces containing immobilised hCD4 (blue) or IgG (red) used as negative control. (a) $Z_{hCD4\text{-}Aa13}$, (b) $Z_{hCD4\text{-}Ab38}$, (c) $Z_{hCD4\text{-}Ba11}$, (d) $Z_{hCD4\text{-}Bb60}$, (e) $Z_{hCD4\text{-}Ab22}$ and (f) $Z_{hCD4\text{-}Aa31}$

6 DISCUSSION

In this work, selections using phage display technology was employed to obtain hCD4-binding proteins. Soluble selection using biotinylated target protein and streptavidin coated magnetic beads is a straightforward method commonly used for selection. However, tendencies to produce streptavidin-binding ligands, possibly at the expense of target specific binders, have been reported (E. Gunneriusson, personal communication). In an attempt to reduce the risk of selecting streptavidin binders, an alternate immobilisation strategy was performed in parallel. By covalently attaching hCD4 directly to tosylactivated beads, both biotin and streptavidin could be abolished from the selection environment, thereby reducing background binding. Another way of reducing the risk of selecting unspecific binders is by introducing a preselection step. By exposing the library to the background factors present during selection, variants binding to for example streptavidin molecules or phage coat proteins are removed from the library prior to the actual selection. In theory, this ought to have significantly reduced the amount of unspecific binding and consequently resulted in higher stringency during the crucial first cycle.

With thirteen variegated positions in the Affibody® scaffold, the total number of possible variants is a staggering 20^{13}. This number is a simplification since it only deals with the number of possible aa, not codon, combinations. Furthermore it does not take the relative aa prevalence into consideration. The $3.3 \cdot 10^9$ variants of the library used in this work makes it a comparatively large phage display library. It is important to keep in mind that since it does not completely fill the 13-dimensional space spanned by the 20^{13} *possible* variants (Fig. 12), one can never be certain that a binding variant is included in the library. However, experience has shown (E. Gunneriusson, unpublished data) that libraries with 10^6 and 10^9 variants generally produce binders with K_D values in the range of μM and nM, respectively. This is considered adequate for many applications. Should higher affinities be required, library maturation could be attempted. If sequence homologies are found among some selected molecules, this may be taken as an indication that they could be important for binding to the target. By locking these particular positions and randomising the remaining ones, a library is created that only spans a fraction of the original space but since it is much denser, the possibilities of finding better binders are increased.

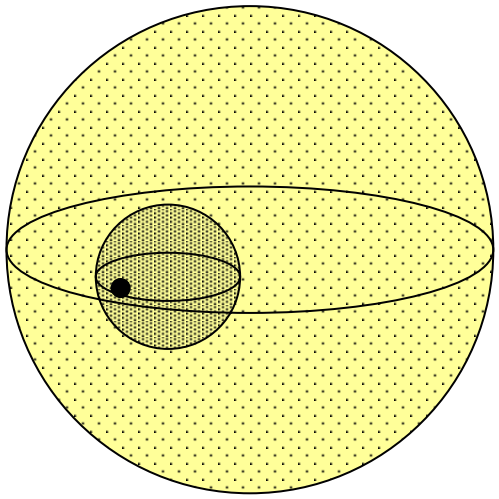

Figure 12. Illustration of the 20^{13} possible variants (whole sphere) partly filled by the $3.3 \cdot 10^9$ variants in the library (dots). Small denser sphere shows possible library after maturation and the black dot represents a hypothetical ideal binder.

Due to the low levels of expressed protein, it is difficult to draw any general conclusions about either the selected Affibody® molecules or the selection strategies that produced them. Nevertheless, six out of twelve investigated variants were shown to bind hCD4 to a greater ($Z_{hCD4\text{-}Aa13}$ and $Z_{hCD4\text{-}Ab38}$) or lesser ($Z_{hCD4\text{-}Ba11}$, $Z_{hCD4\text{-}Bb60}$, $Z_{hCD4\text{-}Ab22}$ and $Z_{hCD4\text{-}}$

$_{Aa31}$) extent. The lower response obtained for $Z_{hCD4\text{-}Aa31}$ could possibly be explained by the lower concentration used in the biosensor binding analysis. However, this does not apply to $Z_{hCD4\text{-}Ab22}$ since enough protein supposedly was available to inject a 10 µM solution over the biosensor chip. Considering the overall low expression levels and the fact that the sensorgram (Fig. 11 e) indicates binding, it might be wise to investigate $Z_{hCD4\text{-}Ab22}$ further before dismissing it as less interesting. The ambiguous results concerning binding variants $Z_{hCD4\text{-}Ab22}$ and $Z_{hCD4\text{-}Aa31}$ was most likely due to insufficient amounts of expressed protein. This may also explain why the binding characteristics of another three variants ($Z_{hCD4\text{-}Ab09}$, $Z_{hCD4\text{-}Ab31}$ and $Z_{hCD4\text{-}Bb64}$) proved difficult to analyse.

Interestingly, only one of the four sequence duplicates (boxed in Tab. 2) showed any binding to hCD4 ($Z_{hCD4\text{-}Ab22}$ and $Z_{hCD4\text{-}Aa39}$). The appearance of matching sequences is generally taken as a token for binding variants as the probability of non-binding sequence homologues merely 'slipping by' all four selection cycles is not very high. The selection of non-binding variants in general and of sequence homologues in particular was not further investigated. It could, at least in part, be explained by background binding of these variants to components other than hCD4 present during selection such as phages and streptavidin (soluble selection).

Again, due to the low amounts of protein, it is difficult to safely assess the relative merits of the two selection strategies. While soluble selection yielded four out of six identified binders, including the two scoring highest in biosensor analysis, there is no indication of significant differences in overall selection effectiveness *per se*. In spite of the poorer enrichment values for solid phase selection (Tab. 1), the strategy did in fact manage to bring about two hCD4-binding Affibody® molecules. Additionally, it produced no streptavidin-binding variants (Fig. 9a). On the other hand, the possibility to preselect the library provided by selection in solution should reduce the risk of unspecific binding and this option is not as readily available when using solid phase selection.

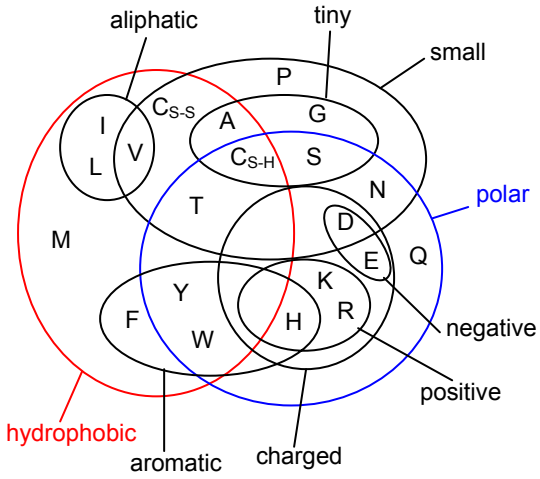

Figure 13. Venn diagram grouping amino acids according to their properties. (concept from Livingstone CD *et al.*, 1993 [33]).

Had more protein been available for analysis, a possible correlation between target concentration during selection and final binding properties could have been evaluated. Since diverged target concentrations only were applied in the final cycle, no significant differences were expected. It is not impossible that variants with better affinity could have been selected if the target concentration had been reduced earlier (increased stringency criterion).

When comparing the characteristics of the thirteen altered residues in the six binding Affibody® molecules, no immediate homologies are revealed to suggest that they bind to the same site on hCD4 (Tab. 4). The overall impression is of an abundance of polar residues intermixed with a noteworthy amount of hydrophobic amino acids (Fig. 13). The

two highest scoring variants ($Z_{hCD4\text{-}Aa13}$ and $Z_{hCD4\text{-}Ab38}$) are very similar in positions one, two and three. Apart from a hydrophobic valine, all residues are positively charged and polar.

Epitope mapping on hCD4 have not been performed but the sequence discrepancies suggest that there could be more than one binding site.

Table 4. Summary of variegated positions in binding Affibody® molecules.

	HELIX 1							HELIX 2					
	Pos 1	Pos 2	Pos 3	Pos 4	Pos 5	Pos 6	Pos 7	Pos 8	Pos 9	Pos 10	Pos 11	Pos 12	Pos13
$Z_{hCD4\text{-}Aa13}$	R	R	V	W	K	V	L	G	Q	Y	K	R	L
$Z_{hCD4\text{-}Ab38}$	K	R	K	E	G	N	T	W	T	L	S	H	I
$Z_{hCD4\text{-}Ba11}$	W	L	M	L	P	A	P	V	S	F	T	Q	Q
$Z_{hCD4\text{-}Bb60}$	N	W	H	I	A	W	K	V	S	I	E	L	P
$Z_{hCD4\text{-}Ab22}$	R	V	F	C	F	S	I	A	D	D	E	G	G
$Z_{hCD4\text{-}Aa31}$	D	K	S	G	M	S	S	E	A	P	D	P	G

7 FUTURE WORK

Most importantly, more protein needs to be expressed in order to enable further analysis. Using the T7-promotor system and expressing the hCD4-binding Affibody® molecules in their final form, i.e. without the ABD fusion partner, could accomplish this. This requires cloning of the binding variants to new vectors as well as subsequent expression and purification. Once sufficient amounts of protein is available, the next successive step towards evaluating cell separation using Affibody® coated magnetic beads would be to characterise the binding variants through a kinetic analysis using a biosensor. This is carried out in order to determine on and off rates so that K_D values can be calculated. Appropriate hCD4 ligands would then be immobilised on paramagnetic beads, either as mono-, di- or multimeric constructs, and assayed for their ability to specifically separate T cells from human blood. Purity and quality of isolated cell populations could be investigated using hCD4-specific Abs and flow cytometry. Finally, if hCD4-binding Affibody® molecules would prove to be more specific than mAbs and/or superior regarding capacity, it will also be necessary to delineate the activating/deactivating potential of the selected Affibody® molecules on the isolated T cells.

The versatility of phage display was demonstrated in this work where Affibody® molecules binding to hCD4 were successfully selected. Once characterised, these affinity ligands open up the possibility to conduct further studies comparing them with existing Abs. Should these Affibody® molecules prove to have binding characteristics equal to or surpassing those of existing hCD4-specific molecules, several applications could be possible. Taking advantage of the robust properties inherent in the Affibody® scaffold, not only cell separation but also techniques such as affinity chromatography could be improved upon by using these binders. Since both hCD4-binding mAbs and hCD4-specific paramagnetic beads are already commercially available, future comparative studies will be greatly facilitated.

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10 APPENDIX A

Human CD4, NCBI acc.no: P01730 [35]

```
1    mnrgvpfrhl llvlqlallp aatqgkkvvl gkkgdtvelt ctasqkksiq fhwknsnqik
61   ilgnqgsflt kgpsklndra dsrrslwdqg nfpliiknlk iedsdtyice vedqkeevql
121  lvfgltansd thllqgqslt ltlesppgss psvqcrsprg kniqggktls vsqlelqdsg
181  twtctvlqnq kkvefkidiv vlafqkassi vykkegeqve fsfplaftve kltgsgelww
241  qaerasssks witfdlknke vsvkrvtqdp klqmgkklpl hltlpqalpq yagsgnltla
301  leaktgklhq evnlvvmrat qlqknltcev wgptspklml slklenkeak vskrekavwv
361  lnpeagmwqc llsdsgqvll esnikvlptw stpvqpmali vlggvaglll figlgiffcv
421  rcrhrrrqae rmsqikrlls ekktcqcphr fqktcspi
```

1. Signal sequence 1-25 (25 aa)
2. Extracellular region 26-396 (371)
    - 26-125 Ig V-type domain
    - 126-203 Ig-like C2-type domain 1
    - 204-317 Ig-like C2-type domain 2
    - 318-374 Ig-like C2-type domain 3
3. Transmembrane region 397-418 (22 aa)
4. Cytoplasmic region 419-458 (40 aa)

11 APPENDIX B

The genetic code

UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys
UUA	Leu	UCA	Ser	UAA	Stop	UGA	Stop
UUG	Leu	UCG	Ser	UAG	Stop	UGG	Trp
CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
CUC	Leu	CCC	Pro	CAC	His	CGC	Arg
CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg
GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly
GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly
GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly

Symbols for Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid or aspartate
E	Glu	Glutamic acid or glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine