

Inhibition of the CD28–CD80 co-stimulation signal by a CD28-binding affibody ligand developed by combinatorial protein engineering

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CD28 is one of the key molecules for co-stimulatory signaling in T cells. Here, novel ligands (affibodies) showing selective binding to human CD28 (hCD28) have been selected by phage display technology from a protein library constructed through combinatorial mutagenesis of a 58-residue three-helix bundle domain derived from staphylococcal protein A. Analysis of selected affibodies showed a marked sequence homology and biosensor analyses showed that all investigated affibodies bound to hCD28 with micromolar affinities (K_D). No cross-reactivity towards the related protein human CTLA-4 could be observed. This lack of cross-reactivity to hCTLA-4 suggests that the recognition site on hCD28 for the affibodies resides outside the conserved MYPPPPY motif. The apparent binding affinity for hCD28 could be improved through fusion to an Fc fragment fusion partner, resulting in a divalent presentation of the affibody ligand. For the majority of selected anti-CD28 affibodies, in co-culture experiments involving Jurkat T-cells and CHO cell lines transfected to express human CD80 (hCD80) or LFA-3 (hLFA-3) on the cell surface, respectively, pre-incubation of Jurkat cells with affibodies resulted in inhibition of IL-2 production when they were co-cultured with CHO (hCD80⁺) cells, but not with CHO (hLFA-3⁺) cells. For one affibody variant denoted Z_{CD28:5} a clear concentration-dependent inhibition was seen, indicating that this affibody binds hCD28 and specifically interferes in the interaction between hCD28 and hCD80.

Keywords: affibody/CD28/CD80/combinatorial/co-stimulation/CTLA-4/Fc-fusion/inhibition/LFA-3/ligands/phage display/selection

Introduction

In the cellular contacts between T-cells and antigen-presenting cells mediated via T-cell receptor (TCR)/peptide antigen-major histocompatibility complex (MHC) recognition, additional interactions are crucial to control activation or induction of tolerance. Of significant importance are those between B7-1 (CD80)/B7-2 (CD86) proteins expressed on antigen-presenting cells and their cognate binding partners CD28 or CTLA-4 (CD152) on T-cells which regulate T-cell proliferation and cell cycle arrest, respectively (Alegre *et al.*, 2001; Chambers, 2001). These intercellular contacts are widely considered for the investigation of novel immunotherapy strategies. Stimulation of these co-stimulatory molecules may, for

instance, be useful for vaccination or immune therapy of cancer. Efforts in this field include, for example, transfection of tumor cell lines for surface expression of CD80/86 (Townsend and Allison, 1993). Alternatively, genetic fusions between CD80/86 or the T-cell receptor binding bacterial superantigen SEA (staphylococcal enterotoxin A) and tumor-specific antibody fragments to provide reagents for tumor-specific immunotherapy via simultaneous tumor cell and T-cell binding have been extensively investigated (Dohlsten *et al.*, 1994; Rohrbach *et al.*, 2000; Hoffman *et al.*, 2001; Marshall and Marks, 2001).

Conversely, inhibition of co-stimulatory signals may be used to induce tolerance in, for example, autoimmune diseases such as psoriasis and rheumatoid arthritis indications and organ transplantation (Aruffo and Hollenbaugh, 2001). To this end, various CD80/86 binding reagents are being investigated for their ability to block co-stimulatory signals, including anti-CD80/86 antibodies (Gottlieb *et al.*, 2002; Montgomery *et al.*, 2002), fusions between the extracellular domain of CTLA-4 and human IgG₁ (CTLA-4.Ig) (Najafian and Sayegh, 2000) and a novel class of ligands based on a human VL scaffold (van der Beucken *et al.*, 2001). Reagents capable of blocking co-stimulation via CD28 binding have also been described (Dong *et al.*, 2002; Otto *et al.*, 2002). Such reagents could be advantageous since unligated CD80/86 would be accessible for CTLA-4 binding, which potentially could result in a further down-regulation of unwanted immune responses (Dong *et al.*, 2002).

A detailed understanding of the structures of the molecular complexes involved in co-stimulation signalling would contribute to the development of novel strategies for intervention. Recently, the co-crystal structures for the complexes between CTLA-4 and CD80/CD86 were described, indicating the formation of highly avid intercellular interactions mediated by bivalent homodimers of both CTLA-4 and CD80/86 molecules arranged in a lattice-like network (Schwartz *et al.*, 2001). Interactions between CD28 and CD80/86 are characterized by lower apparent affinities and have been proposed to occur through non-linked interactions (Chambers, 2001). The CD28/CTLA-4 family of receptors has more recently been expanded with new members. The importance of these receptors, ICOS (Inducible co-stimulator) and PD-1 (Programmed death-1), for co-stimulation remains to be fully elucidated, but could provide interesting targets for immunotherapy applications (Chambers, 2001).

We have earlier described the use of a 58-residue single three-helix bundle domain of staphylococcal protein A as supporting scaffold for the construction of combinatorial protein libraries from which novel binding proteins denoted affibodies can be selected to desired target proteins (Nord *et al.*, 1997, 2000; Hansson *et al.*, 1999; Eklund *et al.*, 2002; Ronnmark *et al.*, 2002a). Identified affibodies, isolated using phage display technology, have been shown to bind their

respective target proteins with high selectivity, also after genetic fusion to other proteins, which has prompted investigations of their use as affinity tools in various biotechnology applications, including diagnostics (Ronmark *et al.*, 2002b), bioseparation (Nord *et al.*, 2000, 2001; Graslund *et al.*, 2002) and therapy (Henning *et al.*, 2002).

Here, we describe the selection and characterization of novel affibodies binding to human CD28 (hCD28), using an hCD28–mouse IgG₁ Fc fusion protein as target during biopannings. Biosensor binding analyses and cell co-culture-based IL-2 production inhibition assays showed that this class of affinity proteins could be used as highly selective reagents for intervention of CD80-specific intercellular contacts.

Materials and methods

Strains, vectors and phagemid library

The amber suppressor *Escherichia coli* strain RR1ΔM15 (Ruther, 1982) was used as bacterial host for phage works. *E. coli* strains RV308 (Maurer *et al.*, 1980) and KS476 (Strauch and Beckwith, 1988) were used for protein production from the phagemid pKN1-derived constructs (Nord *et al.*, 1995) and pEFc3.1-derived constructs (Jendeberg *et al.*, 1997), respectively. The construction of the phagemid library used in this study, ZLIB-2, has been described earlier (Nord *et al.*, 1997).

Preparation of phage stocks

Preparation of phage stocks from the library (ZLIB-2) and between selection rounds was performed using standard procedures involving helper phage M13K07 (New England Biolabs, Beverly, MA), routinely yielding phage titres between 10¹¹ and 10¹² pfu/ml after PEG/NaCl precipitation.

Selections

The target fusion protein hCD28–mIgG₁Fc (hCD28–mFc) was produced and purified by protein A affinity chromatography as described previously (Bennett *et al.*, 2000). The protein was biotinylated *in vitro* using an EZ-Link Sulfo-NHS-LC-Biotin biotinylation kit (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. The biotinylated protein was dialysed against phosphate-buffered saline (PBS; 50 mM phosphate, 100 mM NaCl, pH 7.2) using a 10-k SLIDE-A-LYZER cassette (Pierce Chemical) according to the manufacturer's recommendations. The biotinylated target fusion protein was immobilized on streptavidin-coated paramagnetic beads (SA-beads) (Dynabeads M280-SA; Dynal, Oslo, Norway). For each round of biopanning, a 5 mg portion of the beads was washed three times with PBS supplemented with 0.1% Tween 20 (PBST), followed by incubation with 50 μg of biotinylated hCD28–mFc in 1.2 ml of PBST, pH 7.2, overnight at 4°C under continuous rotation (end over end). The beads with the immobilized hCD28–mFc fusion protein were subsequently washed six times with PBST, pH 7.2. This procedure resulted in an immobilization of ~10 μg of the 100 kDa hCD28–mFc protein on 5 mg of SA-beads, as determined by SDS–PAGE analysis.

The four rounds of biopanning was performed as follows. To avoid unspecific binders, all the tubes used in this procedure were pretreated with PBS supplemented with gelatine (final concentration 0.1%). Additionally, a 150 μl portion of the phagestock (ZLIB-2) was subjected to a 1 h preincubation step with 0.5 mg of plain, dry SA-beads together with gelatine (0.1%). Approximately 100 μl of unbound phagestock were subsequently transferred to another tube containing the

hCD28–mFc immobilized SA-beads in PBS and gelatine (0.1%). To reduce the number of phages binding to the immobilized fusion partner mIgG₁, a non-biotinylated mouse monoclonal of IgG₁ was also added to the mixture (final concentration ~0.9 μM). Biopanning was performed for 3 h at room temperature under continuous rotation. The beads were washed once with PBS, pH 7.2, before being transferred to a new pretreated tube (0.1% gelatine), whereupon the bound phages were eluted with 500 μl of 0.1 M glycine HCl, pH 2.2, for 10 min at room temperature, followed by immediate neutralization with 50 μl of 1 M Tris–HCl. The eluted phage particles were used to infect log phase RR1ΔM15 cells for 20 min at 37°C. The infected cell suspensions were spread on TYE agar plates (per litre: 15 g of agar, 8 g of NaCl, 10 g of Tryptone and 6 g of yeast extract), supplemented with 2% glucose and 100 μg/ml ampicillin, and incubated overnight at 37°C. The colonies were subsequently collected by resuspension in TSB medium (supplemented with 1% glucose and 100 μg/ml ampicillin) and a fraction (10⁹ cells) was used for inoculation, leading to the next generation of phage stock. The procedures in rounds 2, 3 and 4 were the same as in the first round of panning, except that the washing steps were performed with PBST, pH 7.2, and the number of washing steps was increased (3, 6 and 12 washes, respectively).

DNA sequencing

DNA sequencing of phagemid (pKN1) inserts of 10 selected clones was carried out after four rounds of panning using an indodicarbocyanine dye ALFred (Cy5) phosphoramidite-labelled sequencing primer NOKA-3, employing a robotic workstation (Biomek 1000; Beckman Instruments, Fullerton, CA). The Sanger fragments were analysed on an ALFexpress (Pharmacia Biotech, Uppsala, Sweden).

DNA constructions

The gene encoding the variant Z_{CD28:5} was amplified by PCR using primers Zsub1 (5'-CCC CGA ATT TCC GTA GAC AAC AAT TAA CAA-3') and RIJE1 (5'-TTT TGA ATT CCC GCT GCC ACC GCC TTT CGG CGC CTG AGC ATC-3'), containing *EcoRI* recognition sites, using phagemid DNA as template. The fragments were transferred to pGEM-T-vectors (Promega, Madison, WI) and transformed to RR1ΔM15 cells according to the manufacturer's instructions. After standard plasmid preparation using Jetstar miniprep columns (Genomed, Oeyenhausen, Germany), the Z_{CD28:5} encoding insert was excised from the pGEM-T-Vector by *EcoRI* digestion. The fragments were purified using agarose gel before ligation to the vector pEFc3(1) (Jendeberg *et al.*, 1997), previously restricted with *EcoRI*, gel purified and dephosphorylated.

Protein production and purification

The affibodies were expressed either from their phagemid (pKN1) constructs as ABD fusions in a non-suppressor strain (RV308) or from the pEFc3.1 vector as Fc fusions in a *degP* strain (KS476) (Strauch *et al.*, 1988). The affibody–ABD fusions were produced as follows: overnight cultures in TSB–Amp supplemented with yeast extract (5 g/l) at 37°C were used to inoculate (diluted 1:200) 500 ml of TSB–Amp in baffled flasks. The cells were grown until A_{600 nm} reached 1, when cultures were induced with IPTG (final concentration 1 mM) and subsequently grown at 25°C for another 20 h. The Fc fusions, as encoded from the affibody:pEFc3.1 vector, were expressed by the inoculation of a single colony in 500 ml of

TSB medium [supplemented with yeast extract and ampicillin (100 µg/ml)] and grown in baffled flasks for 40 h at 30°C. In both expression systems, periplasmic proteins (ABD and Fc fusions) were released by osmotic shock treatment (Nygren *et al.*, 1988) before subsequent affinity purification. Expression levels were determined by measuring the absorbance at 280 nm and the fractions of interest were pooled and lyophilized.

The affibody–ABD fusion proteins were purified on HSA–Sepharose columns (Nygren *et al.*, 1988). Fractions of the pools (10 mOD) were analysed on high-density phast gel using the Phast system (Pharmacia Biotech) under reducing conditions. Some lyophilized affibody fusion proteins were subsequently dissolved in 20% acetonitrile (ACN) containing 0.25% (v/v) pentafluoropropionic acid (PFPA) and loaded (1 mg for each run) on a Vydac reversed-phase C-18 column (Separations Group, Hesperia, CA). The separation process was performed at room temperature with a flow rate of 1 ml/min. Elution was performed by increasing the ACN concentration from 40 to 55% in 20 min. The absorbance was monitored at both 214 and 280 nm. Relevant fractions were collected and pooled. A fraction of each pool was analysed by SDS–PAGE on a gradient (10–20%) gel (NOVEX; Novel Experimental Technology, San Diego, CA) under reducing conditions.

The affibody–Fc fusion proteins released to the periplasm were purified on a Hi-trap affinity column, pre-packed with protein AG (Eliasson *et al.*, 1989) (according to the manufacturer's instructions) and connected to an ÄKTA explorer 10 system (Amersham Pharmacia Biotech).

Biosensor analyses

A BIAcore 2000 instrument (Biacore, Uppsala, Sweden) was employed for real-time biospecific interaction analysis (BIA). The ligands were immobilized on the carboxylated dextran layer of a CM5 sensor chip (research grade) (Biacore) by amine coupling according to the manufacturer's instructions. Mouse IgG₁ monoclonal, fusion proteins hCD28–mFc and hCTLA-4–hFc [human CTLA-4 extracellular domains fused to human IgG₁ Fc, produced in Chinese hamster ovary (CHO) cells and purified by protein G affinity chromatography] were immobilized on separate flow-cell surfaces, resulting in ~2400, ~1300 and ~1100 resonance units (RU), respectively. One flow cell was activated and deactivated to be used as a blank surface during sample injections. Binding analysis was performed at 25°C with a flow rate of 5 µl/min and injection of 20 µl of either affibody–ABD or affibody–Fc fusion proteins diluted in the running buffer HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) to a final concentration of 15–30 µM. After each injection, the flow cells were regenerated by the injection of 0.05% SDS. The K_D value for the variant Z_{CD28:5} was determined using a CM-5 chip surface containing 1300 RU hCD28–mFc. Samples of Z_{CD28:5}–ABD were injected (duplicates in random order) at different concentrations (1.9–60 µM). Injections were made at a flow rate of 5 µl/min and the surface was regenerated between each injection using 10 mM HCl. The dissociation constant (K_D) was calculated using BIAevaluation 3.1 software (Biacore), assuming a one-to-one binding model.

Jurkat cells and human co-stimulation molecule-transfected cell lines

Jurkat, a human T leukaemia cell line, was maintained in culture in RPMI 1640 supplemented with 2 mM glutamine and 10% FCS. CHO cells were transfected with cDNA encoding

the human CD80 (hCD80) or LFA-3 cell surface molecules as expressed from pEE6–hCMV–GS (glutamine synthetase marker) or pSVL–GS (neomycin marker) vector constructs including hHLA–DR4 expression cassettes. The surface expression of the transfected molecules was confirmed by fluorescence-activated cell sorter analysis. The transfectants were cultured in glutamine-free RPMI 1640 supplemented with 10% FCS, 1 mM MSX and 1 mg/ml G418.

CD28-dependent IL-2 production assay

Jurkat cells (1 × 10⁶/ml) were incubated with different concentrations of testing affibodies for 20 min and then co-cultured with CHO–hHLA–DR4–hCD80 transfectants which were irradiated with 10 000 rad in a ratio with Jurkat cells of 1:5. Jurkat cells co-cultured with CHO–hHLA–DR4–LFA-3 transfectants served as specific controls. Staphylococcal enterotoxin E (SEE; Toxin Technology, Madison, WI) was added at same time at a final concentration of 5 nM. In this assay, SEE bound to hHLA–DR4 and presented to CD3/TCR on Jurkat cells as the first signal for the cell activation. Interaction between hCD80 on CHO cells and CD28 on Jurkat cells delivers the second signal. Jurkat cells do not produce IL-2 when co-cultured with CHO–hHLA–DR4 transfectants in the presence of SEE, demonstrating the necessity for co-stimulation. The cultures were kept in 5% CO₂ at 37°C in a saturated humid atmosphere for 18 h. The supernatants were harvested and immediately determined for IL-2 production with commercial ELISA kits (Duoset; Genzyme). The percentage inhibition was calculated as [(pg/ml in the experimental group – pg/ml in the control group)/pg/ml in the control group] × 100.

Results

Library and selections

Phage display *in vitro* selection technology was used to investigate if novel ligands for the human T-cell surface marker CD28 (hCD28) could be isolated from the combinatorial protein library (ZLIB-2), based on the structure of a single domain (Z domain) derived from staphylococcal protein A (Nord *et al.*, 1997). In this library, comprising ~4.5 × 10⁷ members, variants resulting from combinatorial substitution mutagenesis of 13 positions located at the domain surface responsible for Fc binding activity of the wild-type 58-residue Z domain are displayed on bacteriophage particles using a phagemid vector system (Nord *et al.*, 1997). For use as a target during selection of new ligands (denoted Z-affibodies) to hCD28, the extracellular portion of the protein was recombinantly produced in a dimeric immunoadhesion format, i.e. genetically fused to the Fc fragment of murine IgG₁ (hCD28–mFc) (Figure 1A), allowing for recovery of the fusion protein by protein A affinity chromatography from CHO host cell supernatants (Bennett *et al.*, 2000).

Four rounds of phage display selection were performed using a low pH (pH 3) for phage particle elution from streptavidin microbeads with biotinylated hCD28–mFc target fusion protein. An alignment of deduced amino acid sequences of inserts in 10 randomly picked clones obtained after four rounds of biopannings revealed five different variants showing sequence homology in some of the positions (Table I; see Discussion).

Biosensor binding analyses of selected affibodies

The different affibody variants (Z_{CD28:1}–Z_{CD28:5}) were expressed (1–10 mg per litre of shakeflask culture) from their

respective phagemid vectors in a non-suppressor *E.coli* strain as periplasmically secreted proteins C-terminally fused to a 5 kDa serum albumin binding affinity fusion partner (ABD), allowing for affinity recovery by HSA affinity chromatography. Whereas the $Z_{CD28:5}$ variant was shown to be stably expressed as full-length ABD fusion proteins of high solubility, the other variants showed lower solubility and various degrees of proteolytic degradation between the affibody moiety and the ABD tag, requiring additional purification steps (see Materials and methods).

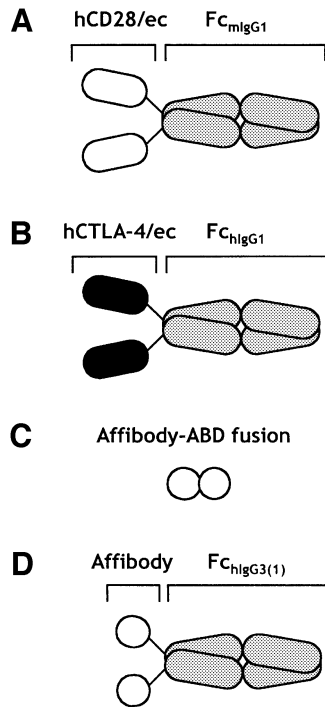


Fig. 1. Schematic description of fusion proteins used in the study. (A) Immunoadhesin construct (hCD28–mFc) between the extracellular portion of human CD28 (hCD28/ec) and an Fc fragment of murine IgG₁ immunoglobulin; (B) immunoadhesin construct (hCTLA-4–hFc) between the extracellular portion of human CTLA-4 (hCTLA-4/ec) and an Fc fragment of human IgG₁ immunoglobulin; (C) fusions between different selected affibodies and a 5 kDa serum albumin binding fragment derived from streptococcal protein G; (D) *E.coli*-produced immunoadhesion between the $Z_{CD28:5}$ affibody and an Fc fragment functionally equivalent to human IgG₁ Fc in terms of staphylococcal protein A binding (Jendberg *et al.*, 1997).

Purified affibody–ABD fusion proteins were analysed for hCD28 binding by real-time biospecific interaction analysis using a BIAcore 2000 biosensor instrument. Samples of the different fusion proteins were injected over a sensor chip flow cell containing immobilized hCD28–mFc fusion protein. To be able to distinguish between CD28 and mouse IgG₁ Fc specific binding, an irrelevant mouse monoclonal antibody of the same isotype (IgG₁) was immobilized in an adjacent flow cell. Since hCD28 and hCTLA-4 share sequence homology and have common natural ligands in the glycoproteins CD80 and CD86, an hCTLA-4–hFc immunoadhesin fusion protein construct (see Materials and methods) was also included in the analysis to investigate if the selected anti-CD28 affibodies showed any cross-reactivity towards this protein.

The biosensor binding study showed that all selected variants bound the hCD28–mFc fusion protein with similar binding profiles, as exemplified in Figure 2A for the $Z_{CD28:5}$ variant. No significant binding responses towards the hCTLA-4–hFc immunoadhesin and mIgG₁ controls could be observed for any of the variants, indicating selective hCD28 binding. For the variant $Z_{CD28:5}$, the affinity (K_D) was determined as $\sim 8.5 \mu\text{M}$ from the calculated kinetic rate constants $k_{\text{on}} = 0.99 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{\text{off}} = 8.4 \times 10^{-3} \text{ s}^{-1}$.

The variant $Z_{CD28:5}$ was also produced genetically fused to an hIgG1 fragment, resulting in divalent $Z_{CD28:5}$ –Fc fusion proteins (Figure 1D) through the homodimerizing Fc fusion partner (Jendberg *et al.*, 1997; Ronnmark *et al.*, 2002b). A biosensor binding analysis of *E.coli*-produced and protein AG affinity-purified $Z_{CD28:5}$ –Fc fusion protein showed that this construct also displayed hCD28-specific binding, indicating that the binding specificity of the affibody moiety was retained after fusion to a different and larger fusion partner. The divalent $Z_{CD28:5}$ –Fc fusion protein also showed significantly slower off-rate kinetics than its monovalent $Z_{CD28:5}$ –ABD fusion protein counterpart, indicating that both affibody moieties were functionally presented and contributed to hCD28 binding (Figure 2B).

IL-2 production inhibition assay

To investigate if an hCD28-binding affibody could interfere with the ligation between cell surface-expressed hCD28 and hCD80 proteins, a cellular assay monitoring IL-2 production was performed (Figure 3A; see Materials and methods for details). Co-culturing the human T-cell line Jurkat (CD28⁺/CD2⁺) with CHO cells transfected for hHLA–DR4 and hCD80 expression [CHO (CD80⁺)] in the presence of SEE resulted in

Table I. Characterization of selected affibody variants

Variant	Amino acid occupancies at variegated positions ^a														Target binding ^b	
	9	10	11	13	14	17	18	24	25	27	28	32	35	hCD28	hCTLA-4	
Z _{WT}	Q	Q	N	F	Y	L	H	E	E	R	N	Q	K	n.d.	n.d.	
Z _{hCD28:1}	K	G	E	N	I	R	R	R	E	G	G	I	R	+	–	
Z _{hCD28:2}	R	V	G	V	L	R	R	V	E	G	G	L	K	+	–	
Z _{hCD28:3}	Q	V	R	P	I	L	H	K	E	G	G	L	A	+	–	
Z _{hCD28:4}	K	Q	M	R	L	I	Q	K	D	G	G	I	G	+	–	
Z _{hCD28:5}	A	R	A	R	I	T	P	R	E	G	G	V	K	+ ^c	–	

^aNote that the 13 variegated positions are distributed over two α -helices of the 58-amino acid three-helix domain. The amino acid occupancies at the corresponding positions of the wild-type Z domain (Z_{WT}) are shown for comparison. Numbers in bold and italics indicate positions located on the first or second helix of the Z scaffold, respectively.

^bBiosensor binding studies; n.d., not determined.

^cThe dissociation constant (K_D) for the $Z_{hCD28:5}$ variant was determined as $8.5 \mu\text{M}$.

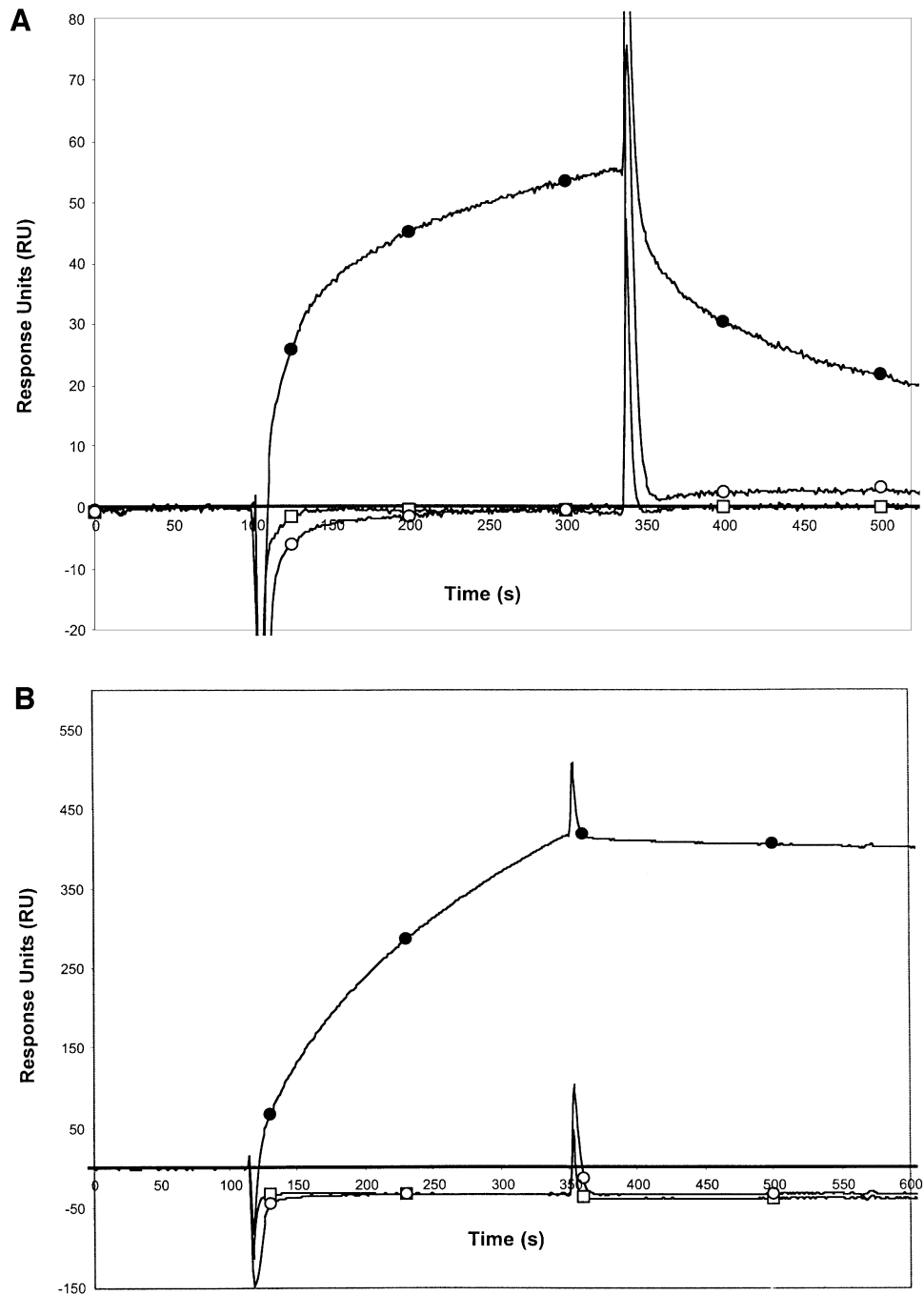


Fig. 2. Biosensor binding studies. Sensorgrams obtained after injection of purified Z_{CD28.5}-ABD fusion protein (**A**) or Z_{CD28.5}-Fc hIgG₁ immunoadhesin analytes (**B**) over sensor chip surfaces containing amine-coupled hCD28-mFc (filled circles); hCTLA-4-hFc (open circles) and a murine IgG₁ monoclonal antibody control (open squares), respectively. The effect from the sample buffer has been subtracted using the response obtained from a blank (activated and deactivated) sensor chip surface. Apparent dissociation rate constants for the Z_{CD28.5}-ABD (monovalent) and Z_{CD28.5}-Fc hIgG₁ (divalent) constructs under these conditions were determined as 8.4×10^{-3} and $1.1 \times 10^{-4} \text{ s}^{-1}$, respectively.

IL-2 production as determined from analyses of supernatant samples using an IL-2 ELISA quantification kit (data not shown). Interleukin-2 production was also observed when Jurkat cells were co-cultured in the presence of SEE with CHO cells transfected for hHLA-DR4 and hLFA-3 expression [CHO (LFA-3⁺)], corresponding to the cognate ligand for CD2 (data not shown). Interestingly, preincubation of Jurkat cells with Z_{CD28.5}-ABD fusion protein, but not with a control affibody-

ABD fusion protein, resulted in a concentration-dependent inhibition of IL-2 production for Jurkat/CHO (CD80⁺) co-cultures (Figure 3B). No significant effect on IL-2 levels from preincubation with Z_{CD28.5}-ABD fusion protein was observed for Jurkat/CHO (LFA-3⁺) co-cultures (Figure 3B). This indicates that the preincubation with the Z_{CD28.5}-ABD fusion protein resulted in selective blocking of the hCD28-hCD80 interaction.

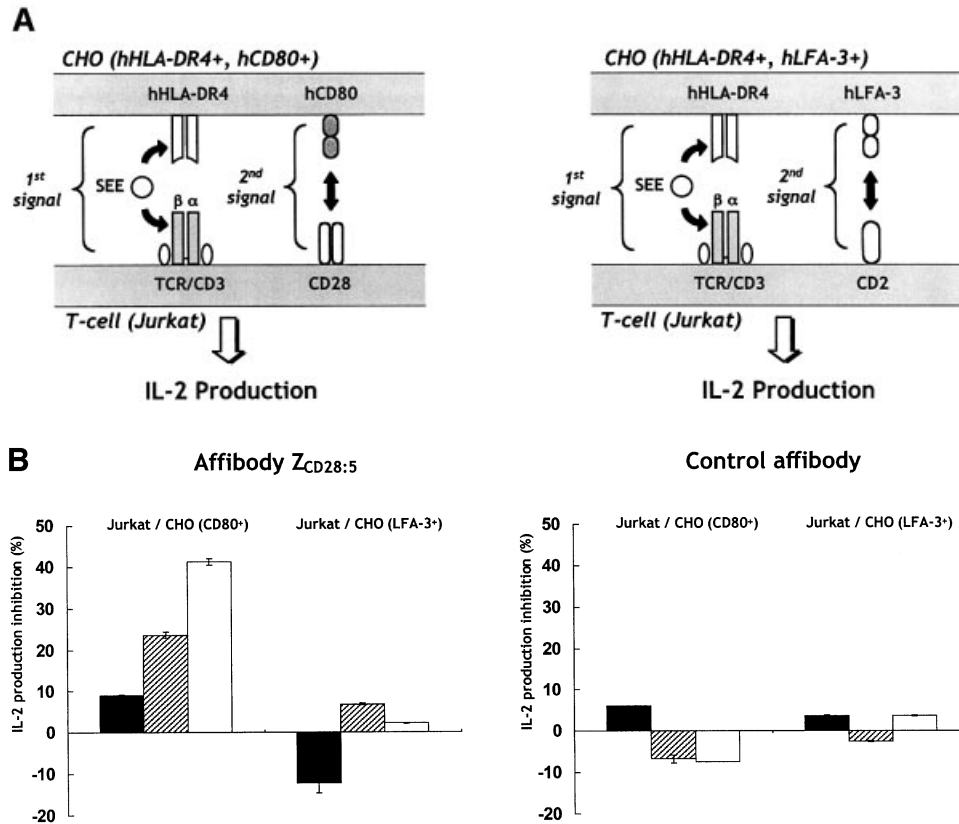


Fig. 3. IL-2 production inhibition assay. **(A)** Schematic description of cell lines used in the assay including interactions between surface expressed proteins. CHO cells transfected for surface expression of hHLA-DR4 together with either hCD80 [CHO (CD80⁺)] or hLFA-3 [CHO (LFA-3⁺)] were co-cultured with Jurkat T-cells (hCD2⁺/hCD28⁺). The effect on IL-2 production from pre-incubation of Jurkat cells with different concentrations of Z_{CD28:5}-ABD fusion protein was investigated. **(B)** Histograms showing the degree of inhibition of IL-2 production obtained from pre-incubation of Jurkat cells with different concentrations (black bars, 4 µg/ml; striped bars, 20 µg/ml; and white bars, 100 µg/ml) of Z_{CD28:5}-ABD fusion protein or a control affibody-ABD fusion [*Taq* DNA polymerase binding affibody (Nord *et al.*, 1997)] for the two co-culture systems investigated (see Materials and methods for details).

Discussion

We have described the characterization of novel binding proteins (affibodies) to hCD28, selected by phage display technology from protein library-based combinatorial engineering of a three-helix bundle domain of bacterial origin. The selected binders thus represent a radically different topology compared with the natural CD28 ligands CD80 and CD86, composed of immunoglobulin-like β -sheet domains. In cell co-culture experiments performed with mixtures of CD80- and CD28-expressing cells, respectively, a preincubation with such anti-CD28 affibody variants generally resulted in inhibition of Jurkat cell IL-2 production, indicating a potential to block the second co-stimulatory signal in this *in vitro* system based on a delivery of the first stimulatory signal by the bacterial superantigen SEE/hHLA-DR4 interaction. However, whereas the Z_{CD28:5} variant showed a clear concentration-dependent inhibitory capacity, the other four variants investigated showed significant fluctuation in their inhibitory capacities with less clear concentration dependences (data not shown). An amino acid sequence analysis of the investigated variants showed a high sequence homology in the second variegated helix, including positions at which several variants had identical amino acid occupancies, suggesting that this region is preferentially involved in the binding to hCD28 in these binders. However, the results from the inhibition experiments indicate

that the sequence of the first helix also influences the performance of the affibodies, either structurally or by direct involvement in the binding.

The observed inhibitory capacity and high selectivity displayed by the Z_{CD28:5} variant could indicate direct blocking of the CD80 binding site on hCD28. It has earlier been shown that an MYPPPY motif on hCD28 is essential for CD80/86 binding, a motif which is also present in hCTLA-4 (Peach *et al.*, 1994; Stamper *et al.*, 2001). However, none of the anti-CD28 affibodies investigated in this work showed any detectable binding to hCTLA-4, suggesting that the MYPPPY motif is not directly included in the binding site for the affibodies.

Although the affibodies described in this study show an inhibitory capacity in the assay format performed, it would be interesting to investigate if they could be used for the development of reagents for T-cell stimulation through genetic fusion to an appropriate anti-tumor antibody. Dense binding of such reagents on a targeted tumor cell could possibly lead to CD28 clustering on recruited T-cells, earlier described as one mechanism leading to stimulation (Greene *et al.*, 1996).

The Z_{CD28:5}-Fc fusion, in which the affibody is presented as a dimer, could be functionally expressed in *E. coli* and showed a higher binding avidity to immobilized hCD28 protein. This Fc fragment gene fusion format, which can result in improved pharmacokinetics (Chamow and Ashkenazi, 1996), has also been evaluated by several groups for CTLA-4 production,

resulting in CTLA-4-Ig immunoadhesins for evaluation as immunosuppressive agents (Najafian and Sayegh, 2000).

In addition to possible therapeutic use, other potential applications for the described affibodies include recovery and detection of CD28-expressing cells. For diagnostic purposes, immunoconjugates produced by direct genetic fusion between recognition proteins and reporter enzymes have been described, including fusions to scFv fragments (Ducancél *et al.*, 1993). Similar fusions between affibodies and reporter enzymes have also been successfully produced, including fusions to alkaline phosphatase, β -galactosidase and enhanced green fluorescence protein fusions (P.A.Nygren, M.Eklund and J.Ronnmark, unpublished work).

In summary, the CD28-specific ligands described in this work, based on a non-cysteine-dependent three-helix bundle scaffold of bacterial origin, offer interesting alternatives to both antibodies and natural CD28-binding ligands for immunotechnology applications.

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