

Electronic Supporting Information

**Receptor mimicking TGF- $\beta$ 1 binding peptide for targeting TGF- $\beta$ 1 signaling**

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## Materials and Methods

### *Synthesis of T $\beta$ R-derived peptides*

Peptides were synthesized by Fmoc solid phase peptide synthesis (SPPS) using an automated microwave peptide synthesizer (Liberty1; CEM) equipped with automated liquid handling (Discover; CEM). All amino acids and Rink Amide MBHA resin were purchased from EMD Novabiochem. Resin was initially swollen in N,N-dimethylformamide (DMF; Fisher) for 10 minutes and subsequently deprotected in 0.1 M 1-hydroxybenzotriazole (HOBt; Advanced Chem Tech) in 20% piperidine (Sigma) in DMF and for 5 minutes under microwave at 70 °C. Resin was washed twice in DMF, and coupling of each amino acid was performed by dissolving amino acids at 0.2 M in DMF followed by automated metering of four molar equivalents of amino acid, four equivalents of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; Advanced Chem Tech), and eight equivalents of diisopropylethylamine (DIPEA; Fisher) in 35% in N-methyl-2-pyrrolidone (NMP; Fisher) to the reaction vessel. Coupling was performed at 70 °C (except for cysteine residues, which were coupled at 50 °C) for 5 minutes under microwave. After final deprotection, resin was washed in DMF and acetone and cleaved as previously described.<sup>42</sup> Peptide purity was determined to be >80% using a C18 column (Shimadzu) with reverse phase high performance liquid chromatography (HPLC; Shimadzu). Peptide identity was verified with matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectroscopy (Bruker), and peptide content was determined by Ellman's free thiol assay (Thermo Scientific). MALDI and HPLC spectra of the synthesized peptides are shown in Figs. S7 -S9.

### *In silico docking analysis of TGF- $\beta$ 1 receptor-derived peptides with TGF- $\beta$ 1*

*In silico* docking simulations were used to predict the relative binding affinity and the docking positions of engineered TGF- $\beta$ 1 binding peptides. The sequences of RIP (and variants), RIIP-1, and RIIP-2 were input into a coarse-grained *de novo* peptide structure prediction algorithm (PEP-FOLD).<sup>36</sup> The best fit models of each peptide were then input into AutoDock Vina. Polar hydrogen atoms were added to the structure, and the side chains and backbone were established as flexible. Finally, the crystal structure of TGF- $\beta$ 1 was simulated as a rigid macromolecular monomer (PDB ID 3KFD).<sup>33</sup> The grid box was set to encompass the full length growth factor *via* fully blind docking on the Opal 2 server. For analysis of RIIP-1 point substitutions, a partial blind docking simulation was utilized between RIIP-1 and the receptor-binding domain of TGF- $\beta$ 1. Docking simulations were performed on the folded structure of each peptide and the crystal structure of TGF- $\beta$ 1 with the appropriate grid-box (for a fully blind or partially blinded study) using AutoDock Vina with an exhaustiveness of 6 *via* the Opal 2 server. Predicted binding affinity results were evaluated by calculating the mean and standard deviation of the energy of the 10 lowest-energy peptide-TGF- $\beta$ 1 structures.

#### *Surface plasmon resonance with immobilized TGF $\beta$ RI-derived peptides*

The affinity of RIP binding to TGF $\beta$ 1 was characterized using a Biorad ProteOn XPR36 system on a GLM sensor chip (Biorad) that was pre-conditioned and initialized according to manufacturer's protocol. RIP, RIP $\Delta$ , and scrambled RIP was dissolved at 5-20  $\mu$ M in a buffer containing 10 mM sodium acetate (Biorad) and 2.5 mM NaOH in DI water. Covalent modification of sensor chip with RIP, RIP $\Delta$ , or scrambled RIP was performed by washing the sensor chip twice with NaOH followed by washing twice with DI water, activating with Sulfo-NHS/EDC (Biorad), flowing RIP, RIP $\Delta$ , or scrambled RIP vertically over the chip, de-activating with ethanolamine-

HCl (Biorad), and regenerating with NaOH. Subsequently, recombinant human TGF- $\beta$ 1 (PeproTech) was reconstituted at 100  $\mu\text{g mL}^{-1}$  in running buffer containing 10 mM HEPES (Acros), 150 mM NaCl (Fisher), 0.005% Tween 20 (Fisher), and 0.01% BSA Fraction V (Fisher). TGF- $\beta$ 1 was diluted from stock into running buffer to a final concentration of 0 to 800 nM in running buffer and flowed horizontally over the sensor chip at 100  $\mu\text{L minute}^{-1}$  for 18 seconds followed by dissociation in running buffer for 600 seconds. After each interaction step with TGF- $\beta$ 1, the sensor chip was regenerated by flowing over with NaOH twice in between runs. Sensogram data were collected from two independent TGF- $\beta$ 1 interaction periods per experiment and double referenced to 0 nM TGF- $\beta$ 1 spots and the blocking control spots. The association and dissociation rate constants and equilibrium dissociation constants were modeled using Scrubber v2 (BioLogic Software).<sup>45</sup>

#### *Conjugation of peptides to bovine serum albumin*

Peptides were covalently conjugated to bovine serum albumin (BSA; Fisher scientific) *via* glutaraldehyde crosslinking as previously reported for protein conjugation to carrier proteins.<sup>46</sup> Briefly, peptides were dissolved at 1 mM in buffer containing 0.1 M sodium acetate (Sigma), 8 mM glutaraldehyde (MP Biomedicals), and DI water for two hours at room temperature. Reaction mixtures for RIP $\Delta$ , scrambled RIP, RIIP-2, and scrambled RIIP-2 were titrated to pH 9 to facilitate dissolution, while reaction mixtures for RIIP-1 and scrambled RIIP-1 were titrated to pH 7. After the coupling reaction, 15-20 mg glycine was added to each tube to quench the reaction, and solutions were diluted in PBS prior to dialysis. BSA-peptide conjugates were dialyzed against PBS in Slide-a-Lyzer dialysis cassettes (10,000 MWCO, 0.5 mL capacity; Thermo Scientific) overnight at 4 °C. BSA-peptide conjugates were further diluted 10-fold in PBS and re-concentrated by

centrifugation at 3000 ×g for 25 minutes using Amicon Ultra filtration units (10,000 MWCO, 15 mL capacity; Millipore). The purified BSA-peptide conjugates were reconstituted to ~0.75 mL (~1 mM peptide) for storage at -20 °C.

#### *Characterization of TGF-β1 binding to immobilized BSA-peptide conjugates*

BSA-peptide conjugates were used for quantification of TGF-β1 binding using a modified enzyme-linked immunosorbent assay (ELISA). BSA-peptide conjugates were adsorbed to a 96-well microplate (R&D Systems) overnight at room temperature. Subsequently, plates were washed with 0.05 % Tween 20 (Fisher) in PBS, blocked with 3 % BSA in PBS, washed, and incubated in recombinant human TGF-β1 (R&D Systems) at concentration range of 0 to 40 ng mL<sup>-1</sup> in a reagent diluent containing 1.4 % delipidized bovine serum (Biocell) and 0.05 % Tween 20 (Fisher) in PBS for 2 hours at room temperature. Subsequently, the amount of TGF-β1 bound to the BSA-peptide on the bottom of the wells was quantified using a standard TGF-β1 ELISA (R&D Systems) according to the manufacturer's instructions. The optimal concentration of immobilized BSA-peptide conjugate was determined as shown in Fig. S3. TGF-β1 binding isotherms were generated using a 1:125 dilution of BSA-peptide conjugate (~10 μM peptide) at a range of TGF-β1 concentrations. Experiments were performed with triplicate samples.

#### *Influence of TβR-derived peptides on TGF-β1 activity in culture*

Soluble peptides were assayed for their influence on TGF-β1 activity using an assay of endothelial cell proliferation. Briefly, human umbilical endothelial cells (HUVECs; Lonza) were thawed and expanded in HUVEC growth medium (HUVEC-GM) containing EGM-2 (Lonza) in M199 (CellGro) supplemented with sodium bicarbonate and 1% penicillin-streptomycin (P/S; Gibco).

Cells were plated at 2,000 cells well<sup>-1</sup> in a 96-well microplate overnight. The following day, HUVEC-GM was replaced with HUVEC assay medium (HUVEC-AM) containing M199, 1 % FBS, and TGF- $\beta$ 1 at the specified concentrations with or without peptides at a final concentration of 10  $\mu$ M in 0.1 % DMSO. After 72 hours, medium was replaced with 10 % buffered formalin for 30 min, and subsequently cells were washed with PBS, stained by a 1:1000 dilution of Hoescht in PBS, and imaged using an epifluorescence microscope (Nikon TiEclipse). The number of DAPI-stained cells in a 4 $\times$ 4 field of view was quantified using automated image processing in NIS Elements (Nikon). Data from two independent experiments were combined for twelve total replicates per condition.

Soluble peptides and peptide-containing microspheres were characterized for their influence on TGF- $\beta$ 1 activity using mouse HT-2 T-lymphocytes. The HT-2 cells proliferate in response to interleukin-2 (IL-2), and TGF- $\beta$ 1 inhibits IL-4-dependent growth.<sup>38</sup> HT-2 cells (ATCC) were thawed, expanded in HT-2 growth medium containing 10 % FBS, 50  $\mu$ M  $\beta$ -mercaptoethanol (Fisher), 10 ng mL<sup>-1</sup> IL-2 (R&D Systems), and 1 % P/S (Gibco) in RPMI 1640 (Lonza), and used between passages 4 to 9. On the day of experiments, HT-2 cells were centrifuged as above, washed in RPMI 1640, and resuspended in HT-2 assay medium, which had the same composition as the HT-2 growth medium except without IL-2. Cells were prepared in HT-2 assay medium at the appropriate density for plating, and immediately before plating, a final concentration of 10 ng mL<sup>-1</sup> of murine IL-4 (R&D Systems) was added to the cells. HT-2 cells were plated at 30,000 cells cm<sup>-2</sup> in a 96-well microplate containing the specified concentration of TGF- $\beta$ 1 with or without peptides at a final concentration of 10  $\mu$ M (unless otherwise specified) in 0.1 % DMSO or with or without peptide-containing microspheres (12.5% peptide content) at a final concentration of 1 mg mL<sup>-1</sup>. HT-2 cells were incubated for 42 hours, and subsequently

CellTiter Blue (Promega) was added and incubated for an additional 8 hours. After 50 total hours of incubation, culture media was transferred to a black microplate and analyzed on a fluorescent microplate reader following manufacturer's instructions. Conditions were assayed in replicates of six. Percent inhibition of TGF- $\beta$ 1 activity with soluble peptides or peptide-conjugated microspheres was calculated by the following formula:

$$\text{Inhibition of TGF-}\beta\text{1 activity (\%)} = \frac{I_{\text{peptide or microsphere with TGF-}\beta\text{1}} - I_{\text{TGF-}\beta\text{1}}}{I_{\text{no TGF-}\beta\text{1}} - I_{\text{TGF-}\beta\text{1}}} \times 100$$

where  $I_{\text{peptide or microsphere with TGF-}\beta\text{1}}$  denotes the fluorescence intensity from the cells cultured with soluble peptide or peptide-conjugated microspheres in presence of TGF- $\beta$ 1, and  $I_{\text{TGF-}\beta\text{1}}$  and  $I_{\text{no TGF-}\beta\text{1}}$  denotes the fluorescence intensity from the cells cultured without peptide or microsphere in the presence and absence of TGF- $\beta$ 1, respectively. The standard deviation was propagated using standard propagation of error techniques.

#### *Fabrication of microspheres conjugated with T $\beta$ RI-derived peptides*

Norbornene-terminated, four-arm poly(ethylene glycol) (PEG-NB) was synthesized as previously described.<sup>42</sup> Briefly, 4-arm poly(ethylene glycol) (PEG;  $M_n = 20,000 \text{ g mol}^{-1}$ ; Jenkem) terminated with hydroxyl groups on each arm was reacted under inert gas with ten molar equivalents (with respect to hydroxyl groups) of 5-norbornene-2-carboxylic acid (Sigma) in dichloromethane (Sigma), five equivalents N,N'-dicyclohexylcarbodiimide (Sigma), a half equivalent 4-diethylaminopyridine (Sigma), and five molar equivalents of pyridine (Sigma). PEG functionalization with norbornene was verified with  $^1\text{H}$  nuclear magnetic resonance (NMR) as described elsewhere (Fig. S10).<sup>47</sup> Peptide-conjugated microspheres were fabricated by thiol-ene reaction of PEG dithiol ( $M_n = 3,400 \text{ g mol}^{-1}$ ; Laysan Bio) or peptides with PEG-NB using an aqueous water-in-water emulsion, where a PEG-rich phase, containing PEG-NB, PEG dithiol and

peptides, was dispersed in a dextran-rich continuous phase, as previously described.<sup>42,48</sup> The PEG-rich phase contained four-arm PEG-NB, 0.5 molar equivalent of PEG dithiol relative to norbornene groups, 0.05 wt% photoinitiator (Irgacure 2959), and either 0.005, 0.014, 0.042, or 0.125 molar equivalents of peptide relative to norbornene groups. A blank microsphere condition was prepared by replacing peptide with DI water. All components of PEG-rich phase were combined and purged with inert gas prior to the addition of a 6-fold volume excess (relative to PEG phase) of a dextran-rich phase, consisting of 40 wt.% dextran ( $M_n = 40,000 \text{ g mol}^{-1}$ ; Alfa Aesar) in KCl buffer at pH 8. The PEG-rich phase was emulsified by vortexing for one minute in the dextran-rich phase, and the emulsion was allowed to settle for 20 minutes before being exposed to UV light ( $1.1 \text{ J cm}^{-2}$ ) to initiate the polymerization reaction. Impurities were removed with a 25-fold dilution in DI water followed by centrifugation at  $1600 \times g$  for 5 minutes and two subsequent washes in DI water. The final product was suspended in DI water, frozen in liquid nitrogen, and lyophilized. Peptide content was verified by dissolving microspheres in 1N NaOH (Fisher) and subsequently measuring the absorbance at 212 nm and 260 nm.

#### *Binding of TGF- $\beta$ 1 to peptide-conjugated PEG microspheres*

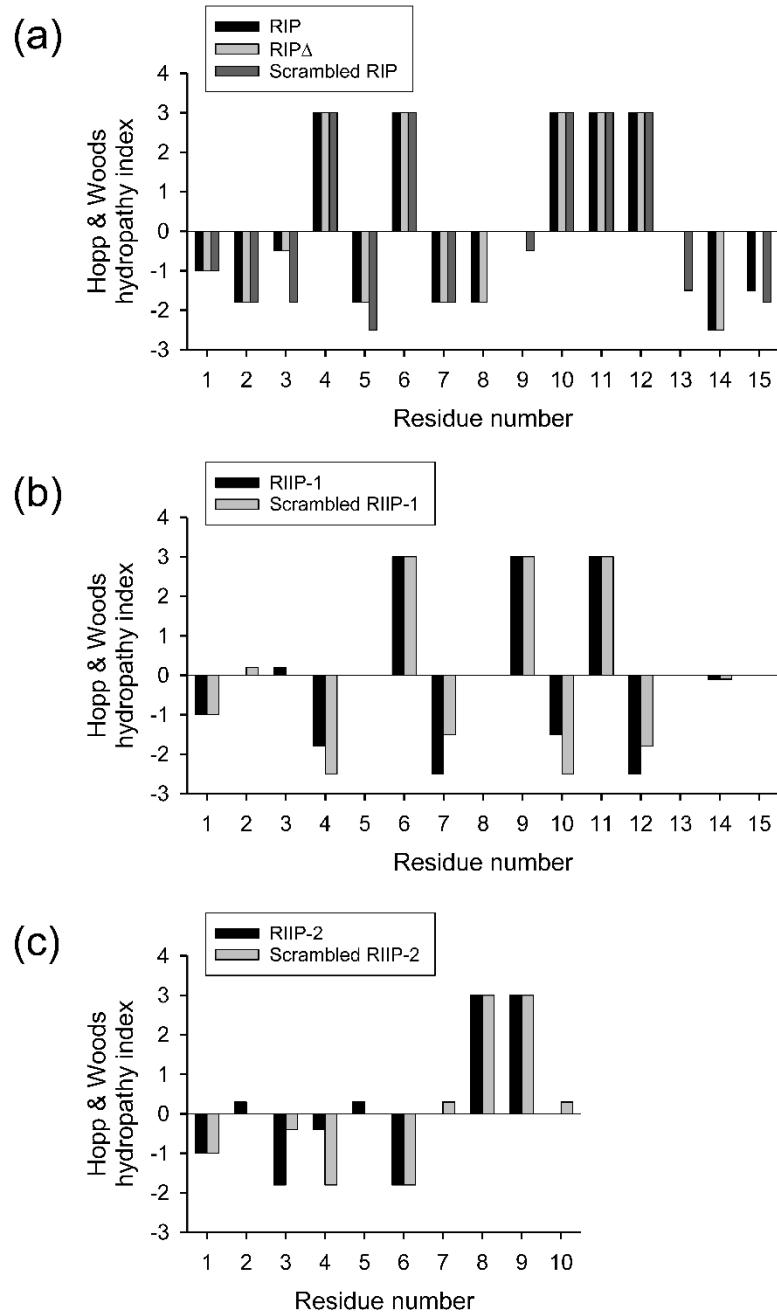
Microspheres conjugated with RIP $\Delta$ , scrambled RIP, or no peptide (blank) were assayed for TGF- $\beta$ 1 binding using  $^{125}\text{I}$ -radiolabeled TGF- $\beta$ 1. Briefly, microspheres were incubated in BSA-blocked tubes with  $0.9 \text{ ng mL}^{-1}$  TGF- $\beta$ 1 and  $0.1 \text{ ng mL}^{-1}$   $^{125}\text{I}$ -labeled TGF- $\beta$ 1 (Perkin-Elmer) in a solution containing 10 % FBS in RPMI 1640 for 1 hour at  $37 \text{ }^\circ\text{C}$ . Subsequently, microspheres were centrifuged at  $10,800 \times g$ , and radioactivity of the supernatant per minute were measured using a  $\gamma$ -counter (Perkin-Elmer, Cobra II Auto-Gamma). The amount of bound TGF- $\beta$ 1 was calculated by quantifying the amount of unbound TGF- $\beta$ 1 in the supernatant using a  $^{125}\text{I}$ -labeled TGF- $\beta$ 1



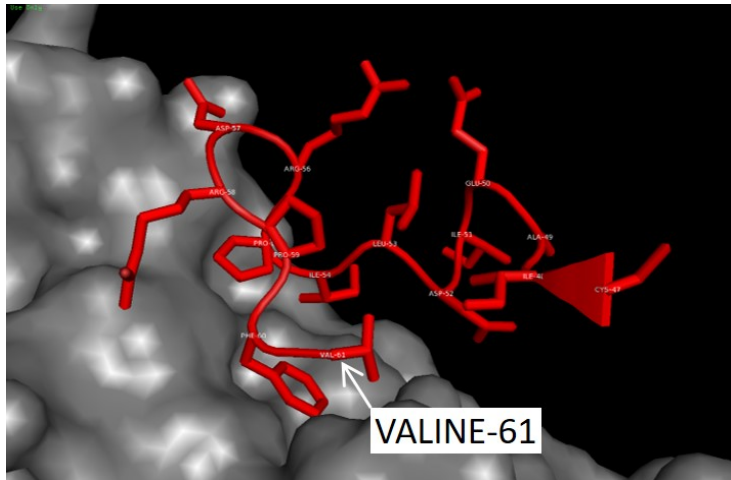
standard curve. Each condition was assayed in quadruplicate. In another set of experiments, RIPΔ-conjugated microspheres were tested for TGF-β1 binding in platelet rich plasma (PRP). PRP ( $1 \times 10^6$  platelets  $\mu\text{L}^{-1}$ ) was activated by three freeze-thaw cycles of 5 minutes in liquid nitrogen and 10 minutes in 37 °C water bath. RIPΔ-conjugated microspheres were added to activated PRP with varying concentrations between 2-32 mg  $\text{mL}^{-1}$ , and incubated at 37 °C for 2 hours under rotation. After centrifuging at 11,000  $\times g$  for 5 minutes, the amount of TGF-β1 in the supernatant was quantified using ELISA (R&D Systems). Each condition was assayed in quadruplicate.

### *Statistical analysis*

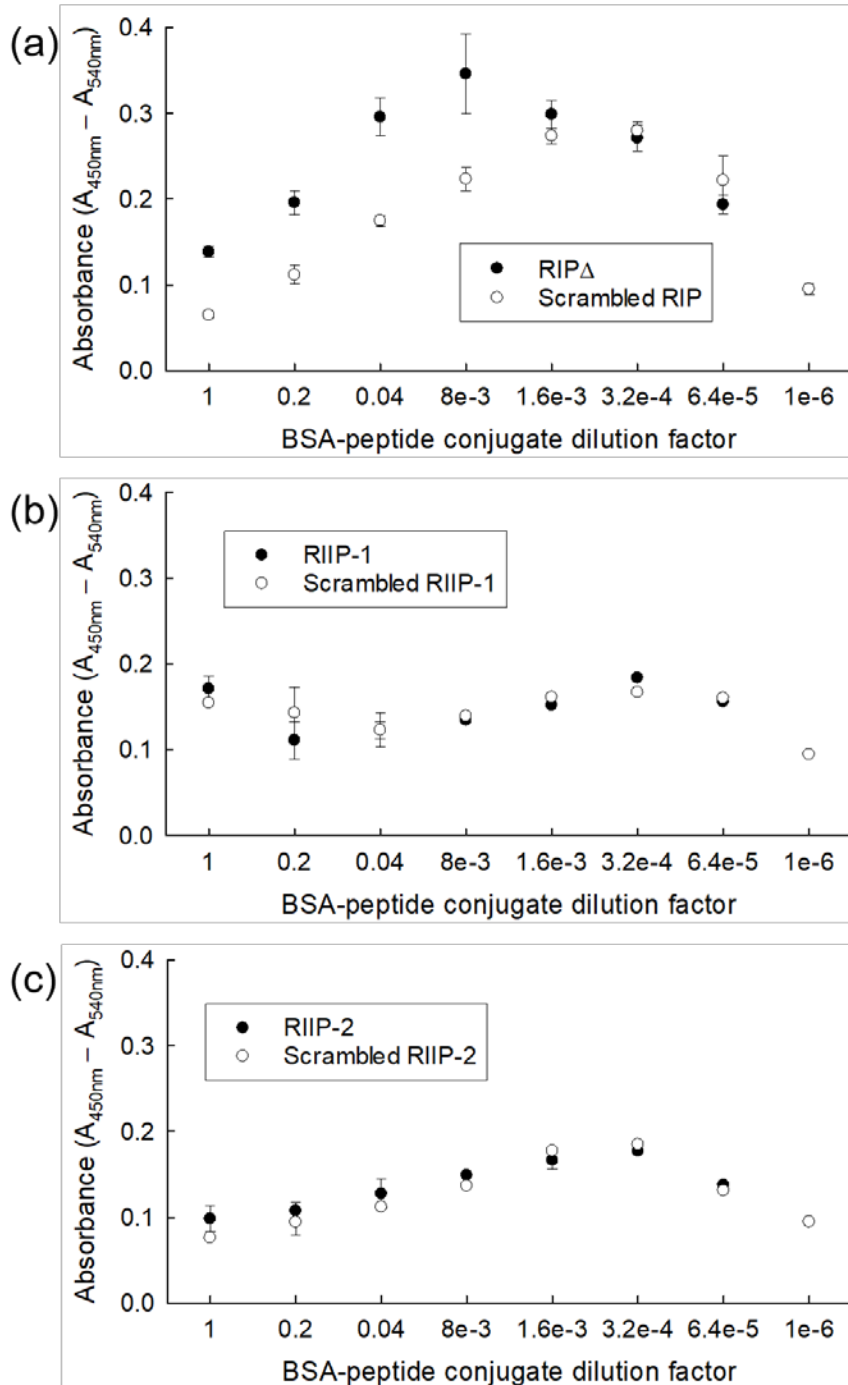
Data are presented as mean  $\pm$  standard deviation unless otherwise stated. Statistical analysis was performed using Student's t-test or two-way ANOVA with Bonferroni or Tukey's post-hoc test, and *p*-values less than 0.05 were considered statistically significant.



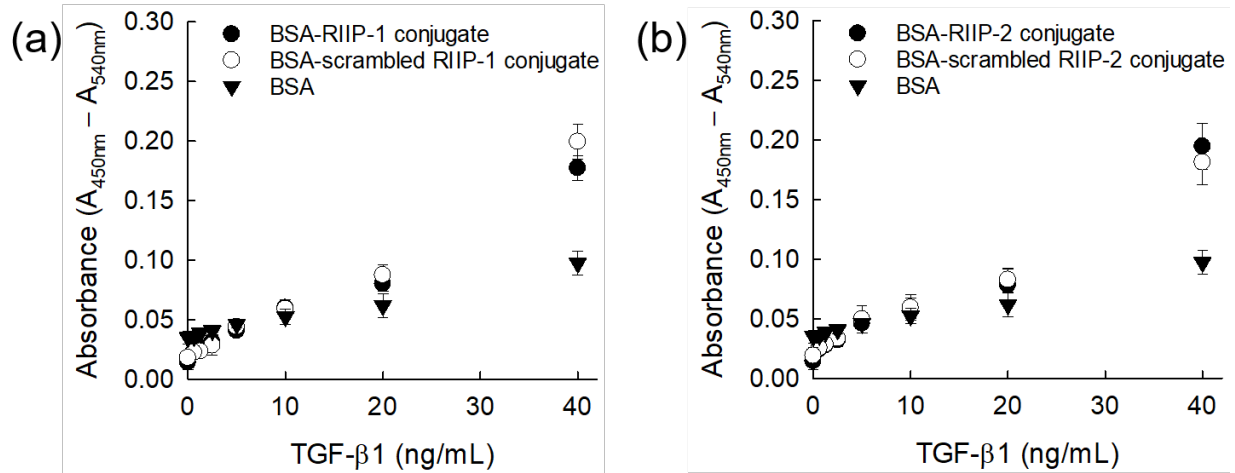
**Figure S1.** Hopp & Woods hydropathy index of TGF- $\beta$ 1 binding peptides. (a) RIP, RIP $\Delta$  and scrambled RIP, (b) RIIP-1 and scrambled RIIP-1, and (c) RIIP-2 and scrambled RIIP-2.



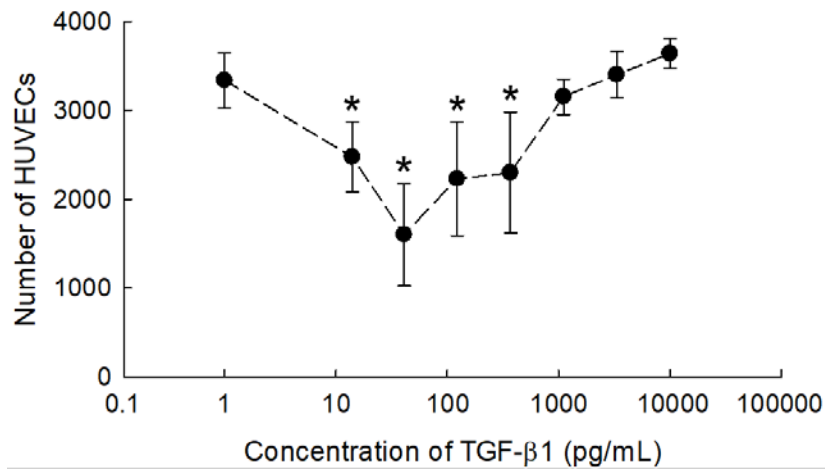
**Figure S2.** Interface of TGF-β type I receptor and TGF-β1 in complex of TGF-β1/TβRI/TβRII. TGF-β1 and RIP are shown in gray and red, respectively. Note that only receptor residues that RIP was derived from are displayed. It shows that the side chain of C-terminal valine of RIP is orientated slightly away from the TGF-β1 binding pocket in the context of the full-length receptor.



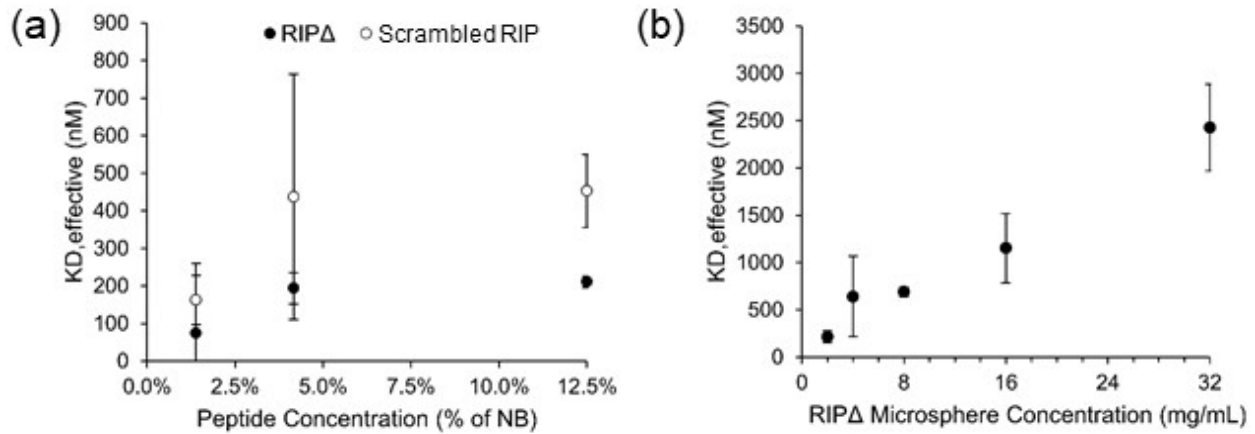
**Figure S3.** Optimizing BSA-peptide immobilization concentration for TGF- $\beta$ 1 ELISA. The BSA-RIP $\Delta$  conjugate bound more TGF- $\beta$ 1 than the BSA-blocking control (no peptide) at all dilutions of the peptide conjugate and more than the BSA-scrambled RIP conjugate at concentrations of 1:125 and higher. Although BSA-RIIP-1 and BSA-RIIP-2 bound more TGF- $\beta$ 1 than BSA-blocking control at concentrations lower than 1:25, neither RIIP-1 nor RIIP-2 bound more TGF- $\beta$ 1 than its scrambled control at any concentration, which suggests that TGF- $\beta$ 1 binding to RIIP-1 and RIIP-2 was not specific.



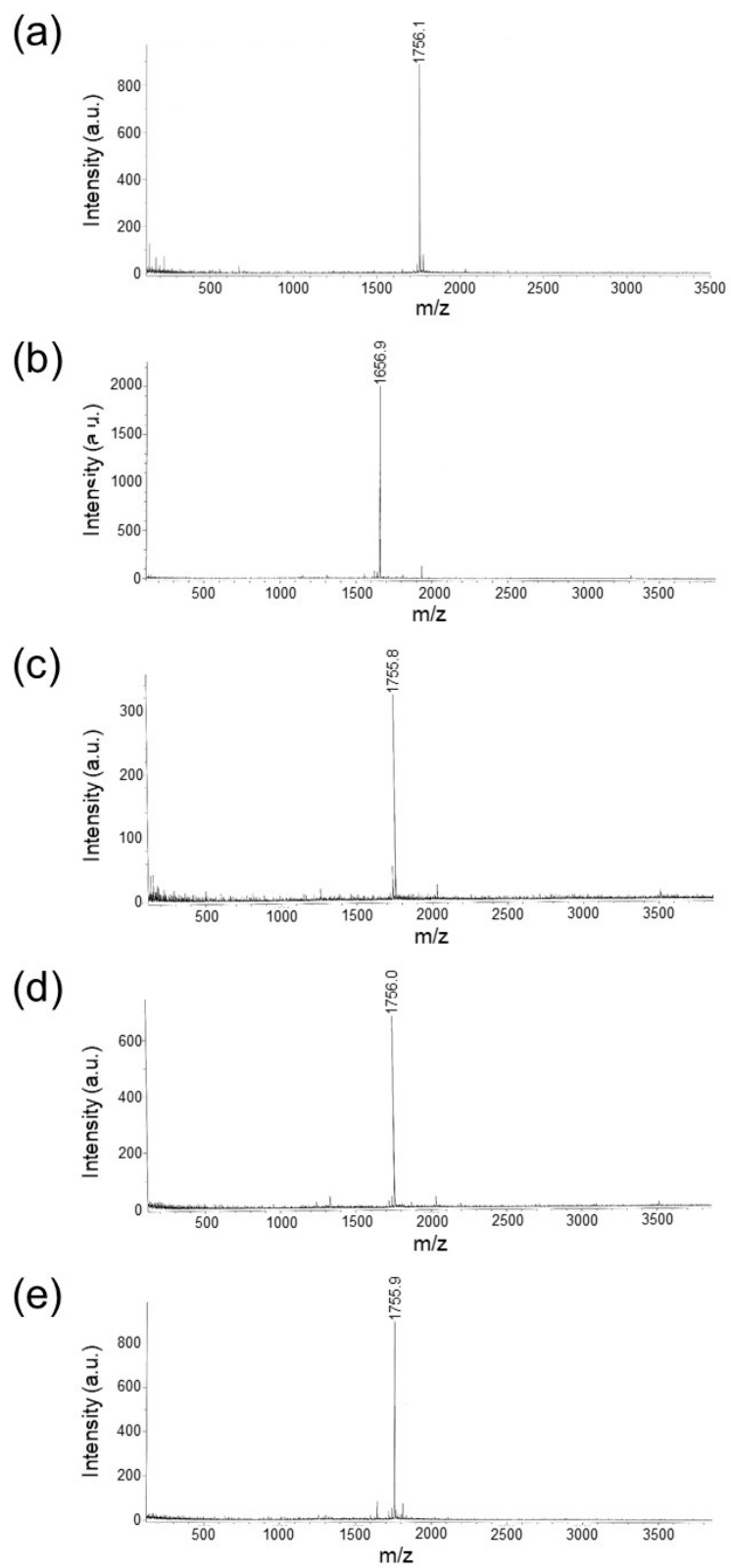
**Figure S4.** TGF- $\beta$ 1 binding to immobilized BSA-conjugated T $\beta$ RII-derived peptides, (a) RIIP-1 and (b) RIIP-2. Absorbance was measured by modified ELISA, where BSA-conjugated peptides were used as a capture antibody. \*  $p < 0.05$  compared to BSA-scrambled RIIP conjugate for a given concentration by post-hoc Bonferroni test.



**Figure S5.** Influence of TGF- $\beta$ 1 on HUVEC proliferation in culture as the mean number of DAPI-positive HUVECs after culture with TGF- $\beta$ 1 at the concentrations specified on the x-axis. Statistical analysis was performed using one-way analysis of variance (ANOVA) with post-hoc Tukey's t-test. \*  $p < 0.05$  compared to no TGF- $\beta$ 1 control shown as 1  $\text{pg mL}^{-1}$  TGF- $\beta$ 1.

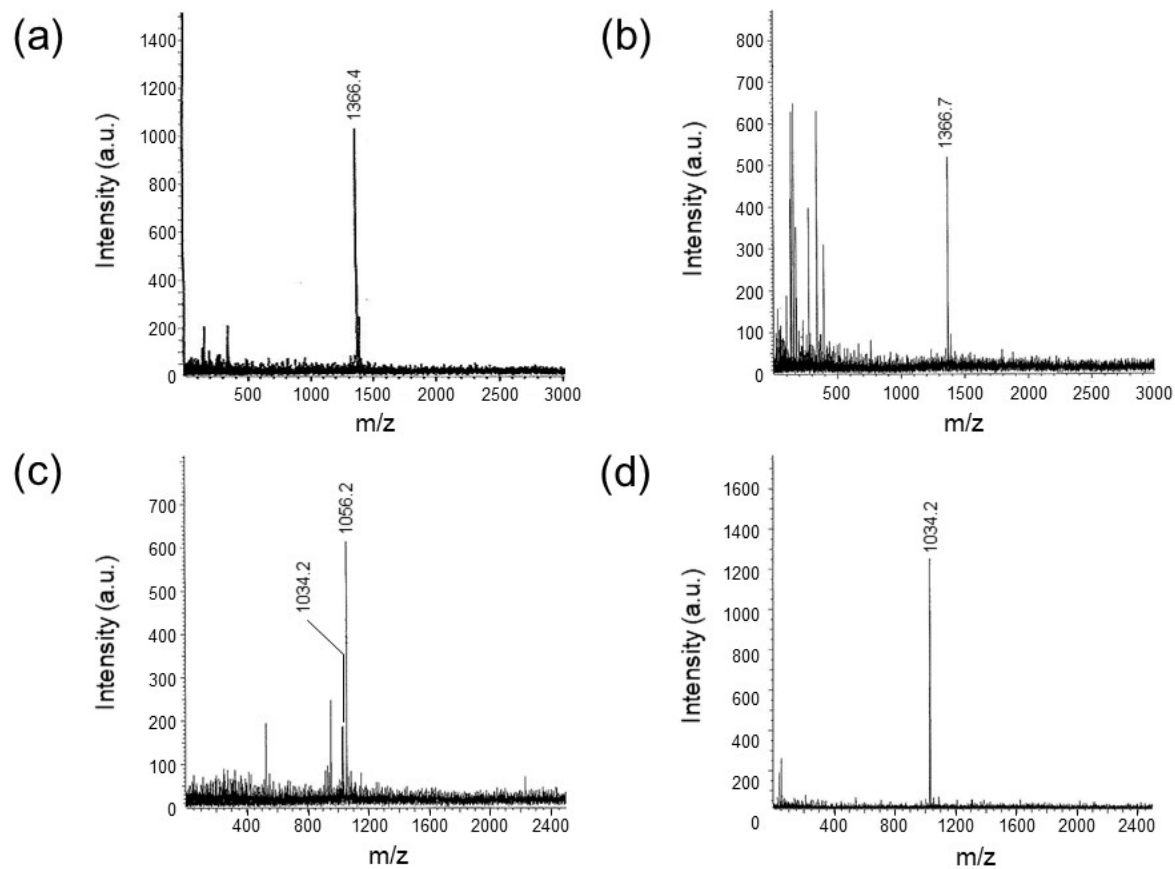


**Figure S6.** Effective equilibrium binding affinity,  $K_{D, \text{effective}}$  of TGF- $\beta$ 1 bound to peptide-conjugated microspheres.  $K_{D, \text{effective}}$  was calculated using the formula  $K_{D, \text{effective}} = L_f \left( \frac{R_t}{L_b} - 1 \right)$  where  $L_f$  is the concentration of free ligand (TGF- $\beta$ 1),  $R_t$  is the concentration of total receptor (RIP $\Delta$  or scrambled RIP) per microsphere mass, and  $L_b$  is the concentration of bound TGF- $\beta$ 1 per microsphere mass. The concentration of peptide in each microsphere condition was assumed based on the molar concentration of peptide added to the reaction mixture. (a)  $K_{D, \text{effective}}$  (in nM) calculated for microspheres, containing various concentration of RIP $\Delta$  or scrambled RIP, incubated in 10% serum in RPMI 1640 supplemented with  $0.09 \text{ ng mL}^{-1}$  TGF- $\beta$ 1 and  $0.1 \text{ ng mL}^{-1}$  [ $^{125}\text{I}$ ]TGF- $\beta$ 1 for 30 min. Microspheres containing 0.5% peptide (molar equivalent to norbornene groups) were omitted from the analysis because the concentration of bound TGF- $\beta$ 1 was negligible in this condition. TGF- $\beta$ 1 in the supernatant was measured by scintillation counting. (b)  $K_{D, \text{effective}}$  (in nM) calculated for each RIP $\Delta$  microsphere concentration upon incubation in PRP for 2 hours. Endogenous TGF- $\beta$ 1 in the supernatant was measured by ELISA.

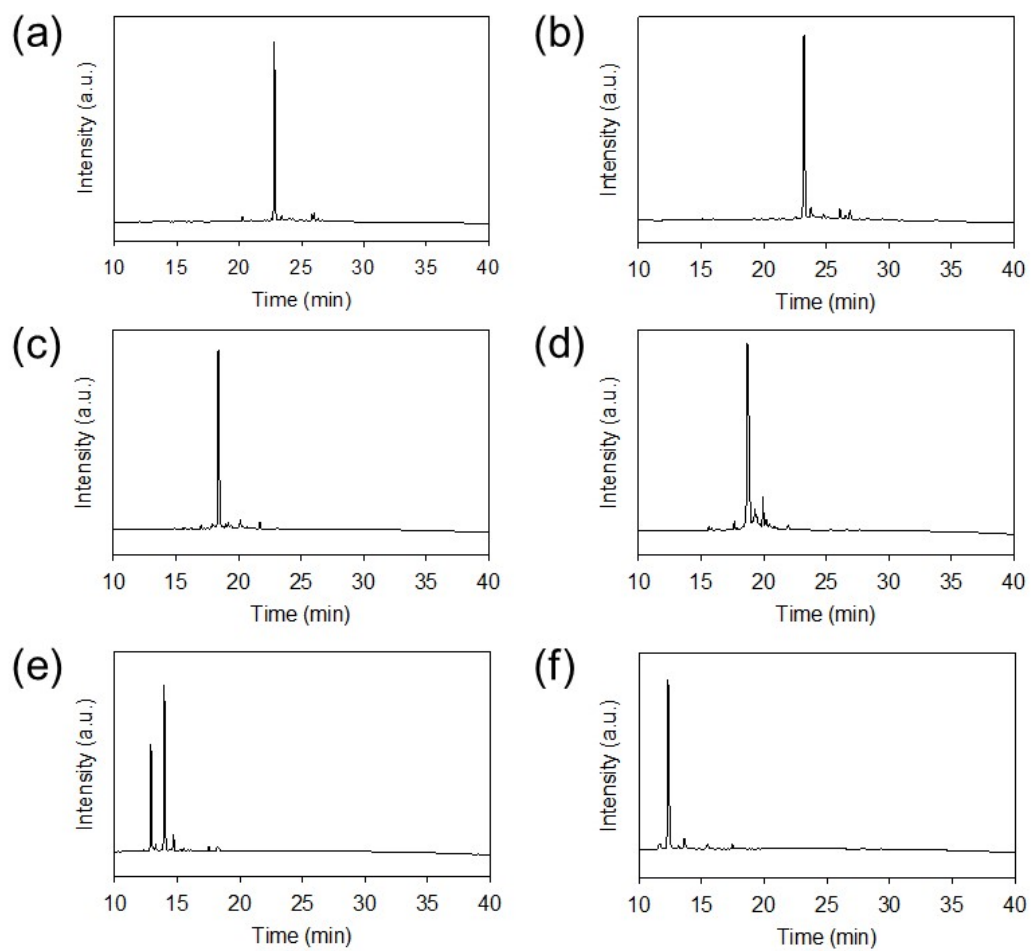


**Figure S7.** MALDI spectra of RIP and RIP variants. (a) RIP, (b) RIP $\Delta$ , (c) scrambled RIP, (d) D-RIP, and (e) RI-RIP.

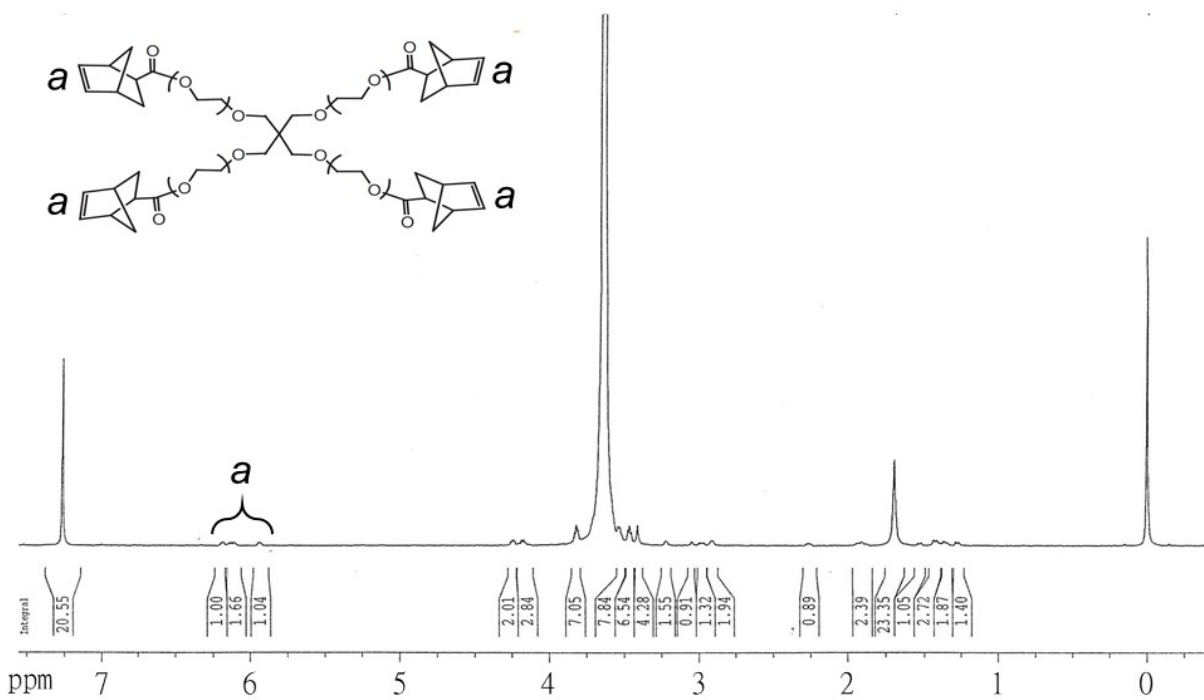




**Figure S8.** MALDI spectra of (a) RIIP-1, (b) scrambled RIIP-1, (c) RIIP-2, and (d) scrambled RIIP-2.



**Figure S9.** C<sub>18</sub> Reverse-Phase HPLC spectra of (a) RIP $\Delta$ , (b) scrambled RIP, (c) RIIP-1, (d) scrambled RIIP-1, (e) RIIP-2, and (f) scrambled RIIP-2.



**Figure S10.** <sup>1</sup>H NMR spectrum of norbornene-terminated 4-arm PEG, showing that over 85% PEG terminal groups were functionalized with norbornene.