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Publisher's version / Version de l'éditeur:
https://doi.org/10.1093/protein/gzw043
Protein Engineering, Design and Selection, pp. 1-5, 2016-09-08

PEDS Advance Access published September 8, 2016

Protein Engineering, Design & Selection, 2016, pp. 1–5
doi: 10.1093/protein/gzw043
Short Communication

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Isolation of TGF-β-neutralizing single-domain antibodies of predetermined epitope specificity using next-generation DNA sequencing

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Edited by Laurent Jespers
NRC-HHT publication number: 53319

Received 27 May 2016; Revised 19 July 2016; Accepted 20 July 2016

Abstract

The epitope specificity of therapeutic antibodies is often critical to their efficacy and mode of action. Here, we report the isolation of single-domain antibodies (sdAbs) against a pre-specified epitope of TGF-β3: namely, the site of interaction between the cytokine and its cell-surface type II receptor. By panning a phage-displayed immune llama VHH library against TGF-β3 using competitive elution with soluble dimeric type II receptor ectodomain in tandem with next-generation DNA sequencing, we identified several sdAbs that competed with the receptor for TGF-β3 binding and neutralized TGF-β3 in *in vitro* cellular assays. In contrast, all other sdAbs identified using conventional panning approaches (*i.e.*, without regard to epitope specificity) did not target the site of receptor:cytokine interaction. We expect this strategy to be generally applicable for identifying epitope-specific sdAbs when binding reagents directed against the epitope of interest are available. The sdAbs identified here are of potential interest as cancer immunotherapeutics.

Key words: antibody, single-domain antibody, V_HH, TGF-β, next-generation DNA sequencing, phage display

Introduction

Antibody (Ab) targeting of epitopes on folded proteins is a complex immunological process involving many factors (reviewed in El-Manzalawy and Honavar, 2010; Sela-Culang *et al.*, 2013). Thirty years of experience in the therapeutic Ab pipeline has shown that some epitopes are clearly of higher therapeutic value than others – for instance, those involved in receptor agonism, receptor down-regulation by internalization, or receptor interaction with a soluble ligand (reviewed in Scott *et al.*, 2012). Isolation of Abs against epitopes of therapeutic interest can be challenging if such Abs are rare and/or of low affinity, and may require extensive screening. Therefore, strategies for isolation of Abs against pre-specified epitopes are desirable.

In conjunction with Ab library technology, next-generation DNA sequencing (NGS) can be a powerful tool for 'function-first' discovery of Abs with complex phenotypes (Fischer, 2011; Henry *et al.*, 2015; Ravn *et al.*, 2013). Here, using a combination of phage-display selection and NGS, we present a strategy for isolating single-domain Abs (sdAbs) against pre-specified epitopes on folded proteins, using the cytokine transforming growth factor beta-3 (TGF-β3) as a model target antigen. Specifically, we constructed a phage-displayed sdAb library from the lymphocytes of a llama immunized with

TGF-β3 and panned the library in a single round against TGF-β3. We eluted sdAbs competitively with either soluble TGF-β receptor ectodomain (Zwaagstra *et al.*, 2012) or an irrelevant competitor, used NGS of panning outputs to identify sdAbs specifically displaced by the former biomolecule, and characterized the resulting sdAbs using binding and *in vitro* cytokine neutralization assays (Fig. 1). The TGF-β-neutralizing sdAbs identified here are promising immunotherapeutics in a variety of malignant and fibrotic conditions in which signaling by the pleiotrophic TGF-β cytokine family becomes dysregulated, and offer clear advantages in stability and manufacturability over receptor ectodomain ligand traps.

Materials and Methods

Llama immunization, library construction and panning

A male llama (*Lama glama*) was immunized five times with 180 μg TGF-β3 (a gift from Andrew Hinck, University of Texas Health Science Center, San Antonio, TX) as previously described (Henry *et al.*, 2015), except that the antigen was formulated in 100 mM glycine buffer, pH 2.5. Acidic buffered solution was used due to TGF-β3's hydrophobicity and tendency to aggregate at physiological pH (Pellaud *et al.*, 1999). After the third and fourth immunizations, serum and peripheral blood mononuclear cells (PBMCs) were obtained from the animal. Serum ELISAs were conducted as described in Supplementary Material.

A phage-displayed sdAb library was constructed from the heavy-chain-only IgG repertoire of the TGF-β3-immunized llama as previously described (Arbabi-Ghahroudi *et al.*, 2009a; Baral *et al.*, 2013; Henry *et al.*, 2015; Hussack *et al.*, 2011). Briefly, total cellular RNA was extracted from approximately 8×10^7 PBMCs using a PureLink® RNA Mini Kit (Life Technologies, Carlsbad, CA), reverse transcribed using qScript™ cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD) as per the manufacturer's instructions and then pooled. Rearranged and expressed V_HH genes were amplified using semi-nested PCR and cloned into the pMED1 phagemid vector, then phage were rescued from library-bearing *Escherichia coli* TG1 cells by superinfection with M13KO7 helper phage (Life Technologies) and purified by polyethylene glycol precipitation.

The phage-displayed sdAb library was panned in a single round against immobilized TGF-β3 as previously described (Arbabi-Ghahroudi *et al.*, 2009a; Baral *et al.*, 2013; Henry *et al.*, 2015; Hussack *et al.*, 2011) with some modifications. Briefly, wells of NUNC MaxiSorp™ microtiter plates (Thermo-Fisher, Ottawa, ON, Canada) were coated overnight at 4°C with 10 μg of TGF-β3 in 35 μL of PBS. The next day, wells were blocked for 1 h at 37°C with 200 μL of PBS containing 5% (w/v) skim milk, then ~10^{12} phage particles (diluted in 100 μL PBS containing 1% (w/v) bovine serum albumin (Sigma-Aldrich, St Louis, MO) and 0.1% (v/v) Tween-20) were applied to each well and incubated at room temperature for 2 h. The wells were washed five times with PBS containing 0.05% Tween-20 (PBS-T), five times with PBS, then bound phage were eluted for 1 h with 50 μL PBS containing either 100 μg/mL soluble dimeric type II TGF-β receptor ectodomain ($(T\beta RII)_2$; Zwaagstra *et al.*, 2012) or 100 μg/mL anti-RSV glycoprotein F Ab (Synagis®; Creative Biolabs, Shirley, NY).

Next-generation DNA sequencing and analysis

Approximately 10^6 library phage particles or phage particles eluted from the single round of panning were used directly as templates for NGS as previously described (Henry *et al.*, 2015). Indexed amplicon libraries were produced in two rounds of PCR, pooled, purified and then sequenced on a MiSeq Sequencing System (Illumina, San Diego, CA) using a 500-cycle MiSeq Reagent Kit V2 and a 5% PhiX genomic DNA spike. From each sample, 0.3–1.6 million reads were generated, of which 0.1–0.6 million were used for analysis after assembly using FLASH (default parameters; Magoc and Salzberg, 2011) and quality filtering using the FAST-X toolkit with a stringency of Q30 over ≥ 95% of each read (Schmieder and Edwards, 2011; Supplementary Table SI). The DNA sequence of each sdAb was then translated *in silico*, and the CDR3 sequence (IMGT positions 105–117) parsed using conserved N-terminal amino acid consensus sequences (YYC).

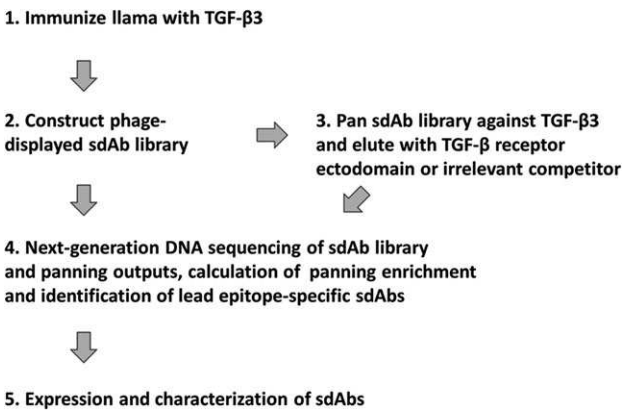

Fig. 1 Strategy and workflow for identification of epitope-specific TGF-β3-neutralizing sdAbs using panning with competitive elution and NGS.

Expression and characterization of sdAbs

The DNA sequences of seven sdAbs were synthesized commercially in the pSJF2 expression vector (Arbabi-Ghahroudi *et al.*, 2009b; GenScript, Piscataway, NJ) and each construct was expressed in *E. coli* as previously described (Arbabi-Ghahroudi *et al.*, 2009a; Baral *et al.*, 2013; Henry *et al.*, 2015; Hussack *et al.*, 2011). Briefly, 10 mL 2 × YT starter cultures containing 100 μg/mL ampicillin were inoculated with single plasmid-bearing *E. coli* TG1 colonies and grown overnight at 37°C with 220 rpm shaking. The next day, 250 mL 2 × YT cultures containing 100 μg/mL ampicillin, 0.1% (w/v) glucose and 0.5 mM IPTG were inoculated with 0.2 mL of starter culture and grown overnight at 37°C with 220 rpm shaking. The next morning, periplasmic proteins were extracted by osmotic shock, dialyzed and purified on a HiTrap IMAC HP column (GE Healthcare, Piscataway, NJ) using an ÄKTA FPLC protein purification system (GE Healthcare) with yields ranging from 1.2 to 14 mg/250 mL.

Prior to surface plasmon resonance (SPR), the sdAbs were purified by size exclusion chromatography as previously described (Arbabi-Ghahroudi *et al.*, 2009a; Baral *et al.*, 2013) using a Superdex™ 75 10/300 GL column (GE Healthcare) on an ÄKTA FPLC protein purification system (GE Healthcare). To determine the affinities of the sdAbs to TGF-β isoforms (all gifts from A. Hinck), a total of 369–530 resonance units (RUs) of each TGF-β protein were immobilized in 10 mM acetate buffer, pH 4.5, on CM5 sensor chips (GE Healthcare) using an amine coupling kit (GE Healthcare). Steady-state analyses were carried out on a Biacore T200 instrument (GE Healthcare) at 25°C by injecting sdAbs at concentrations ranging from 500 to 4 nM, in HBS-EP + buffer (10 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.05% (v/v)

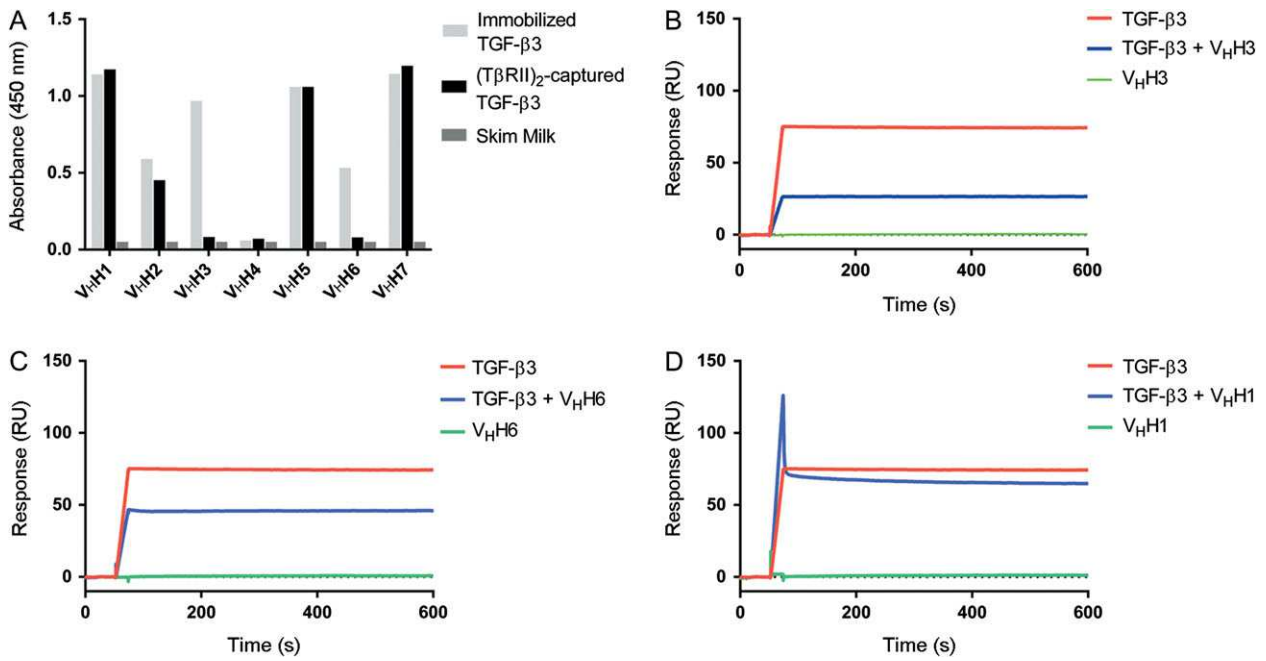

Fig. 2 (**A**) Binding of crude sdAb-containing periplasmic extract to immobilized or $(T\beta RII)_2$ trap-captured TGF-β3 in ELISA. Wells of NUNC MaxiSorp™ microtiter plates were coated overnight at 4°C with either 1 μg TGF-β3 or 1 μg $(T\beta RII)_2$ trap in 50 μL PBS. The next day, wells were blocked with 200 μL of PBS containing 5% skim milk for 1 h at 37°C and washed 3× with PBS; 1 μg TGF-β3 in 50 μL PBS was then added to wells containing $(T\beta RII)_2$ trap for 1 h at room temperature. Twenty-five μL of periplasmic extract were mixed with an equal volume of PBS containing 1% BSA and 0.1% Tween-20 (PBS-BT), added to wells and incubated for 2 h at room temperature. Wells were washed 5× with PBS containing 0.1% Tween-20 (PBS-T), incubated with 50 μL mouse anti-c-Myc Ab (Santa Cruz Biotechnology, Dallas, TX) diluted 1:1000 in PBS-BT, washed again 5× with PBS-T, and then incubated with 50 μL horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:1500 in PBS-BT. After a final wash (5× with PBS-T), wells were developed with 50 μL of tetramethylbenzidine (TMB) substrate (Mandel Scientific, Guelph, ON, Canada) for 5 min, stopped with 50 μL of 1 M H_2SO_4 and absorbance at 450 nm was measured using a Multiskan™ FC photometer (Thermo-Fisher). (**B**) Binding of 10 nM TGF-β3 to immobilized $(T\beta RII)_2$ trap in the presence or absence of 300 nM V_HH3 sdAb in SPR. (**C**) Binding of 10 nM TGF-β3 to immobilized $(T\beta RII)_2$ trap in the presence or absence of 1.4 μM V_HH6 sdAb in SPR. (**D**) Binding of 10 nM TGF-β3 to immobilized $(T\beta RII)_2$ trap in the presence or absence of 7.1 μM V_HH1 sdAb in SPR.

surfactant P20) and at a flow rate of 50 μL/min. Data were analyzed using Biacore™ T200 Software v3.0 (GE Healthcare) and fitted to a 1:1 binding model.

For competition SPR experiments, we immobilized 276 RUs of $(T\beta RII)_2$ trap in 10 mM acetate buffer, pH 4.5, on a CM5 sensor chip using an amine coupling kit. Next, either (i) 10 nM of TGF-β3 alone, (ii) 10 nM of TGF-β3 preincubated for 30 min at room temperature with sdAbs (V_HH1, V_HH3 or V_HH6) at concentrations equivalent to 25-fold their K_D, or (iii) sdAbs alone at concentrations equivalent to 25-fold their K_D, were injected over immobilized $(T\beta RII)_2$ trap on a Biacore T200 instrument at 10°C in HBS-EP+ buffer using a flow rate of 50 μL/min. Injections lasted for 20 s, followed by 10 min dissociation.

Results

ELISAs using polyclonal serum IgGs collected from the immunized llama on day 42 showed that: (i) immunization with TGF-β3 elicited serum Abs that bound all three isoforms of TGF-β (Supplementary Fig. S1) and (ii) a subset of the Abs elicited in response to immunization competed with soluble dimeric type II TGF-β receptor ectodomain [$(T\beta RII)_2$; Zwaagstra *et al.*, 2012] for TGF-β3 binding, since binding of $(T\beta RII)_2$ to TGF-β3 was diminished in the presence of serum from the TGF-β3-immunized llama (Supplementary Fig. S2). These data provided preliminary evidence that Abs (including heavy-chain-only Abs) directed against the site of $(T\beta RII)_2$:TGF-β interaction comprised some proportion of polyclonal serum IgG.

For both pannings (using competitive elution with either $(T\beta RII)_2$ or an irrelevant competitor), the set of CDR3 sequences derived from the output phage was compared to the set from the unpanned sdAb library; for each shared CDR3 sequence, an enrichment score was calculated as the frequency in the output phage divided by the frequency in the library. Using these enrichment scores, we identified seven putative epitope-specific sdAbs using two sets of criteria: (i) ≥ 50-fold enrichment of the sdAb in $(T\beta RII)_2$-eluted output phage along with ≤10-fold enrichment in anti-RSV Ab-eluted output phage (V_HH1–V_HH3), or (ii) largest absolute difference in enrichment scores between $(T\beta RII)_2$ and anti-RSV Ab elutions (V_HH4–V_HH7; Table I). The rationale for using these two criteria to identify epitope-specific sdAbs was as follows: (i) if epitope-specific sdAbs are infrequent in the library or have slow off-rates, then they should be enriched by panning using competitive elution and not enriched by panning using an irrelevant competitor; (ii) if epitope-specific sdAbs are frequent in the library or have fast off-rates, then they may be appreciably enriched even using an irrelevant competitor, and thus the expected signal of competitive replacement would be the magnitude of differences in enrichment scores, comparing elution with epitope-specific competitor to elution with irrelevant competitor. None of the sdAbs were clonally related, as their CDR3 sequences differed in length and shared no sequence homology (Supplementary Table SII).

We studied the binding of the expressed sdAbs to TGF-β3 by ELISA and SPR. Prior to purification, ELISA was conducted using crude sdAb-containing periplasmic extracts to assess binding to

Table I. Enrichment of sdAbs in single-round panning against TGF-β3 using competitive elution with $(T\beta RII)_2$ trap or irrelevant competitor (α-RSV Ab), as determined by NGS

sdAb	Fold-enrichment		Δ Fold-enrichment[a]	Frequency (%)[b]
	$(T\beta RII)_2$	anti-RSV Ab		
V_HH1	101	6	95	1.4
V_HH2	105	5	100	0.6
V_HH3	60	7	53	0.3
V_HH4	158	20	138	1.0
V_HH5	217	37	180	3.4
V_HH6	224	24	200	1.8
V_HH7	207	60	147	0.1

[a]Calculated as fold-enrichment ($(T\beta RII)_2$ elution) – fold-enrichment (anti-RSV Ab elution).

[b]Absolute frequency in $(T\beta RII)_2$-eluted output phage.

either directly immobilized or $(T\beta RII)_2$ trap-captured TGF-β3 (Fig. 2A). Six of seven sdAbs bound specifically to immobilized TGF-β3 but two sdAbs (V_HH3 and V_HH6) did not bind $(T\beta RII)_2$ trap-captured TGF-β3, indicating that they may recognize epitopes identical to or overlapping that of the $(T\beta RII)_2$ trap; in purified form, these sdAbs also competed with $(T\beta RII)_2$ trap for TGF-β3 binding in ELISA (Supplementary Fig. S3). One sdAb, V_HH4, did not bind either immobilized or $(T\beta RII)_2$ trap-captured TGF-β3. As shown in Table II, the six sdAbs bound TGF-β3 with affinities ranging from 12 to 284 nM; three of the sdAbs (V_HH2, V_HH3 and V_HH6) bound TGF-β2 with slightly weaker affinity but none cross-reacted significantly with TGF-β1.

To confirm the epitope specificity of the V_HH3 and V_HH6 sdAbs, we performed competitive SPR experiments in which mass transport-limited binding of soluble TGF-β3 to immobilized $(T\beta RII)_2$ trap was assessed in the presence or absence of excess soluble sdAb. As shown in Fig. 2B–D, V_HH3 and V_HH6, but not V_HH1, clearly inhibited binding of TGF-β3 to the $(T\beta RII)_2$ trap. In all cases, sdAbs alone did not bind to immobilized $(T\beta RII)_2$ trap (green line) and injecting 10 nM of TGF-β3 produced a response of ~75 RUs (red line) with little detectable dissociation from the bivalent $(T\beta RII)_2$ trap. Preincubation of V_HH3 and V_HH6, but not V_HH1, with TGF-β3 reduced the amount of TGF-β3 available to bind immobilized $(T\beta RII)_2$ trap (blue line), indicating that both of these sdAbs are capable of blocking the TGF-β3:$(T\beta RII)_2$ interaction. V_HH1 bound to immobilized $(T\beta RII)_2$ trap as a sdAb-TGF-β3 complex with the sdAb quite rapidly dissociating from the trimolecular complex after the injection. None of > 20 other sdAbs selected for binding to TGF-β3 without regard to epitope specificity competed with $(T\beta RII)_2$ trap for TGF-β3 binding (Supplementary Fig. S4).

Finally, two sdAbs directed against the site of the TGF-β3:$(T\beta RII)_2$ interaction (V_HH3, V_HH6) and two sdAbs directed against other sites (V_HH2, V_HH5) were expressed by transient transfection of HEK293-6E cells as fusions to human IgG1 F_c and hinge region as previously described (Zhang *et al.*, 2009). The sdAb-F_cs were assessed for their ability to neutralize TGF-β1 and TGF-β3 *in vitro* using IL-11 secretion by TGF-β-sensitive A549 cells as a readout of TGF-β signaling (Rapoza *et al.*, 2006). V_HH3-F_c neutralized TGF-β3 most potently in a dose-dependent manner, although all of the V_HH-F_cs neutralized TGF-β3 at high concentrations (Fig. 3). Neutralization of TGF- β3 appeared to be primarily affinity-dependent (Table II, Fig. 3). Only V_HH3-F_c neutralized TGF-β1.

Table II. Steady-state affinities of sdAbs to TGF-β1, TGF-β2 and TGF-β3 (pH 7.4, 25°C)

sdAb	K_D (nM)		
	TGF-β1	TGF-β2	TGF-β3
V_HH1	n.b.	n.b.	284
V_HH2	n.b.	49	25
V_HH3	520	28	12
V_HH4	n.d.	n.d.	n.d.
V_HH5	n.b.	n.b.	251
V_HH6	n.b.	44[a]	55[a]
V_HH7	n.b.	670	17

n.b., no binding observed.

n.d., not determined.

[a]Calculated from a single concentration injection.

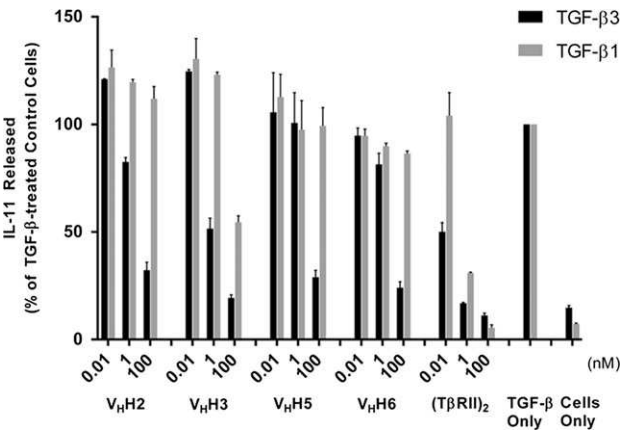

Fig. 3 Neutralization of TGF-β1 and TGF-β3 by sdAb-F_cs. A549 lung cancer cells were seeded in 96-well plates (5×10^6 cells/well). The following day, 10 pM TGF-β1 or TGF-β3 was preincubated in complete media with the indicated concentrations of sdAb-F_cs or $(T\beta RII)_2$ trap for 30 min at room temperature, then the mixture was added to cells for 18 h at 37°C. Aliquots of conditioned media were added to MSD® Streptavidin Gold plates (Meso Scale Diagnostics, Gaithersburg, MD) coated with 2 μg/mL biotinylated mouse anti-human IL-11 antibody (MAB618, R&D Systems, Minneapolis, MN) for 18 h at room temperature, washed with PBS containing 0.05% Tween-20, treated with 2 μg/mL SULFO-tagged goat anti-human IL-11 antibody (AF-218-NA, R&D Systems) for 1 h at RT. After a final wash, plates were read on a MESO QuickPlex SQ 120 imager (Meso Scale Diagnostics). The results were normalized to cell numbers/well (CyQUANT®, Thermo Fisher) and expressed as % IL-11 release compared to control cells treated with TGF-β1 or TGF-β3 alone.

Discussion

High-affinity immune Abs directed against specific epitopes of folded proteins can be difficult to obtain in cases where epitopes are not inherently immunogenic. While alternative strategies for selection of epitope-specific Abs have been described (*e.g.*, *de novo* protein design (Sormanni *et al.*, 2015); screening of naïve or synthetic sources of Ab diversity (Sidhu and Fellouse, 2006); recombinant protein scaffolds bearing 'grafted' exogenous epitopes as immunization or screening reagents (Burton, 2010)), these can be laborious and often result in Abs that are deficient in affinity or other properties. For therapeutic Abs against well-characterized targets, epitopes of functional importance are sometimes known and thus the ability to select Abs for desired epitope specificity during early stages of development is a major advantage.

Using a single round of panning and NGS analysis, we were able to isolate two sdAbs that competed with soluble dimeric type II TGF-β receptor ectodomain for TGF-β3 binding. Neither panning of the same phage-displayed sdAb library against TGF-β3 with non-specific elution of sdAbs using triethylamine (Supplementary Fig. S4), nor direct screening of Ab-secreting cells for binding to any TGF-β3 epitope (data not shown), yielded sdAbs against this site. We speculate that since the expression yields and binding affinities of the two sdAbs against this site were not unusual, failure to recover them through conventional panning approaches may instead relate to their frequencies in the library (in comparison to non-site-specific sdAbs), as well as to peculiarities of their epitope (*e.g.*, conformational lability; potential steric hindrance of sdAb-phage binding to this site).

The principle underlying this strategy (competitive replacement of sdAb-bearing phage by a protein interacting with the same site) has several implications for the types of Abs we expect to recover and the circumstances under which it will be successful. First, competitive replacement can be a very weak force of selection, depending on the binding affinities and kinetics as well as the relative concentrations of the competitor and phage-displayed sdAb; here, we used high concentrations of bivalent $(T\beta RII)_2$ trap (subnanomolar apparent affinity for TGF-β3) to maximize the signal of competitive replacement. However, epitope-specific sdAbs did not rise to high frequency even after multiple panning rounds with the high-affinity competitor used here (data not shown), necessitating comparative approaches using NGS. Second, the selection is primarily off-rate dependent, and epitope-specific sdAbs with faster off-rates will be easier to detect using this method. Since sdAb-bearing phage, regardless of epitope specificity, will dissociate from immobilized antigen regardless of the presence or absence of a competitor, comparison of the relative amounts of sdAb-phage eluted using site-specific and irrelevant competitors is critical. Third, since competition approaches are inextricably linked to the sizes of the competing molecules, competitive binding of sdAb-phage may not accurately predict the behavior of soluble sdAbs or sdAb-F_cs. Finally, binding of the competitor may under some circumstances induce conformational changes at a distance from the site of interaction, displacing sdAb-phage directed elsewhere on the antigen. Thus, some degree of type I error may be unavoidable, but in our hands this strategy has reliably yielded epitope-specific sdAbs against several targets if these are present in the library (data not shown).

There are several limitations and caveats to the work presented here. First, we present only one case study of a single antigen and epitope of interest, and the results may vary substantially depending on properties of the antigen, the Abs present in the library and the competitor used. Second, this strategy is valid only for epitopes against which Abs or other affinity reagents such as receptor ectodomains are available. Third, in many cases this strategy may be unnecessary, as traditional panning of phage-displayed libraries with non-specific elution of all binding Abs may yield Abs directed against the desired epitope without any special effort. Finally, the necessity of targeting specific epitopes depends largely on the application, as shown here: the epitope specificity of sdAb monomers turned out to be largely irrelevant to their neutralization potency as sdAb-F_c fusions, presumably due to the larger size of the latter molecules.

In summary, we report a strategy for rapid identification of sdAbs to pre-specified epitopes on folded proteins, using TGF-β3 as a model antigen. Using a single round of panning in the presence or absence of an epitope-specific competitor followed by NGS, we identified two TGF-β3-specific sdAbs capable of blocking the interaction between TGF-β3 and its cell-surface receptor and neutralizing TGF-β3 in cell-based assays. The sdAbs isolated here are being developed as immunotherapeutics for TGF-β-driven diseases including cancer and fibrosis (Hawinkels and Ten Dijke, 2011). More generally, panning of phage-displayed Ab libraries with competitive elution followed by NGS can be a useful tool for discovery of biobetter Abs.

Supplementary data

Supplementary data are available at *PEDS* online.

Acknowledgments

We thank Yonghong Guan (NRC) for performing protein expression and size exclusion and Shalini Raphael (NRC) for performing SPR experiments, as well as Maureen O'Connor-McCourt for instigating TGF-β sdAb discovery efforts.

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