Supplementary Material Swers et al.

SUPPLEMENTARY MATERIALS AND METHODS

Tn3 Library Generation and TRAILR2 Selections

A synthetic gene encoding a fragment of the 3rd fibronectin type III domain from human tenascin C was cloned into an in-house M13 phage display vector. Coding modifications were made to the fibronectin type III module to introduce a pair of cysteine substitutions that form a stabilizing disulfide bond, and add an N-terminal alanine to the processed protein. The amino acid sequence of the displayed scaffold protein, termed Tn3, was as follows:

^{1}AIEVKDVTDTTALITWFKPLAEIDGCELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDT EYEVSLICRRGDMSSNPAKETFTT^{84}

A phage displayed Tn3 library was then constructed to randomize codons in the BC (residues 17–25), DE (residues 45–50) and FG (residues 69–78) loop coding regions. In addition to sequence diversity, length diversity was also introduced, such that BC loops of 9, 11 and 12 amino acids in length were created, and FG loops of 9, 10 and 11 amino acids. All selections of phage display libraries against TRAILR2-Fc were performed with biotinylated human TRAILR2 ECD/Fc fusion protein (R&D Systems, Minneapolis, MN) immobilized onto magnetic streptavidin beads (Dynal; Invitrogen, Carlsbad, CA), after first depleting the library for clones that bound human IgG1 Fc or the streptavidin-coated magnetic beads.

For affinity maturation, phage display libraries were generated by saturation mutagenesis of regions within the BC, DE and FG loops of TRAILR2-specific Tn3s, using degenerate oligonucleotides and either Kunkel mutagenesis (1), or overlap extension PCR. Affinity maturation of 1C12 was carried out in three consecutive steps, beginning with optimization of the C-terminal portion of the BC loop. The DE and N-terminal regions of BC and FG loops of clone G3 were then optimized by panning individual loop libraries for two rounds against TRAILR2-Fc, then recombining the output diversity by PCR into a single BC/DE/FG library for additional TRAILR2-Fc selections. A final library was generated to optimize clone 1E11, by randomizing the C-terminal region of the FG loop. This led to clones C4 and G6, which are described in Table 1.

Screening of TRAILR2-Binding Tn3 Proteins

Identification of TRAILR2-binding clones from the naiive Tn3 library was performed by phage ELISA (2). Screening of affinity maturation outputs was performed with soluble, unpurified Tn3 proteins in a competitive ELISA assay. Here, serial dilutions of culture supernatants containing secreted Tn3 proteins were used to displace the interaction between biotinylated TRAILR2-Fc and immobilized 1C12-Ig fusion protein. The media titer required to inhibit 50% of TRAILR2-Fc/1C12-Ig binding was used as a relative measure of TRAILR2 affinity, and for ranking of selection outputs. As 1C12 and its affinity matured variants expressed to similar levels in *E. coli*, the relationship between media titer and affinity was assumed to be free of expression level bias.

Preparation of Tandem Tn3 Multivalent Constructs

A type IIS restriction enzyme (3) cloning strategy was used to assemble gene fragments encoding tandem repeats of Tn3 modules flanked by Gly4Ser-encoding linkers. Briefly, gene sequences encoding a single Tn3 module were PCR amplified and digested with either BpmI or AcuI (New England Biolabs, Ipswich, MA). Digested products were ligated, gel purified, and cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) to generate a tandem Tn3-Tn3 gene cassette. Cassettes encoding 4 tandem Tn3 modules were generated by amplifying the tandem Tn3-Tn3 repeat, digesting with either BpmI or AcuI, ligating, then cloning into pCR 2.1-TOPO. For preparation of tandem sequences containing more than 4 Tn3 modules, an adapter cassette was first cloned downstream of the tandem 4 repeats. Unique BamHI and KpnI sites within the adaptor allowed for insertion of additional downstream tandem Tn3 gene repeats generated as described above.

Expression, Purification and Characterization of TRAILR2-Binding Tn3 Proteins

Bacterial secretion of C-terminal polyhistidine-tagged proteins was used to produce monomeric and tandem Tn3 proteins. Overexpression of tagged Tn3 proteins in *E. coli* results in leakage of periplasmic contents into the culture media, from which the His-tagged proteins were purified by metal chelating chromatography using Ni-NTA Superflow resin (Qiagen, Valencia, CA). Expression of Tn3-Ig and Tn3-Fc fusion proteins was by transient transfection of HEK293F cells. Transfection, cell culture, and protein A purification was as described elsewhere (4).

All purified proteins were analyzed by SDS-PAGE on NuPage Novex 4-12% Bis-Tris gels in MES buffer without reducing agent (Invitrogen, Carlsbad, CA). Size exclusion chromatography was also used to analyze purified proteins, and where necessary, aggregated material was removed on either a Superdex 75 10/300GL or Superdex 200 10/300GL column (GE Healthcare, Piscataway, NJ) in PBS buffer, to a final level below 4% of total protein. An Acrodisc unit with a Mustang E membrane (Pall Corporation, Port Washington, NY) was used as indicated by the manufacturer to remove endotoxin from bacterially expressed protein preparations. Binding affinity measurements were performed on a ProteOn XPR36 protein interaction array system (Bio-Rad, Hercules, CA). Affinity determinations were performed at 25ºC where TRAILR2-Fc was covalently immobilized on a GLC sensor chip via amine coupling. Samples of TRAILR2-binding Tn3's were prepared in running buffer (PBS, pH 7.4/0.005% Tween 20/0.5 mg/mL bovine serum albumin) and injected over the TRAILR2-Fc surface for 5 minutes at 30 μL/min. K_D values were determined using ProteOn Manager software, by fitting data to a simple 1:1 steady-state Langmuir binding model.

G6T8 Binding ELISA

A direct-binding ELISA was used to evaluate the specificity of G6T8 binding to a panel of TRAIL receptors. G6T8 was coated overnight onto a 96-well ELISA plate (Nunc, Neptune, NJ, USA). After the plate was blocked with PBS containing 4% w/v skim milk powder, serial dilutions of different TRAIL receptor fusion proteins were added

(human TRAILR1-Fc, TRAILR2-Fc, TRAILR3-Fc, TRAILR4-Fc, OPG-Fc and murine TRAILR2-Fc; all sourced from R&D Systems, Minneapolis, MN). Bound receptor-Fc fusion protein was detected using a horseradish peroxidase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA). The plates were developed using tetramethyl benzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), and the reaction was stopped using 1M H_3PO_4. The plates were read at 450 nm with a 620 nm reference.

SUPPLEMENTARY REFERENCES

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