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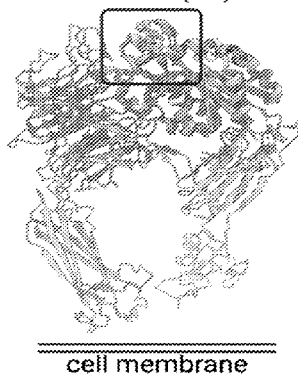
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(54) Title: DE NOVO DESIGNED ALPHA (V) BETA (8) INTEGRIN SELECTIVE MINIBINDER

Figure 1

a.

Integrin $\alpha v\beta 6/\beta 8$ +
RGDLXX(L/I)



(57) Abstract: Alpha(v) beta (8) integrin ($\alpha v\beta 8$)-selective binding polypeptides and their use for treating or inhibiting cancer or tissue fibrosis are provided, where the polypeptides have an amino acid sequence at least 50% identical to the amino acid sequence selected from SEQ ID NO: 1-6, not including any amino acid insertions, wherein the polypeptide selectively binds to $\alpha v\beta 8$, and wherein: (a) residues 10-12 relative to the reference sequence are RGD (b) residue 13 relative to the reference sequence is F, M, or L; (c) residue 16 relative to the reference sequence is Y or V; and (d) residue 40 relative to the reference sequence is D, E, or P.

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De Novo designed alpha (v) beta (8) integrin selective minibinder

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Sequence Listing Statement

A computer readable form of the Sequence Listing is filed with this application by electronic submission and is incorporated into this application by reference in its entirety. The
10 Sequence Listing is contained in the file created on June 5, 2024 having the file name “23-0157-WO_SequenceListing” and is 17,313 bytes in size.

Background

The RGD (Arg-Gly-Asp)-binding integrin $\alpha v\beta 6$ and $\alpha v\beta 8$ are clinically validated
15 cancer and fibrosis targets of considerable therapeutic importance. Compounds that can discriminate between the two closely related integrin proteins and other RGD integrins, stabilize specific conformational states, and have sufficient stability enabling tissue restricted administration could have considerable therapeutic utility. Existing small molecules and antibody inhibitors do not have all of these properties, and hence there is a need for new
20 approaches.

Summary

In one aspect, the disclosure provides alpha(v) beta (8) integrin ($\alpha v\beta 8$)-selective
25 binding polypeptides, comprising or consisting of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from SEQ ID NO:1-6, not including any amino acid insertions, wherein the polypeptide selectively binds to $\alpha v\beta 8$, and wherein:

- (a) residues 10-12 relative to the reference sequence are RGD
- 30 (b) residue 13 relative to the reference sequence is F, M, or L;
- (c) residue 16 relative to the reference sequence is Y or V; and
- (d) residue 40 relative to the reference sequence is D, E, or P.

In one embodiment, residue 41 relative to the reference sequence is D, E, Y, or N. In another embodiment, residue 15 relative to the reference sequence is W, V, T, N, K, or Q. In
35 a further embodiment, residue 16 is Y, relative to the reference sequence. In one embodiment, residue 13, relative to the reference sequence, is M or L. In another

embodiment, residue 15, relative to the reference sequence, is T or V. In further embodiments, residues 13-16, relative to the reference sequence, are LATY (SEQ ID NO:9) or MAVY (SEQ ID NO:14). In other embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or all 15 of residues 13-16, 39-42, and 63-69, relative to the reference sequence, are identical to the reference sequence. In one embodiment, the polypeptides comprise or consist of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from SEQ ID NO:2-3, not including any amino acid insertions. In another embodiment, amino acid changes from the reference protein are conservative amino acid substitutions. In a further embodiment, amino acid substitutions relative to the reference sequence are selected from the amino acid substitutions in Table 2. In other embodiments, the polypeptides further comprise one or more additional functional domains added at the N-terminus, the C-terminus of the polypeptide, and/or one or more insertion sites selected from residues 28-31, 38, 48, and 50-52 relative to the reference sequence.

The disclosure also provides nucleic acids encoding the polypeptide of any embodiment herein, expression vectors comprising the nucleic acid operatively linked to a control sequence, such as a promoter, host cells comprising the nucleic acid and/or the expression vector of any embodiment, recombinant cells expressing the polypeptide of any embodiment, and pharmaceutical compositions, comprising the polypeptide, nucleic acid, expression vector, host cell, or recombinant cell of any embodiment; and a pharmaceutically acceptable carrier.

In another aspect, the disclosure provides methods for treating or inhibiting an $\alpha\text{v}\beta 8(+)$ tumor, comprising administering to a subject in need thereof an amount of the polypeptide, nucleic acid, expression vector, host cell, and/or pharmaceutical composition of any embodiment herein effective to treat or inhibit the tumor in the subject. In one embodiment, the method further comprises administering to the subject one or more other therapeutic agents selected from the group consisting of radiation therapy, an angiogenesis inhibitor, a protein kinase inhibitor, a proteasome inhibitor, an immune checkpoint inhibitor, an mTOR inhibitor, a PI3K inhibitor, a histone deacetylase inhibitor, and a Hedgehog pathway blocker. In a further embodiment, the tumor is selected from the group consisting of a colorectal tumor, a glioblastoma, and a melanoma.

The disclosure also provides methods for detecting an $\alpha\text{v}\beta 8(+)$ tumor or immune cell population, comprising administering to a subject suspected of having an $\alpha\text{v}\beta 8(+)$ tumor or

immune cell population an amount of the polypeptide of any embodiment herein effective to detect the tumor in the subject.

In another aspect, the disclosure provides methods for treating or inhibiting tissue fibrosis, comprising administering to a subject in need thereof an amount of the polypeptide, nucleic acid, expression vector, host cell, and/or pharmaceutical composition of any
 5 embodiments herein effective to treat or inhibit the tissue fibrosis in the subject. In one embodiment, the tissue fibrosis is selected from the group consisting of pulmonary fibrosis, liver fibrosis, and renal fibrosis.

10 Description of the Figures

Figure 1. Computational design of $\alpha\beta 8$ selective minibinders. **a)** Crystal structure of $\beta 8$ (PDB ID 6OM2) overlaid on the structure of $\alpha\beta 6$ integrin in complex with the L-TGF- $\beta 3$ peptide RGD $LXX(L/I)$ (SEQ ID NO: 7)(PDB ID 4UM9). **b)** Polar interactions between the RGD motif and $\alpha\beta 8$ integrin (PDB ID 6OM2). **c)** Hydrophobic packing of $LXX(L/I)$
 15 (SEQ ID NO: 8) motif of L-TGF- $\beta 3$ peptide with SDL2 of $\alpha\beta 6$ (PDB ID 4UM9). Leu247 packs optimally against Y185 from SDL2 of $\beta 6$. **d)** Hydrophobic packing of $LXX(L/I)$ (SEQ ID NO: 8) of L-TGF- $\beta 1$ peptide with SDL2 of $\alpha\beta 8$ (PDB ID 6OM2). I221 of L-TGF- $\beta 1$ peptide packs less tightly against L174 on SDL2 of $\beta 8$ compared to the homologous interactions in panel d. **e)** Charge reversal on β subunit: $\beta 8$ contains K304 whereas the
 20 equivalent position on $\beta 6$ is E316. **f)** Small α/β ferredoxin folds were able to scaffold the RGD $LXX(L/I)$ (SEQ ID NO: 7) binding loop without clashing with the integrin while making additional contacts with both α and β subunit (α - and β -loop respectively) **g)** Loop extension strategy to design an $\alpha\beta 8$ selective minibinder: to make more extensive contacts to the $\beta 8$ subunit the β -loop was resampled by one residue insertion (surface representation for
 25 $\beta 8$ subunit, PDB ID 6OM2). In addition to the loop extension, the $LXX(L/I)$ (SEQ ID NO: 8) motif was redesigned using Rosetta. **h)** Partial sequence alignment of SDL2 of the $\beta 6/\beta 8$ subunits is shown highlighting two key positions packing against the $LXX(L/I)$ (SEQ ID NO: 8) motif of the L-TGF- β ligand (I183 and Y185 in SLD2- $\beta 6$, and Y172 and L174 in SDL2- $\beta 8$) (top to bottom SEQ ID NOs: 15-16).

Figure 2. Competition assay using yeast surface display technique to determine selectivity of designed $\alpha\beta 8$ binders. **a)** Designs were expressed using yeast surface display technique and incubated with 50 nM of FLAG-tagged $\alpha\beta 8$ (to confirm binding to $\alpha\beta 8$) and in the presence or absence of 625nM of unlabelled $\alpha\beta 6$ (the purpose of this unlabeled $\alpha\beta 6$ integrin is to compete away non- $\alpha\beta 8$ -selective binders that also bind to $\alpha\beta 6$). This assay
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setup allows for identification of $\alpha\text{v}\beta 8$ -selective binders, which are verified only if a binding population is present in the top right quadrant in both the “no_ $\alpha\text{v}\beta 6$ ” and “625 nM $\alpha\text{v}\beta 6$ ” conditions (the only construct in which two clear binding populations are present in the top right quadrant is avb8_#3 [MAVY]). LATI is the amino acid sequence of the residues that immediately follow the RGD motif in endogenous human L-TGF β (latent TGF β). Binding was detected by anti-FLAG-PE conjugated antibody from Abcam (Product # ab72469). Based on these data, avb8_#3 was identified as a lead candidate that exhibited exquisite selectivity for integrin $\alpha\text{v}\beta 8$. **b)** Zoomed in data of bold box in FIG. 2A. Effect of RGD $\underline{\text{LXXL}}$ (SEQ ID NO: 11) motif on selectivity of the designed binders. The -RGD $\underline{\text{MAVY}}$ (SEQ ID NO: 12) motif, present in avb8_#3, also referred to as B8_BP_dslf; (SEQ ID NO:2) selectively binds to $\alpha\text{v}\beta 8$ as shown by the two populations in the top right quadrant (left panel): one population is in the presence of 625 nM $\alpha\text{v}\beta 6$ and the other population has no $\alpha\text{v}\beta 6$ present to compete for binding. Mutation of the RGD $\underline{\text{MAVY}}$ (SEQ ID NO: 12) motif to RGD $\underline{\text{LATI}}$ (SEQ ID NO: 13) (present in avb8_#12) completely abrogates selectivity towards $\alpha\text{v}\beta 8$. B8_BP_dslf (SEQ ID NO:2) is highly selective to $\alpha\text{v}\beta 8$. $\alpha\text{v}\beta 8$ -selective B8_BP_dslf does not bind to any other RGD binding integrins as confirmed by BLI (Fig 3a).

Figure 3. Binding selectivity studies. a) Binding for the B8_BP_dslf and its point mutants against $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 8$ and selectivity for B8_BP_dslf for RGD integrins. **b)** Binding affinities (Kd) of B8_BP_dslf (SEQ ID NO:2) point mutants in the MAVY (SEQ ID NO: 14) motif (residues 13-16 in SEQ ID NO:2) to integrins $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 8$, determined by BLI, and binding affinities (Kd) and fold-selectivity values of B8_BP-LATY (SEQ ID NO:3; the LATY motif is present at residues 13-16 in SEQ ID NO: 9) to all eight RGD integrins.

Figure 4. Binding selectivity studies. a, b) Competitive inhibition of h-LAP I binding to (a) $\alpha\text{v}\beta 6$ and (b) $\alpha\text{v}\beta 8$ by designed inhibitors and control small molecule PLN-74809. **c)** Heatmap of IC $_{50}$ values for h-LAP I binding assays in a and b.

Figure 5. Structural characterization. a) Representative 2D class averages of integrin with and without minibinder. For $\alpha\text{v}\beta 8$, no open headpieces were observed, with or without minibinder. **b)** CryoEM density map of $\alpha\text{v}\beta 8$ bound to minibinder B8_BP_dslf. B8_BP_dslf binds the integrin ligand binding cleft between the αv and $\beta 8$ subunits, however the conformation of $\alpha\text{v}\beta 8$ remains in the closed headpiece conformation. The sharpened, locally refined cryoEM map is shown, superimposed with the unsharpened map showing all domains in the $\alpha\text{v}\beta 8$ ectodomain construct in semi-transparent white. **c, d)** An overlay of the designed $\alpha\text{v}\beta 8$ + B8_BP_dslf model (gray) and the experimentally determined model. Although the overall angle of the minibinder is shifted, the RGD loop positioning is as

predicted. Insets in c) and d) are magnified in panels e) and f), respectively. **e)** E41 from β -loop makes backbone level hydrogen bond with I216 from β 8 subunit and D40 makes salt bridge interaction with K304 from β 8 subunit (panel i). **f)** Experimental vs designed (gray) packing pattern of the MAVY (SEQ ID NO: 14) motif and SDL2 of $\alpha\beta$ 8.

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Detailed Description

All references cited are herein incorporated by reference in their entirety. Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), “Guide to Protein Purification” in *Methods in Enzymology* (M.P. Deutscher, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), RosettaCommons.org, and the Ambion 1998 Catalog (Ambion, Austin, TX).

As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

As used herein, the amino acid residues are abbreviated as follows: alanine (Ala; A), asparagine (Asn; N), aspartic acid (Asp; D), arginine (Arg; R), cysteine (Cys; C), glutamic acid (Glu; E), glutamine (Gln; Q), glycine (Gly; G), histidine (His; H), isoleucine (Ile; I), leucine (Leu; L), lysine (Lys; K), methionine (Met; M), phenylalanine (Phe; F), proline (Pro; P), serine (Ser; S), threonine (Thr; T), tryptophan (Trp; W), tyrosine (Tyr; Y), and valine (Val; V).

In all embodiments of polypeptides disclosed herein, any N-terminal methionine residues are optional (i.e.: the N-terminal methionine residue may be present or may be deleted).

All embodiments of any aspect of the disclosure can be used in combination, unless the context clearly dictates otherwise.

Unless the context clearly requires otherwise, throughout the description and the claims, the words ‘comprise’, ‘comprising’, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of

“including, but not limited to”. Words using the singular or plural number also include the plural and singular number, respectively. Additionally, the words “herein,” “above,” and “below” and words of similar import, when used in this application, shall refer to this application as a whole and not to any particular portions of the application

5 In one aspect, the disclosure provides alpha(v) beta (8) integrin ($\alpha\text{v}\beta 8$)-selective binding polypeptides comprising or consisting of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from SEQ ID NO:1-6, not including any amino acid insertions, wherein the polypeptide selectively binds to $\alpha\text{v}\beta 8$, and wherein:

- 10 (a) residues 10-12 relative to the reference sequence are RGD
 (b) residue 13 relative to the reference sequence is F, M, or L;
 (c) residue 16 relative to the reference sequence is Y or V; and
 (d) residue 40 relative to the reference sequence is D, E, or P.

The polypeptides disclosed here are hyperstable RGD-containing miniproteins that
 15 are highly selective for the human $\alpha\text{v}\beta 8$ integrin, such that they bind with a picomolar affinity (K_d) to the human $\alpha\text{v}\beta 8$ integrin that is at least 1,000-fold higher affinity than the affinity at which they bind to the other human RGD integrins (e.g., $\alpha\text{v}\beta 1$, $\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 5$, $\alpha\text{v}\beta 6$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha \text{IIb}\beta 3$), as determined by biolayer interferometry in which protein binders are immobilized on Ni-NTA and streptavidin sensor tips. The tips were then dipped into wells
 20 containing different concentrations of integrin, and association and dissociation steps were recorded for 900 s and 1200 s respectively.

The amino acid sequence of SEQ ID NO:1-6 are shown in Table 1. The proteins comprise a rigid backbone formation of an RGD loop-beta sheet-alpha helical domains connected by amino acid linkers. Within SEQ ID NO:1-6, the RGD-loop domain (binds to
 25 the ligand binding site at the interface between the alpha and beta chains) comprises residues 10 to 16, the beta-loop domain (binds to the beta chain on the integrin) comprises residues 39 to 42, and the alpha-loop domain (binds to the alpha chain on the integrin) comprises residues 63 to 69. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or all 15 of residues 13-16, 39-42, and 63-69, relative to the reference sequence, are identical to the reference
 30 sequence. These are the interface residues, in addition to residues 10-12

In one embodiment, the polypeptides comprise or consist of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from SEQ ID NO:2-3, not including any amino acid insertions, wherein the polypeptide selectively binds to

$\alpha\text{v}\beta 8$. In various embodiments, the polypeptides comprise an amino acid sequence at least 60% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; or at least 70% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; or at least 80% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; or at least 90% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; or at least 95% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; in all embodiments not including any insertions, wherein residues 10-12 relative to the reference polypeptide are RGD, and wherein the polypeptide selectively binds to integrin $\alpha\text{v}\beta 8$. In other embodiments, the polypeptides consist of an amino acid sequence at least 60% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; or at least 70% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; or at least 80% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; or at least 90% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; or at least 95% identical to the amino acid sequence selected from SEQ ID NO:1-6 or SEQ ID NO:2-3; in all embodiments not including any insertions, wherein residues 10-12 relative to the reference polypeptide are RGD, and wherein the polypeptide selectively binds to integrin $\alpha\text{v}\beta 8$.

One or more amino acid insertions can be made at residues 28-31, 38, 48, and 50-52 without affecting $\alpha\text{v}\beta 8$ binding. These residues are referred to as “insertion sites”. Insertions are permissible at these regions because they are (1) flexible loop regions that can accommodate residue insertions without altering the rigid backbone conformation of beta sheet and alpha helix secondary structures and (2) they are not involved in interactions with the $\alpha\text{v}\beta 8$ integrin. Amino acid insertions may be single residues, multiple residues, or functional domains. In one embodiment, an amino acid insertion is present, but does not result in elimination of any residues in the polypeptide. In another embodiment, an amino acid insertion is present, and does result in elimination of 1, 2, 3, or 4 contiguous insertion sites.

The position of residues in the polypeptides of the disclosure are “relative to” the position of residues in the reference sequence; this does not necessarily mean that the residue number in the polypeptide of the disclosure will be identical to the residue number in the reference sequence. For example, residues 10-12 of the reference sequences of SEQ ID NO:1-6 are RGD. Polypeptides of the disclosure have an RGD tripeptide that is at a position corresponding to residues 10-12 in the reference sequence, but not necessarily at residues 10-

12 in the polypeptides of the disclosure. Those of skill in the art will understand that the polypeptides of the disclosure may be fused to other functional domains, including N-terminal domains, such that the RGD residues in the polypeptides will not be at positions 10-12, but the polypeptides will still have the RGD triad at residues 10-12 relative to the reference polypeptide selected from SEQ ID NO:1-14. Similarly, the polypeptides of the disclosure may include insertions at residues 28-31, 38, 48, and 50-52 relative to the reference sequence, as noted above.

Table 1

SEQ ID NO	Name	α v β 8 Binding affinity	Rel. selectivity	Amino acid sequence
1	avb8_2	+	+	TKCVVRFIFRGDFAWVALRAVKDHLKKEGPHWNITETNQDY MQLVVVIRGIHESDAKRIAKWVENRFPYARVETQCD
2	avb8_3 (B8_BP_dslf)	++++	++++	TKCVVRFNFRGDMAVYALKAVKDHLKKEGPHWNITTHNDDE VYLVVIRGIHESDAKRIAKWVESTIPGISVETQCD
3	avb8_3_LATY	++++	++++	TKCVVRFNFRGDLATYALKAVKDHLKKEGPHWNITTHNDDE VYLVVIRGIHESDAKRIAKWVESTIPGISVETQCD
4	avb8_5	+	++	TKCVVRFIFRGDMANYALKAVKDHLKKEGPHWNITTQEEP NQLVVVIRGIHESDAKRIAKWVEKRFPFAGVETQCD
5	avb8_6	++	++	TKCVVRFVFRGDFAKYMLKAVKDHLKKEGPHWNITEGESEE NQLVVVIRGIHESDAKRIAKWVQTRFPYIRVETQCD
6	avb8_9	++	++	TKCVVRFIFRGDMAQYALKAVKDHLKKEGPHWNITTQEEP NQLVVVIRGIHESDAKRIAKWVEKRFPFAGVETQCD

In one embodiment, residue 41 relative to the reference sequence is D, E, Y, or N. Residue 41 is present in the β -loop and makes a backbone level hydrogen bond with I216 from the β 8 subunit.

In another embodiment, residue 15 relative to the reference sequence is W, V, T, N, K, or Q. In another embodiment, residue 15 relative to the reference sequence is T or V, which are smaller residues that allow optimal packing of when residues 15 is Y, and improves α v β 8 selectivity.

In a further embodiment, residue 16, relative to the reference sequence, is Y. Residue 16 was shown to be important for binding affinity and selectivity for $\alpha\text{v}\beta 8$, as Y16 forms stabilizing interactions with A115 of the $\beta 8$ subunit and interacts with the less bulky L174 in the $\beta 8$ -SDL2 loop.

5 In various further embodiments, one of the following is true:

(a) relative to the reference sequence, residue 13 is F, residue 15 is W, residue 16 is V, residue 40 is D, and residue 41 is Y;

(b) relative to the reference sequence, residue 13 is M, residue 15 is V, residue 16 is Y, residue 40 is D, and residue 41 is E;

10 (c) relative to the reference sequence, residue 13 is L, residue 15 is T, residue 16 is Y, residue 40 is D, and residue 41 is E;

(d) relative to the reference sequence, residue 13 is M, residue 15 is N, residue 16 is Y, residue 40 is P, and residue 41 is N;

(e) relative to the reference sequence, residue 13 is F, residue 15 is K, residue 16 is Y, residue 40 is E, and residue 41 is E; or

(f) relative to the reference sequence, residue 13 is M, residue 15 is Q, residue 16 is Y, residue 40 is P, and residue 41 is N.

In one embodiment, residue 13, relative to the reference sequence, is Met (M) or Leu (L). Binders residues 13-16 identified as LATY and MAVY were optimally selective for $\alpha\text{v}\beta 8$ (Fig. 3B).

Residue 40 was shown to form salt bridge interaction with K304 of the $\beta 8$ subunit.

In specific embodiments, one of the following is true:

(a) relative to the reference sequence, residue 13 is M, residue 15 is V, residue 16 is Y, residue 40 is D, and residue 41 is E; or

25 (b) relative to the reference sequence, residue 13 is L, residue 15 is T, residue 16 is Y, residue 40 is D, and residue 41 is E.

In one embodiment, amino acid substitutions relative to the reference sequence are selected from those disclosed in Table 2. Potential substitutions represent shared chemical properties of the amino acids. Terms used in Table 2 have the following meaning

30 Small Polar or hydrophobic: S, T, Q, N, V, D, or E;

Hydrophobic: A, V, L, I, F, Y, W, M, P, or G;

Polar (Charged or Neutral): S, T, Q, N, D, E, K, R, or Y;

Polar (Negatively charged or Neutral): S, T, Q, N, D, E, or Y;

Polar (Positively charged or Neutral); S, T, Q, N, K, R, or Y;

Small Polar: S, T, Q, or D;

Affinity, Hot spot: Mutation causes loss of binding;

Affinity, Selectivity: Contributes to affinity and selectivity;

Affinity: Contributes to affinity; and

5 **Insertion:** amino acid insertions may be incorporated at the identified position.

Table 2

Position	Identity	Function	May be substituted with
1	T	Structural	small polar or hydrophobic
2	K	Structural	Polar (charged or neutral)
3	C	Structural, disulfide	Hydrophobic
4	V	Structural	Hydrophobic
5	V	Structural	Hydrophobic
6	R	Structural	Polar
7	F	Structural	Hydrophobic
8	N	Structural	Polar
9	F	Structural	Hydrophobic
10	R	Affinity, Hot spot Interface residue	N/A
11	G	Affinity, Hot spot Interface residue	N/A
12	D	Affinity, Hot spot Interface residue	N/A
13	M	Affinity, Selectivity Interface residue	Hydrophobic, specifically F, M, or L
14	A	Structural Interface residue	small polar or hydrophobic
15	V	Structural Interface residue	polar or hydrophobic, specifically W, V, T, N, K, or Q
16	Y	Affinity, Selectivity Interface residue	V
17	A	Structural	small hydrophobic
18	L	Structural	Hydrophobic
19	K	Structural	Polar
20	A	Structural	hydrophobic
21	V	Structural	Hydrophobic

22	K	Structural	Polar (charged or neutral)
23	D	Structural	Polar (neutral or negatively charged)
24	H	Structural	Polar (charged or neutral)
25	L	Structural	Hydrophobic
26	K	Structural	Polar (charged or neutral)
27	K	Structural	Polar (charged or neutral)
28	E	Structural	Polar (negatively charged or neutral) Insertion
29	G	Structural	small polar or hydrophobic Insertion
30	P	Structural	small polar or hydrophobic Insertion
31	H	Structural	Polar (charged or neutral) Insertion
32	W	Structural	Hydrophobic
33	N	Structural	Polar (charged or neutral)
34	I	Structural	Hydrophobic
35	T	Structural	polar or hydrophobic
36	T	Structural	polar or hydrophobic
37	H	Structural	polar or hydrophobic
38	N	Structural	Polar (charged or neutral) Insertion
39	D	Structural Interface residue	Polar (charged or neutral)
40	D	Affinity Interface residue	Polar (Negatively charged or neutral), specifically D, E, or P
41	E	Affinity Interface residue	Polar (Negatively or neutral), specifically D, E, Y, or N
42	V	Structural Interface residue	Hydrophobic
43	Y	Structural	Hydrophobic
44	L	Structural	Hydrophobic
45	V	Structural	Hydrophobic
46	V	Structural	Hydrophobic
47	R	Structural	Polar (charged or neutral)
48	G	Structural	P, or small polar Insertion

49	I	Structural	Hydrophobic
50	H	Structural	Polar (charged or neutral) Insertion
51	E	Structural	Polar (charged or neutral) Insertion
52	S	Structural	Polar Insertion
53	D	Structural	Polar (charged or neutral)
54	A	Structural	small polar or hydrophobic
55	K	Structural	Polar (charged or neutral)
56	R	Structural	Polar (charged or neutral)
57	I	Structural	Hydrophobic
58	A	Structural	Hydrophobic
59	K	Structural	Polar (charged or neutral)
60	W	Structural	Hydrophobic
61	V	Structural	small hydrophobic
62	E	Structural	Polar (charged or neutral)
63	S	Structural Interface residue	Small polar or hydrophobic
64	T	Structural Interface residue	Polar
65	I	Structural Interface residue	Hydrophobic
66	P	Structural Interface residue	Glycine, small polar
67	G	Structural Interface residue	Proline, small polar
68	I	Structural Interface residue	Hydrophobic
69	S	Structural Interface residue	small polar or hydrophobic
70	V	Structural	Hydrophobic
71	E	Structural	Polar (charged or neutral)
72	T	Structural	small polar or hydrophobic
73	Q	Structural	Polar (charged or neutral)
74	C	Structural, disulfide	Hydrophobic
75	D	Structural	Polar (charged or neutral)

In one embodiment of each of the above aspects, amino acid substitutions relative to the reference peptide domains are conservative amino acid substitutions. As used herein, “conservative amino acid substitution” means a given amino acid can be replaced by a residue having similar physiochemical characteristics, e.g., substituting one aliphatic residue for another (such as Ile, Val, Leu, or Ala for one another), or substitution of one polar residue for another (such as between Lys and Arg; Glu and Asp; or Gln and Asn). Other such conservative substitutions, e.g., substitutions of entire regions having similar hydrophobicity characteristics, are known. Polypeptides comprising conservative amino acid substitutions can be tested in any one of the assays described herein to confirm that a desired activity, e.g. antigen-binding activity and specificity of a native or reference polypeptide is retained.

Amino acids can be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in *Biochemistry*, second ed., pp. 73-75, Worth Publishers, New York (1975)): (1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M); (2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q); (3) acidic: Asp (D), Glu (E); (4) basic: Lys (K), Arg (R), His (H). Alternatively, naturally occurring residues can be divided into groups based on common side-chain properties: (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; (6) aromatic: Trp, Tyr, Phe. Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Particular conservative substitutions include, for example; Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

In a further embodiment, the polypeptides may comprise an insertion in one or more loop regions of the polypeptide (i.e., residues 28-31, 38, 48, and 50-52). The insertion may be any one or more amino acid, and may comprise a functional domain as described in the next paragraph, or one or more amino acids for additional spacing or for any other purpose. In one embodiment, an insertion in the loop regions is 1-3, 1-2, 1, 2, or 3 amino acids in length. In certain embodiments, amino acids or amino acid domains (such as a functional domain) may be inserted in the loop region. The polypeptides of the disclosure may include any such insertion, and in these embodiments the polypeptide would still comprise the reference amino acid sequence, with an interruption at the site of insertion.

In another embodiment of any of the above aspects, the polypeptide further comprises one or more additional functional domains added at the N-terminus and/or the C-terminus of the polypeptide, and/or one or more insertion site (i.e., residues 28-31, 38, 48, and 50-52).

Any suitable functional domain(s) may be added as suitable for an intended purpose,

- 5 including but not limited to albumin (to improve serum half-life), Fc domains, a receptor targeting domain, therapeutics, diagnostics, molecular probes such as fluorescent proteins, a tag (including but not limited to a polyhistidine tag), etc.

- In another embodiment, the polypeptide binds to the human $\alpha\text{v}\beta 8$ integrin with a picomolar affinity (K_a) that is at least 1,000-fold higher than the affinity at which the
10 polypeptide binds to any one of another human RGD integrins selected from the group consisting of $\alpha\text{v}\beta 1$, $\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 5$, $\alpha\text{v}\beta 6$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, and $\alpha \text{IIb}\beta 3$.

- In a further aspect, the present disclosure provides nucleic acids, including isolated nucleic acids, encoding the polypeptides of the present disclosure. The isolated nucleic acid sequence may comprise RNA or DNA. Such isolated nucleic acid sequences may comprise
15 additional sequences useful for promoting expression and/or purification of the encoded protein, including but not limited to polyA sequences, modified Kozak sequences, and sequences encoding epitope tags, export signals, and secretory signals, nuclear localization signals, and plasma membrane localization signals. It will be apparent to those of skill in the art, based on the teachings herein, what nucleic acid sequences will encode the polypeptides
20 of the invention.

- In another aspect, the present disclosure provides expression vectors comprising the nucleic acid of any aspect of the invention operatively linked to a suitable control sequence. "Expression vector" includes vectors that operatively link a nucleic acid coding region or gene to any control sequences capable of effecting expression of the gene product. "Control
25 sequences" operably linked to the nucleic acid sequences of the invention are nucleic acid sequences capable of effecting the expression of the nucleic acid molecules. The control sequences need not be contiguous with the nucleic acid sequences, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the nucleic acid sequences and
30 the promoter sequence can still be considered "operably linked" to the coding sequence. Other such control sequences include, but are not limited to, polyadenylation signals, termination signals, and ribosome binding sites. Such expression vectors include but are not limited to, plasmid and viral-based expression vectors. The control sequence used to drive expression of the disclosed nucleic acid sequences in a mammalian system may be

constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). The expression vector must be replicable in the host organisms either as an episome or by integration into host chromosomal DNA. In various embodiments, the expression vector may comprise a plasmid, viral-based vector (including but not limited to a retroviral vector or oncolytic virus), or any other suitable expression vector. In some embodiments, the expression vector can be administered in the methods of the disclosure to express the polypeptides *in vivo* for therapeutic benefit.

In a further aspect, the present disclosure provides host cells that comprise the expression vectors, polypeptides, and/or nucleic acids disclosed herein, wherein the host cells can be either prokaryotic or eukaryotic. The cells can be transiently or stably engineered to incorporate the expression vector of the invention, using techniques including but not limited to bacterial transformations, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. (See, for example, *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press); *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney, 1987, Liss, Inc. New York, NY)). A method of producing a polypeptide according to the invention is an additional part of the invention. The method comprises the steps of (a) culturing a host according to this aspect of the invention under conditions conducive to the expression of the polypeptide, and (b) optionally, recovering the expressed polypeptide. The expressed polypeptide can be recovered from the cell free extract, but preferably they are recovered from the culture medium.

In another embodiment, the disclosure provides pharmaceutical compositions, comprising:

- (a) the polypeptide, nucleic acid, expression vector, host cell, or recombinant cell of any preceding claims; and
- (b) a pharmaceutically acceptable carrier.

In certain embodiments, the carrier in a pharmaceutical composition can be either aqueous or non-aqueous in nature. For example, in certain embodiments, a suitable vehicle or carrier can be water for injection, physiological saline solution or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. In certain embodiments, the saline comprises isotonic phosphate-buffered saline. In certain embodiments, neutral buffered saline or saline mixed with serum albumin

are further exemplary vehicles. In certain embodiments, pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which can further include sorbitol or a suitable substitute therefore. In certain embodiments, a composition comprising an immunomodulatory fusion protein is prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents (Remington's Pharmaceutical Sciences, *supra*) in the form of a lyophilized cake or an aqueous solution. Further, in certain embodiments, a composition comprising an immunomodulatory fusion protein is formulated as a lyophilizate using appropriate excipients such as sucrose.

The compositions may be used, for example, in the methods disclosed herein. The compositions may further comprise for example, (a) a lyoprotectant; (b) a surfactant; (c) a bulking agent; (d) a tonicity adjusting agent; (e) a stabilizer; (f) a preservative and/or (g) a buffer. In some embodiments, the buffer in the pharmaceutical composition is a Tris buffer, a histidine buffer, a phosphate buffer, a citrate buffer or an acetate buffer. The composition may also include a lyoprotectant, e.g. sucrose, sorbitol or trehalose. In certain embodiments, the composition includes a preservative e.g. benzalkonium chloride, benzethonium, chlorohexidine, phenol, m-cresol, benzyl alcohol, methylparaben, propylparaben, chlorobutanol, o-cresol, p-cresol, chlorocresol, phenylmercuric nitrate, thimerosal, benzoic acid, and various mixtures thereof. In other embodiments, the composition includes a bulking agent, like glycine. In yet other embodiments, the composition includes a surfactant e.g., polysorbate-20, polysorbate-40, polysorbate-60, polysorbate-65, polysorbate-80 polysorbate-85, poloxamer-188, sorbitan monolaurate, sorbitan monopalmitate, sorbitan monostearate, sorbitan monooleate, sorbitan trilaurate, sorbitan tristearate, sorbitan trioleate, or a combination thereof. The composition may also include a tonicity adjusting agent, e.g., a compound that renders the formulation substantially isotonic or isoosmotic with human blood. Exemplary tonicity adjusting agents include sucrose, sorbitol, glycine, methionine, mannitol, dextrose, inositol, sodium chloride, arginine and arginine hydrochloride. In other embodiments, the composition additionally includes a stabilizer, e.g., a molecule which substantially prevents or reduces chemical and/or physical instability of the nanostructure, in lyophilized or liquid form. Exemplary stabilizers include sucrose, sorbitol, glycine, inositol, sodium chloride, methionine, arginine, and arginine hydrochloride.

The polypeptide, fusion protein, nucleic acid, expression vector, and/or host cell may be the sole active agent in the composition, or the composition may further comprise one or more other agents suitable for an intended use. In various non-limiting embodiments, such

other agents may include angiogenesis inhibitors (including but not limited to axitinib, bevacizumab, cabozantinib, everolimus, lenalidomide, lenvatinib mesylate, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, thalidomide, vandetanib, and ziv-aflibercept), immune checkpoint inhibitors (including, but not limited to, pembrolizumab, nivolumab, and cemiplimab as anti-PD-1 antibodies, ipilimumab as an anti-CTLA-4 antibody, and atezolizumab, avelumab, and durvalumab as anti-PD-L1 antibodies), and other cancer growth inhibitors including but not limited to tyrosine kinase inhibitors (including but not limited to alectinib, brigatinib, ceritinib, crizotinib, entrectinib, lorlatinib, ALK, I, bosutinib, dasatinib, imatinib, nilotinib, ponatinib, afatinib, dacomitinib, erlotinib, gefitinib, lapatinib, neratinib, osimertinib, vandetanib, gilteritinib, midostaurin, erdafitinib, ruxolitinib, larotrectinib, axitinib, carbozantinib, lenvatinib, pazopanib, regorafenib, sorafenib, sunitinib, dabrafenib, encorafenib, vemurafenib, acalabrutinib, ibrutinib, binimetinib, cobimetinib, trametinib, abemaciclib, palbociclib, or ribociclib), proteasome inhibitors (including but not limited to ortezomib, carfizomib, ixazomib, delanzomib, oprozomib, and marizomib), mTOR inhibitors (including but not limited to everolimus, sirolimus, temsirolimus, everolimus, sirolimus, sirolimus protein-bound, and everolimus), PI3K inhibitors (including but not limited to copanlisib, alpelisib, idelalisib, duvelisib and umbralisib), histone deacetylase inhibitors (including but not limited to vorinostat, romidepsin, panobinostat, and belinostat), and Hedgehog pathway blockers (including but not limited to vismodegib, sonidegib, and glasdegib).

The polypeptide, nucleic acid, expression vector, host cell, recombinant cell, or pharmaceutical composition of any embodiment may be used for various purposes, including but not limited to treating and/or detecting $\alpha\beta8(+)$ tumors or immune cells in vivo; blocking $\alpha\beta8$ -mediated TGF- β signaling in vitro or in vivo; use in combination immuno-oncology therapeutic regimens that include immune checkpoint blockade (e.g., anti-PD1 or anti-PD-L1), chemotherapy, and/or radiotherapy; and treating pulmonary fibrosis such as Idiopathic Pulmonary Fibrosis (IPF) or any other fibrotic tissue pathology such as in cancer or inflammatory and autoimmune diseases.

In one embodiment, the disclosure provides methods for treating or inhibiting an $\alpha\beta8(+)$ tumor, comprising administering to a subject in need thereof an amount of the polypeptide, nucleic acid, expression vector, host cell, and/or pharmaceutical composition of any embodiment or combination of embodiments disclosed herein effective to treat or inhibit the tumor in the subject. The methods can be used to treat any suitable tumor, including but not limited to colorectal cancer, glioblastoma, and melanoma. See, for example, Liu et al.,

Pharmacology and Therapeutics, Volume 247, July 2023, 108458, which is incorporated herein by reference in its entirety.

The polypeptides, fusion proteins, nucleic acids, expression vectors, and/or host cells made be administered as the sole therapeutic agent, or may be administered together with
 5 (i.e.: combined or separately) one or more other therapeutic agents, including but not limited to tumor resection, chemotherapy, radiation therapy, and immunotherapy (such as checkpoint inhibitors). In various non-limiting embodiments, other agents that may be administered in the methods of the disclosure for treating cancer include angiogenesis inhibitors (including but not limited to axitinib, bevacizumab, cabozantinib, everolimus, lenalidomide, lenvatinib
 10 mesylate, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, thalidomide, vandetanib, and ziv-aflibercept), immune checkpoint inhibitors (including, but not limited to, pembrolizumab, nivolumab, and cemiplimab as anti-PD-1 antibodies, ipilimumab as an anti-CTLA-4 antibody, and atezolizumab, avelumab, and durvalumab as anti-PD-L1 antibodies), and other cancer growth inhibitors including but not limited to tyrosine kinase inhibitors
 15 (including but not limited to alectinib, brigatinib, ceritinib, crizotinib, entrectinib, lorlatinib, ALK, I, bosutinib, dasatinib, imatinib, nilotinib, ponatinib, afatinib, dacomitinib, erlotinib, gefitinib, lapatinib, neratinib, osimertinib, vandetanib, gilteritinib, midostaurin, erdafitinib, ruxolitinib, larotrectinib, axitinib, carbozantinib, lenvatinib, pazopanib, regorafenib, sorafenib, sunitinib, dabrafenib, encorafenib, vemurafenib, acalabrutinib, ibrutinib,
 20 binimetinib, cobimetinib, trametinib, abemaciclib, palbociclib, or ribociclib), proteasome inhibitors (including but not limited to orteomib, carfizomib, ixazomib, delanzomib, oprozomib, and marizomib), mTOR inhibitors (including but not limited to everolimus, sirolimus, temsirolimus, everolimus, sirolimus, sirolimus protein-bound, and everolimus), PI3K inhibitors (including but not limited to copanlisib, alpelisib, idelalisib, duvelisib and
 25 umbralisib), histone deacetylase inhibitors (including but not limited to vorinostat, romidepsin, panobinostat, and belinostat), and Hedgehog pathway blockers (including but not limited to vismodegib, sonidegib, and glasdegib).

In various embodiments, the methods for treating or inhibiting tumors comprise also administering immune checkpoint inhibitors (including, but not limited to, pembrolizumab,
 30 nivolumab, and cemiplimab as anti-PD-1 antibodies, ipilimumab as an anti-CTLA-4 antibody, and atezolizumab, avelumab, and durvalumab as anti-PD-L1 antibodies, and/or radiotherapy.

In another embodiment, the disclosure provides methods for detecting an $\alpha\beta8(+)$ tumor or immune cell population, comprising administering to a subject suspected of having

an $\alpha\beta8(+)$ tumor or immune cell population an amount of the polypeptide of any embodiment or combination of embodiments disclosed herein effective to detect the tumor in the subject. Such methods can be used, for example, in fluorescence-activated cell sorting (FACS) or in vivo imaging studies of $\alpha\beta8(+)$ tumor or immune cell populations. Binding of
5 the polypeptide to the $\alpha\beta8(+)$ tumor or immune cell population can be detected via standard methods.

In another embodiment, the disclosure provides methods for treating or inhibiting tissue fibrosis, comprising administering to a subject in need thereof an amount of the polypeptide, nucleic acid, expression vector, host cell, and/or pharmaceutical composition of
10 any embodiment or combination of embodiments disclosed herein effective to treat or inhibit the tissue fibrosis in the subject. The methods may be used to treat any tissue fibrosis, including but not limited to pulmonary fibrosis (for example, to treat or inhibit chronic obstructive pulmonary disease and asthma, or to treat or inhibit Idiopathic Pulmonary Fibrosis (IPF)), liver fibrosis (for example, liver fibrosis resulting from colorectal carcinoma),
15 and/or renal fibrosis (see, for example, McCarthy, J Cell Sci (2020) 133 (12): jcs239434.

As used herein, "treat" or "treating" means accomplishing one or more of the following: (a) reducing the severity of the disorder; (b) limiting or preventing development of symptoms characteristic of the disorder(s) being treated; (c) inhibiting worsening of symptoms characteristic of the disorder(s) being treated; (d) limiting or preventing recurrence
20 of the disorder(s) in patients that have previously had the disorder(s); and (e) limiting or preventing recurrence of symptoms in patients that were previously symptomatic for the disorder(s).

As used herein, "inhibit" or "inhibiting" means reducing the amount, rate, and/or rate of increase of what is being inhibited (for example, metastasis or fibrosis).

25 When the method comprises treating cancer, "treat" or "treating" means accomplishing one or more of the following: (a) reducing the size or volume of tumors and/or metastases in the subject; (b) limiting any increase in the size or volume of tumors and/or metastases in the subject; (c) increasing survival; (d) reducing the severity of symptoms associated with cancer; (e) limiting or preventing development of symptoms associated with
30 cancer; and (f) inhibiting worsening of symptoms associated with cancer.

The subject may be any subject that has a relevant disorder. In one embodiment, the subject is a mammal, including but not limited to humans, dogs, cats, horses, cattle, etc.

As used herein, an "effective" amount refers to an amount of the polypeptide, fusion protein, nucleic acid, expression vector, and/or host cell that is effective for treating the

disorder. The polypeptides, fusion proteins nucleic acids, expression vectors, and/or host cells are typically formulated as a pharmaceutical composition, such as those disclosed above, and can be administered via any suitable route, including but not limited to orally, by inhalation spray, ocularly, intravenously, subcutaneously, intraperitoneally, and
 5 intravesicularly in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles.

Any suitable dosage range may be used as determined by attending medical personnel. Dosage regimens can be adjusted to provide the optimum desired response. A suitable dosage range for the polypeptides or fusion proteins may, for instance, be 0.1 ug/kg-
 10 100 mg/kg body weight; alternatively, it may be 0.5 ug/kg to 50 mg/kg; 1 ug/kg to 25 mg/kg, or 5 ug/kg to 10 mg/kg body weight. In some embodiments, the recommended dose could be lower than 0.1 mcg/kg, especially if administered locally (such as by intra-tumoral injection). In other embodiments, the recommended dose could be based on weight/m² (i.e. body surface area), and/or it could be administered at a fixed dose (e.g., .05-100 mg). The polypeptides,
 15 fusion proteins, nucleic acids, expression vectors, and/or host cells can be delivered in a single bolus, or may be administered more than once (e.g., 2, 3, 4, 5, or more times) as determined by an attending physician.

The polypeptides, fusion proteins, nucleic acids, expression vectors, and/or host cells made be administered as the sole therapeutic agent, or may be administered together with
 20 (i.e.: combined or separately) one or more other therapeutic agents, including but not limited to tumor resection, chemotherapy, radiation therapy, and immunotherapy (such as checkpoint inhibitors). In various non-limiting embodiments, other agents that may be administered in the methods of the disclosure for treating cancer include angiogenesis inhibitors (including but not limited to axitinib, bevacizumab, cabozantinib, everolimus, lenalidomide, lenvatinib
 25 mesylate, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, thalidomide, vandetanib, and ziv-aflibercept), immune checkpoint inhibitors (including, but not limited to, pembrolizumab, nivolumab, and cemiplimab as anti-PD-1 antibodies, ipilimumab as an anti-CTLA-4 antibody, and atezolizumab, avelumab, and durvalumab as anti-PD-L1 antibodies), and other cancer growth inhibitors including but not limited to tyrosine kinase inhibitors
 30 (including but not limited to alectinib, brigatinib, ceritinib, crizotinib, entrectinib, lorlatinib, ALK, I, bosutinib, dasatinib, imatinib, nilotinib, ponatinib, afatinib, dacomitinib, erlotinib, gefitinib, lapatinib, neratinib, osimertinib, vandetanib, gilteritinib, midostaurin, erdafitinib, ruxolitinib, larotrectinib, axitinib, carbozantinib, lenvatinib, pazopanib, regorafenib, sorafenib, sunitinib, dabrafenib, encorafenib, vemurafenib, acalabrutinib, ibrutinib,

binimetinib, cobimetinib, trametinib, abemaciclib, palbociclib, or ribociclib), proteasome inhibitors (including but not limited to orteomib, carfizomib, ixazomib, delanzomib, oprozomib, and marizomib), mTOR inhibitors (including but not limited to everolimus, sirolimus, temsirolimus, everolimus, sirolimus, sirolimus protein-bound, and everolimus),
5 PI3K inhibitors (including but not limited to copanlisib, alpelisib, idelalisib, duvelisib and umbralisib), histone deacetylase inhibitors (including but not limited to vorinostat, romidepsin, panobinostat, and belinostat), and Hedgehog pathway blockers (including but not limited to vismodegib, sonidegib, and glasdegib).

10 Examples

Abstract

The RGD (Arg-Gly-Asp)-binding integrins $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 8$ are clinically validated cancer and fibrosis targets of considerable therapeutic importance. Compounds that can
15 discriminate between the two closely related integrin proteins and other RGD integrins, stabilize specific conformational states, and have sufficient stability enabling tissue restricted administration could have considerable therapeutic utility. Existing small molecules and antibody inhibitors do not have all of these properties, and hence there is a need for new approaches. Here a method is described for computationally designing hyperstable RGD-
20 containing miniproteins that are highly selective for a single RGD integrin heterodimer and conformational state, and use this strategy to design inhibitors of $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 8$ with high selectivity. The $\alpha\text{v}\beta 8$ inhibitors have picomolar affinities for their target, and >1000-fold selectivity over other RGD integrins. CryoEM structures are within 0.6-0.7Å root-mean-square deviation (RMSD) to the computational design models, and the $\alpha\text{v}\beta 8$ inhibitor
25 maintains the constitutively fixed extended-closed $\alpha\text{v}\beta 8$ conformation.

Binding data was collected on an Octet RED96 (Forte Bio) and processed using the instrument's software. His Tagged and avi-tagged protein binders were immobilized on Ni-NTA and streptavidin sensor tips. The tips were then dipped into wells containing different concentrations of $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 8$. Association and dissociation steps were recorded for 900 s
30 and 1200 s respectively. Dissociation constant (KD) was calculated using ForteBio software. An empty sensor with no loaded binding protein was included to discard any non-specific binding of $\alpha\text{v}\beta 6$ to the octet tip.

Computational Design Strategy:

To overcome the limitations of integrin-targeted small molecules and antibodies, a computational approach was developed that generates small (<75 amino acids) hyperstable *de novo* integrin binding proteins that have high integrin selectivity and specific receptor binding interfaces optimal for treating disease. Integrin $\alpha\beta6$ and $\alpha\beta8$ both bind to a RGD $LXX(L/I)$ (SEQ ID NO: 7) motif in the pro-domains of L-TGF- $\beta1$ and $\beta3$ with low nM affinity (Fig. 1a).^{1,2} As in other structures of RGD-containing peptides bound to integrins, the arginine and aspartate side chains make multiple hydrogen bond and salt-bridge interactions to residues at the interface between the integrin alpha and beta subunits (Fig. 1b). For both $\alpha\beta6$ and $\alpha\beta8$, C-terminal to the RGD, the peptide adopts an alpha-helix-like turn with two leucines (or Ile for $\beta8$) fitting into a hydrophobic pocket formed by a $\beta6/\beta8$ subunit specificity determining loop 2 (SDL2, Figure 1c, 1d).¹⁻³ In the unliganded state, SDL2 of $\alpha\beta6$ is ordered with multiple backbone hydrogen bonds (PDB ID 4UM8), whereas SDL2 of unliganded $\alpha\beta8$ is mainly flexible.¹⁻³ To engineer selectivity, two main areas on the β subunit were assessed that differ between the two targets: the region that contacts the $LXX(L/I)$ (SEQ ID NO: 8) motif in the L-TGF- $\beta3$ peptide (Fig. 1c, 1d) and a charge reversal on the β subunit (Fig. 1e). There are several key differences in the hydrophobic packing pattern of $LXX(L/I)$ (SEQ ID NO: 8) motif and SDL2 of $\beta6$ compared to $\beta8$ (Fig. 1d, 1d, 1h). Y185 from SDL2 of $\alpha\beta6$ packs optimally with Leu ($LXX(L/I)$ (SEQ ID NO: 8), L247) of the L-TGF- $\beta3$ peptide (PDB ID 4UM9, Fig. 1c), while the equivalent position on the SDL2 of $\alpha\beta8$ (L174) packs much less tightly with Ile ($LXX(L/I)$ (SEQ ID NO: 8), I221) of L-TGF- $\beta1$ (PDB ID 6OM2, Fig. 1d). There is also a key charge reversal on the β subunit; $\beta8$ contains K304 whereas the equivalent position on $\beta6$ is E316 (Fig. 1e). It was hypothesized that minibinders interacting with the Y185/L174 and E316/K304 regions of $\alpha\beta6$ and $\alpha\beta8$, respectively, might be able to achieve selectivity between the two proteins.

Small α/β ferredoxin folds (Fig. 1f) were able to scaffold the RGD $LXX(L/I)$ (SEQ ID NO: 7) binding loop without clashing with the integrin while making additional contacts with both α and β subunit (α - and β -loop respectively, Fig. 1f).

To achieve selectivity for the $\beta8$ subunit, the β -loop was redesigned to take advantage of the K304 charge reversal on the β subunit (Fig. 1g). We generated 200 models with different lengths and conformations of the β -loop using RosettaRemodel⁴ and the resulting models were superimposed on the L-TGF- $\beta1$ / $\alpha\beta8$ complex structure (PDB ID 6OM2) by superposition on the RGD peptide (Fig. 1g). The packing of the L-TGF- $\beta1$ $LXX(L/I)$ (SEQ ID NO: 8) motif with SDL2 of $\alpha\beta8$ integrin is suboptimal (Fig. 1d), it was hypothesized that a minibinder mimicking this interaction would be able to accommodate bulkier residues at

these positions, giving additional selectivity. Both the β -loop and LXX(L/I) (SEQ ID NO: 8) motif were redesigned using Rosetta and a total of 9 designs with lowest predicted binding energy were selected following structure prediction using AlphaFold.⁵

Five out of 9 designs showed preferential binding to $\alpha v\beta 8$ integrin with B8_BP_dslf (SEQ ID NO:2) showing the highest affinity and selectivity towards $\alpha v\beta 8$ (Fig 2a). These five designs are: avb8_#2 (SEQ ID NO. 1), avb8_#3 (SEQ ID NO. 3), avb8_#5 (SEQ ID NO. 4), avb8_#6 (SEQ ID NO. 5), and avb8_#9 (SEQ ID NO. 6) (Fig. 2a and Table 2). As shown in Fig. 2a, only these five designs have two populations in the top right quadrant of their flow cytometry panels. avb8_#3 shows the highest binding affinity to $\alpha v\beta 8$ in the presence and absence of $\alpha v\beta 6$ competition, and the remaining four designs show lower binding affinity to $\alpha v\beta 8$ (Fig. 2a). Qualitative $\alpha v\beta 8$ binding affinity for these five designs is listed as a column in Table 2. Mutations from the B8_BP_dslf (SEQ ID NO:2) sequence motif MAVY (SEQ ID NO: 14) to LATI (SEQ ID NO: 10) (present in avb8_#12, which corresponds to the L-TGF- $\beta 1$ peptide sequence) completely abrogated selectivity towards $\alpha v\beta 8$ on the yeast surface displayed design (Fig. 2b), indicating these residues are critical for selectivity against $\alpha v\beta 8$ vs. $\alpha v\beta 6$. B8_BP_dslf is a monomeric and hyperstable protein when expressed in *E. coli* and binds to human $\alpha v\beta 8$ with 1.9 nM affinity, with no appreciable binding to human $\alpha v\beta 6$ up to 1 μ M (**Fig. 3a**). In agreement with yeast surface display data, purified avb8_12 with the reversion mutations loses selectivity towards $\alpha v\beta 8$ and binds to $\alpha v\beta 6$ with a Kd of 1.13 nM (**Fig. 3a**), confirming the importance of the LXX(L/I) (SEQ ID NO: 8) motif for selectivity. B8_BP_dslf has a MAVY (SEQ ID NO: 14) motif that packs against SDL2 of $\alpha v\beta 8$. Each position within the LATI (SEQ ID NO: 10) motif was systematically varied to determine which residue plays a critical role in determining selectivity (**Fig. 3b**). A single mutation was found containing the LATY (SEQ ID NO: 9) (B8_BP-LATY; SEQ ID NO:3) motif binds to $\alpha v\beta 8$ with an affinity of 500 pM with no appreciable binding to $\alpha v\beta 6$ at 500 nM concentration (**Fig. 3b**).

Selectivity profiles of B8_BP_dslf binder

The potency and selectivity of the designed $\alpha v\beta 6$ and $\alpha v\beta 8$ minibinders were compared to the small-molecule dual $\alpha v\beta 6/\alpha v\beta 1$ inhibitor (PLN-74809) currently in clinical trials as an oral IPF therapy,⁶ by assessing their ability to outcompete binding of hLAP1, the endogenous ligand of $\alpha v\beta 6$ and $\alpha v\beta 8$. For $\alpha v\beta 6$ integrin, BP2_disulf had the lowest IC₅₀ (1.84 nM), followed by BP1_disulf (dual inhibitor, 3.79 nM), PLN-74809 (4.36 nM), and no significant binding of B8_BP_dslf to $\alpha v\beta 6$ integrin was detected (Fig. 4a, 4c). For $\alpha v\beta 8$,

B8_BP_dslf outcompeted hLAP₁ with the lowest IC₅₀ (2.68 nM), followed in order of potency by BP1_disulf (8.48 nM), PLN-74809, and BP2_disulf (Fig. 4b, 4c). Taken together, these data confirm that B8_BP_dslf has exquisite selectivity and affinity for αvβ8 integrin.

5 CryoEM model of αvβ8 - B8_BP_dslf complex

αvβ8 has been shown to bind and activate L-TGF-β while exclusively occupying the extended-closed conformation.³ B8_BP_dslf does not have an effect on the global conformation of αvβ8 and the headpiece remains closed (Fig. 5a).

The cryoEM model of αvβ8 - B8_BP_dslf complex is also very close to the computational design model (Fig. 5b, 5c and 5d, complex RMSD 0.7 Å). In the cryoEM model of the αvβ8 - B8_BP_dslf complex, there are 12 interacting pairs of residues between integrin and the minibinder; as in the design model, the α-loop interacts with αv, β-loop with β8, and the RGD loop spans the two subunits (Fig. 3e). Y172 of the β8-SDL2 loops bends inward to form a hydrophobic patch similar to the conformation in L-TGF-β-bound structures (Fig. 5f).³ Altering the known binding motif LXX(L/I) (SEQ ID NO: 8) to LATY (SEQ ID NO: 9) was shown to confer selectivity for αvβ8 (Fig. 3b) and the cryoEM structure reveals the molecular basis for this selectivity: Y16 forms stabilizing interactions with A115 of the β8 subunit and interacts with the less bulky L174 in the β8-SDL2 loop (Fig. 5f). The equivalent position in the β6-SDL2 loop, Y185, is too bulky and it is hypothesized that the steric clash would interfere with binding.

Referencecs

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- 25 2. Wang, J., Su, Y., Iacob, R. E., Engen, J. R. & Springer, T. A. General structural features that regulate integrin affinity revealed by atypical αVβ8. *Nat. Commun.* **10**, 5481 (2019).
3. Campbell, M. G. *et al.* Cryo-EM Reveals Integrin-Mediated TGF-β Activation without Release from Latent TGF-β. *Cell* **180**, 490–501.e16 (2020).
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We claim

1. An alpha(v) beta (8) integrin ($\alpha v\beta 8$)-selective binding polypeptide comprising or consisting of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%,
5 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from SEQ ID NO: 1-6, not including any amino acid insertions, wherein the polypeptide selectively binds to $\alpha v\beta 8$, and wherein:
 - (a) residues 10-12 relative to the reference sequence are RGD
 - (b) residue 13 relative to the reference sequence is F, M, or L;
 - 10 (c) residue 16 relative to the reference sequence is Y or V; and
 - (d) residue 40 relative to the reference sequence is D, E, or P.
2. The polypeptide of claim 1, wherein residue 41 relative to the reference sequence is D, E, Y, or N.
- 15 3. The polypeptide of claim 1 or 2, wherein residue 15 relative to the reference sequence is W, V, T, N, K, or Q.
4. The polypeptide of any one of claims 1-3, wherein residue 16 relative to the reference
20 sequence is Y.
5. The polypeptide of any one of claims 1-4, wherein, relative to the reference sequence, one of the following is true:
 - (a) relative to the reference sequence, residue 13 is F, residue 15 is W, residue 16
25 is V, residue 40 is D, and residue 41 is Y;
 - (b) relative to the reference sequence, residue 13 is M, residue 15 is V, residue 16 is Y, residue 40 is D, and residue 41 is E;
 - (c) relative to the reference sequence, residue 13 is L, residue 15 is T, residue 16 is Y, residue 40 is D, and residue 41 is E;
 - 30 (d) relative to the reference sequence, residue 13 is M, residue 15 is N, residue 16 is Y, residue 40 is P, and residue 41 is N;
 - (e) relative to the reference sequence, residue 13 is F, residue 15 is K, residue 16 is Y, residue 40 is E, and residue 41 is E; or

(f) relative to the reference sequence, residue 13 is M, residue 15 is Q, residue 16 is Y, residue 40 is P, and residue 41 is N.

6. The polypeptide of any one of claims 1-4, wherein, relative to the reference sequence,
5 one of the following is true:

(a) relative to the reference sequence, residue 13 is M, residue 15 is V, residue 16 is Y, residue 40 is D, and residue 41 is E; or

(b) relative to the reference sequence, residue 13 is L, residue 15 is T, residue 16 is Y, residue 40 is D, and residue 41 is E.

10

7. The polypeptide of any one of claims 1-6, wherein residue 13, relative to the reference sequence, is M or L.

8. The polypeptide of any one of claims 1-7, wherein residue 15, relative to the reference
15 sequence, is T or V.

9. The polypeptide of any one of claims 1-8, wherein residues 13-16, relative to the reference sequence, are LATY (SEQ ID NO:9).

20 10. The polypeptide of any one of claims 1-8, wherein residues 13-16, relative to the reference sequence, are MAVY (SEQ ID NO:14).

11. The polypeptide of any one of claims 1-6, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or all 15 of residues 13-16, 39-42, and 63-69, relative to the reference sequence, are
25 identical to the reference sequence.

12. The polypeptide of any one of claims 1-11, comprising or consisting of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from
30 SEQ ID NO:2-3, not including any amino acid insertions.

13. The polypeptide of any one of claims 1-12, wherein amino acid changes from the reference protein are conservative amino acid substitutions.

14. The polypeptide of any one of claims 1-12, wherein amino acid substitutions relative to the reference sequence are selected from the amino acid substitutions in Table 2.
15. The polypeptide of any one of claims 1-14, further comprising one or more additional
5 functional domains added at the N-terminus, the C-terminus of the polypeptide, and/or one or more insertion sites selected from residues 28-31, 38, 48, and 50-52 relative to the reference sequence.
16. The polypeptide of any one of claims 1-15, wherein the polypeptide binds to the
10 human $\alpha v \beta 8$ integrin with a picomolar affinity (K_d) that is at least 1,000-fold higher affinity than the affinity at which the polypeptide binds to any one of another human RGD integrins selected from the group consisting of $\alpha v \beta 1$, $\alpha v \beta 3$, $\alpha v \beta 5$, $\alpha v \beta 6$, $\alpha 5 \beta 1$, $\alpha 8 \beta 1$, and $\alpha IIb \beta 3$.
17. A nucleic acid encoding the polypeptide of any one of claims 1-15.
15
18. An expression vector comprising the nucleic acid of claim 17 operatively linked to a control sequence.
19. A host cell comprising the nucleic acid of claim 17 and/or the expression vector of
20 claim 18.
20. A recombinant cell expressing the polypeptide of any one of claims 1-16.
21. A pharmaceutical composition comprising:
25 (a) the polypeptide, nucleic acid, expression vector, host cell, or recombinant cell of any one of claims 1-20; and
(b) a pharmaceutically acceptable carrier.
22. A method for treating or inhibiting an $\alpha v \beta 8(+)$ tumor, comprising administering to a
30 subject in need thereof an amount of the polypeptide, nucleic acid, expression vector, host cell, and/or pharmaceutical composition of any one of claims 1-21 effective to treat or inhibit the tumor in the subject.

23. The method of claim 22, wherein the method further comprises administering to the subject one or more other therapeutic agents selected from the group consisting of radiation therapy, an angiogenesis inhibitor, a protein kinase inhibitor, a proteasome inhibitor, an immune checkpoint inhibitor, an mTOR inhibitor, a PI3K inhibitor, a histone deacetylase inhibitor, and a Hedgehog pathway blocker.

24. The method of claim 23, wherein the angiogenesis inhibitors is selected from the group consisting of axitinib, bevacizumab, cabozantinib, everolimus, lenalidomide, lenvatinib mesylate, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, thalidomide, vandetanib, and ziv-aflibercept; the immune checkpoint inhibitor is selected from the group consisting of pembrolizumab, nivolumab, and cemiplimab as anti-PD-1 antibodies, ipilimumab as an anti-CTLA-4 antibody, and atezolizumab, avelumab, and durvalumab as anti-PD-L1 antibodies), the tyrosine kinase inhibitor is selected from the group consisting of alectinib, brigatinib, ceritinib, crizotinib, entrectinib, lorlatinib, ALK, I, bosutinib, dasatinib, imatinib, nilotinib, ponatinib, afatinib, dacomitinib, erlotinib, gefitinib, lapatinib, neratinib, osimertinib, vandetanib, gilteritinib, midostaurin, erdafitinib, ruxolitinib, larotrectinib, axitinib, carbozantinib, lenvatinib, pazopanib, regorafenib, sorafenib, sunitinib, dabrafenib, encorafenib, vemurafenib, acalabrutinib, ibrutinib, binimetinib, cobimetinib, trametinib, abemaciclib, palbociclib, or ribociclib, the proteasome inhibitor is selected from the group consisting of orteomib, carfizomib, ixazomib, delanzomib, oprozomib, and marizomib; the mTOR inhibitor is selected from the group consisting of everolimus, sirolimus, temsirolimus, everolimus, sirolimus, sirolimus protein-bound, and everolimus; the PI3K inhibitor is selected from the group consisting of copanlisib, alpelisib, idelalisib, duvelisib and umbralisib; the histone deacetylase inhibitor is selected from the group consisting of vorinostat, romidepsin, panobinostat, and belinostat; and/or the Hedgehog pathway blocker is selected from the group consisting of vismodegib, sonidegib, and glasdegib.

25. The method of any one of claims 22-24, wherein the tumor is selected from the group consisting of a colorectal tumor, a glioblastoma, and a melanoma

26. A method for detecting an $\alpha\beta8(+)$ tumor or immune cell population, comprising administering to a subject suspected of having an $\alpha\beta8(+)$ tumor or immune cell population an amount of the polypeptide of any one of claims 1-16 effective to detect the tumor in the subject.

27. A method for treating or inhibiting tissue fibrosis, comprising administering to a subject in need thereof an amount of the polypeptide, nucleic acid, expression vector, host cell, and/or pharmaceutical composition of any one of claims 1-21 effective to treat or inhibit the tissue fibrosis in the subject.

28. The method of claim 27, wherein the tissue fibrosis is selected from the group consisting of pulmonary fibrosis, liver fibrosis, and renal fibrosis.

29. The method of claim 28, wherein the tissue fibrosis comprises Idiopathic Pulmonary Fibrosis (IPF).

30. Use of the polypeptide, nucleic acid, expression vector, host cell, and/or pharmaceutical composition of any one of claims 1-21 for treating or inhibiting an $\alpha v \beta 8(+)$ tumor in a subject.

31. The use of claim 30, wherein the tumor is selected from the group consisting of a colorectal tumor, a glioblastoma, and a melanoma.

32. The use of claim 30 or 31, wherein the use further comprise use of one or more other therapeutic agents selected from the group consisting of radiation therapy, angiogenesis inhibitors, immune checkpoint inhibitors, mTOR inhibitors, PI3K inhibitors, histone deacetylase inhibitors, and Hedgehog pathway blockers

33. The use of claim 32, wherein the angiogenesis inhibitor is selected from the group consisting of axitinib, bevacizumab, cabozantinib, everolimus, lenalidomide, lenvatinib mesylate, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, thalidomide, vandetanib, and ziv-aflibercept; the immune checkpoint inhibitor is selected from the group consisting of pembrolizumab, nivolumab, and cemiplimab as anti-PD-1 antibodies, ipilimumab as an anti-CTLA-4 antibody, and atezolizumab, avelumab, and durvalumab as anti-PD-L1 antibodies), the tyrosine kinase inhibitor is selected from the group consisting of alectinib, brigatinib, ceritinib, crizotinib, entrectinib, lorlatinib, ALK, I, bosutinib, dasatinib, imatinib, nilotinib, ponatinib, afatinib, dacomitinib, erlotinib, gefitinib, lapatinib, neratinib, osimertinib, vandetanib, gilteritinib, midostaurin, erdafitinib, ruxolitinib, larotrectinib,

axitinib, carbozantinib, lenvatinib, pazopanib, regorafenib, sorafenib, sunitinib, dabrafenib, encorafenib, vemurafenib, acalabrutinib, ibrutinib, binimetinib, cobimetinib, trametinib, abemaciclib, palbociclib, or ribociclib, the proteasome inhibitor is selected from the group consisting of orteomib, carfizomib, ixazomib, delanzomib, oprozomib, and marizomib; the
 5 mTOR inhibitor is selected from the group consisting of everolimus, sirolimus, temsirolimus, everolimus, sirolimus, sirolimus protein-bound, and everolimus; the PI3K inhibitor is selected from the group consisting of copanlisib, alpelisib, idelalisib, duvelisib and umbralisib; the histone deacetylase inhibitor is selected from the group consisting of vorinostat, romidepsin, panobinostat, and belinostat; and/or the Hedgehog pathway blocker is
 10 selected from the group consisting of vismodegib, sonidegib, and glasdegib

34. Use of the polypeptide of any one of claims 1-16 for detecting an $\alpha v \beta 8(+)$ tumor or immune cell population.

15 35. Use of the polypeptide, nucleic acid, expression vector, host cell, and/or pharmaceutical composition of any one of claims 1-21 for treating or inhibiting tissue fibrosis.

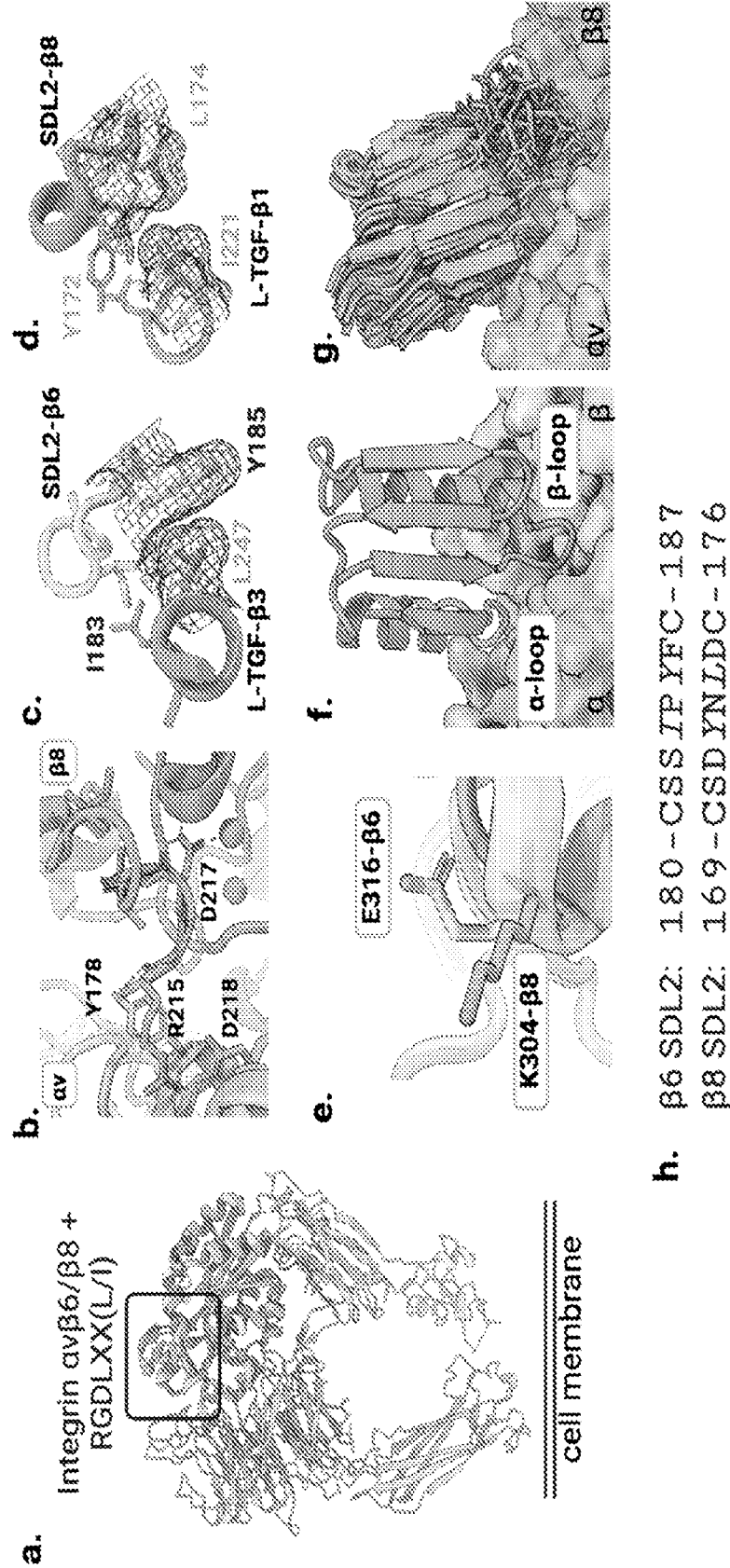
36. The use of claim 35, wherein the tissue fibrosis is selected from the group consisting
 20 of pulmonary fibrosis, liver fibrosis, and renal fibrosis.

37. The use of claim 36, wherein the tissue fibrosis comprises Idiopathic Pulmonary Fibrosis (IPF).

25

30

Figure 1



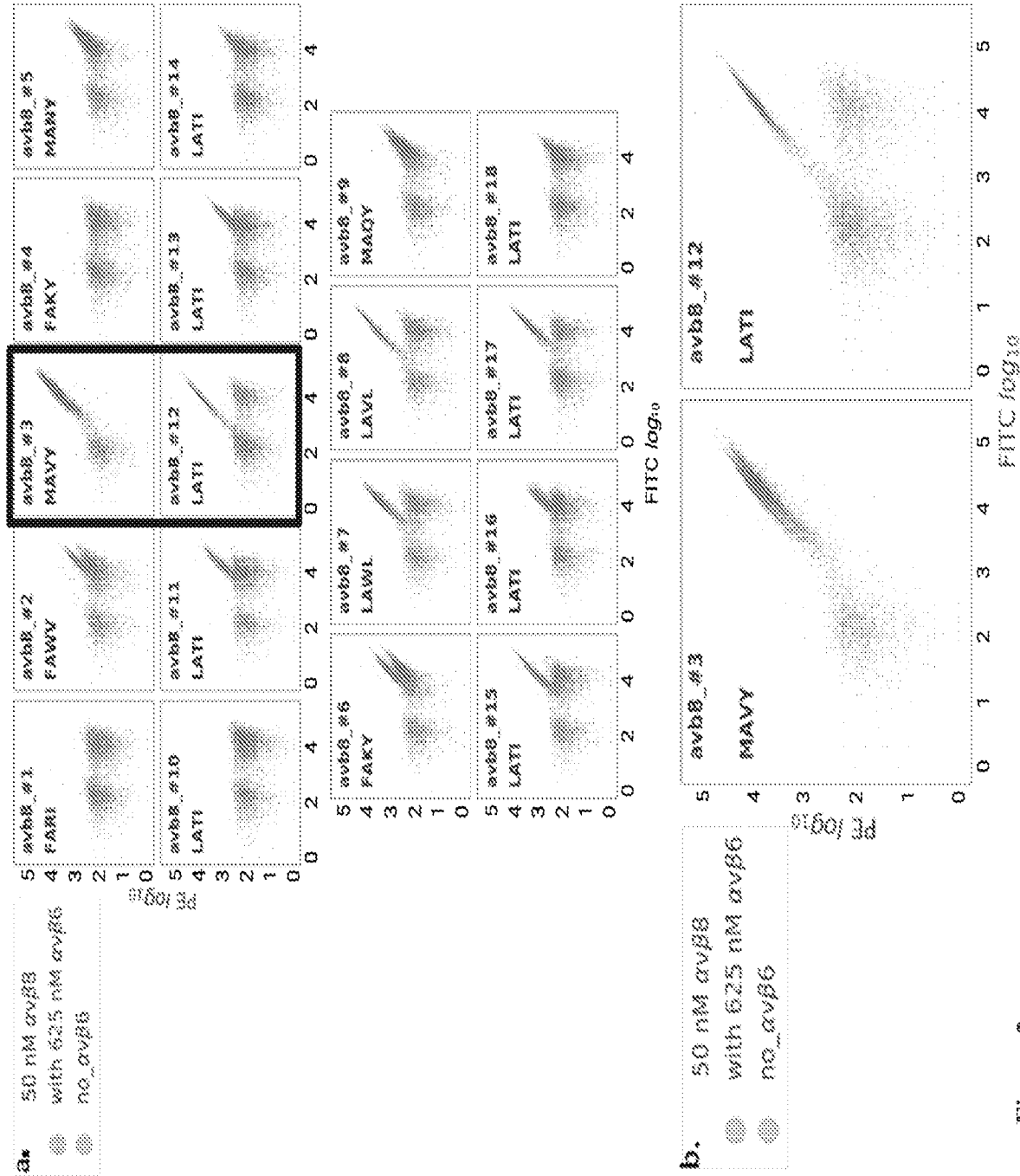


Figure 2

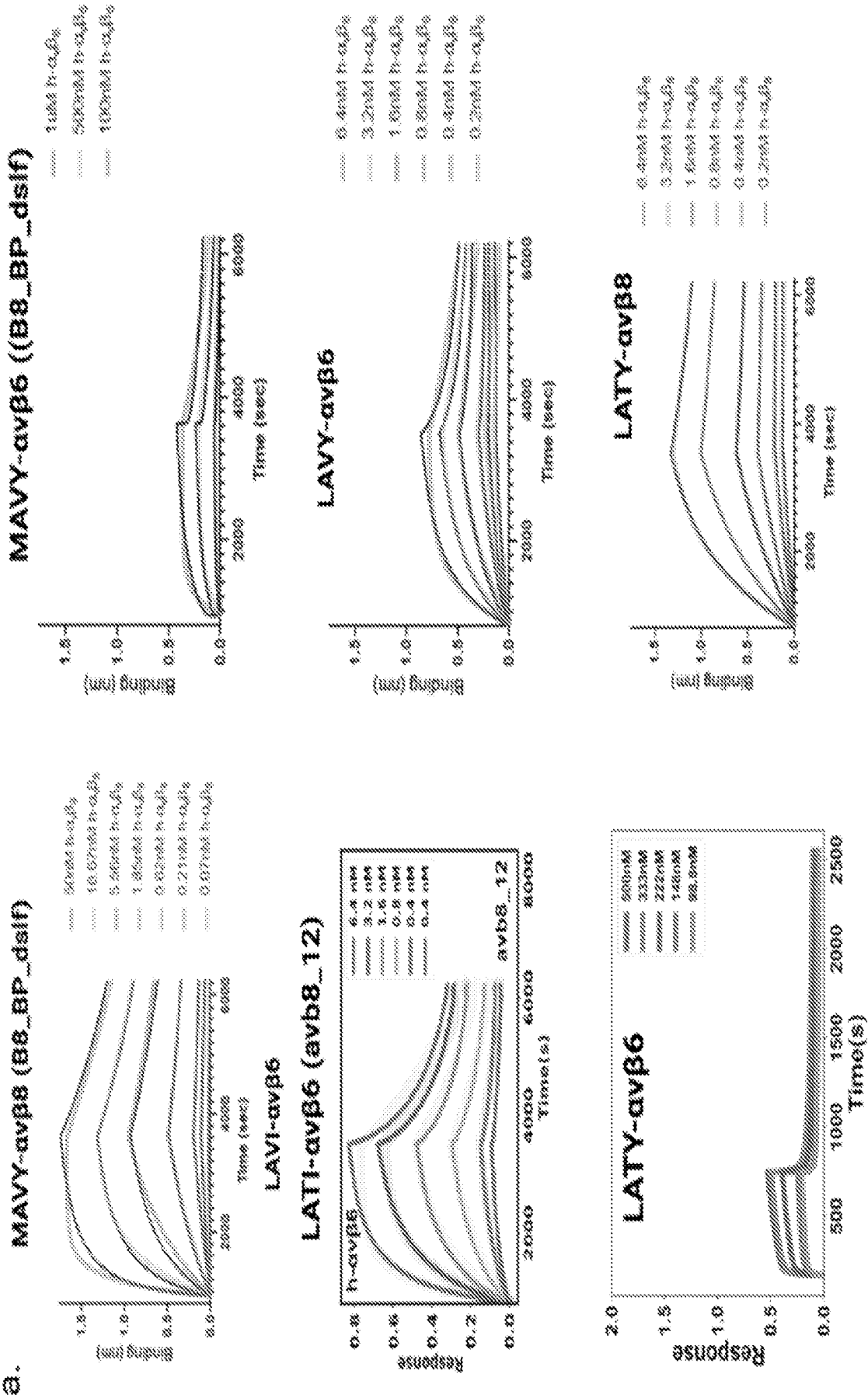


Figure 3

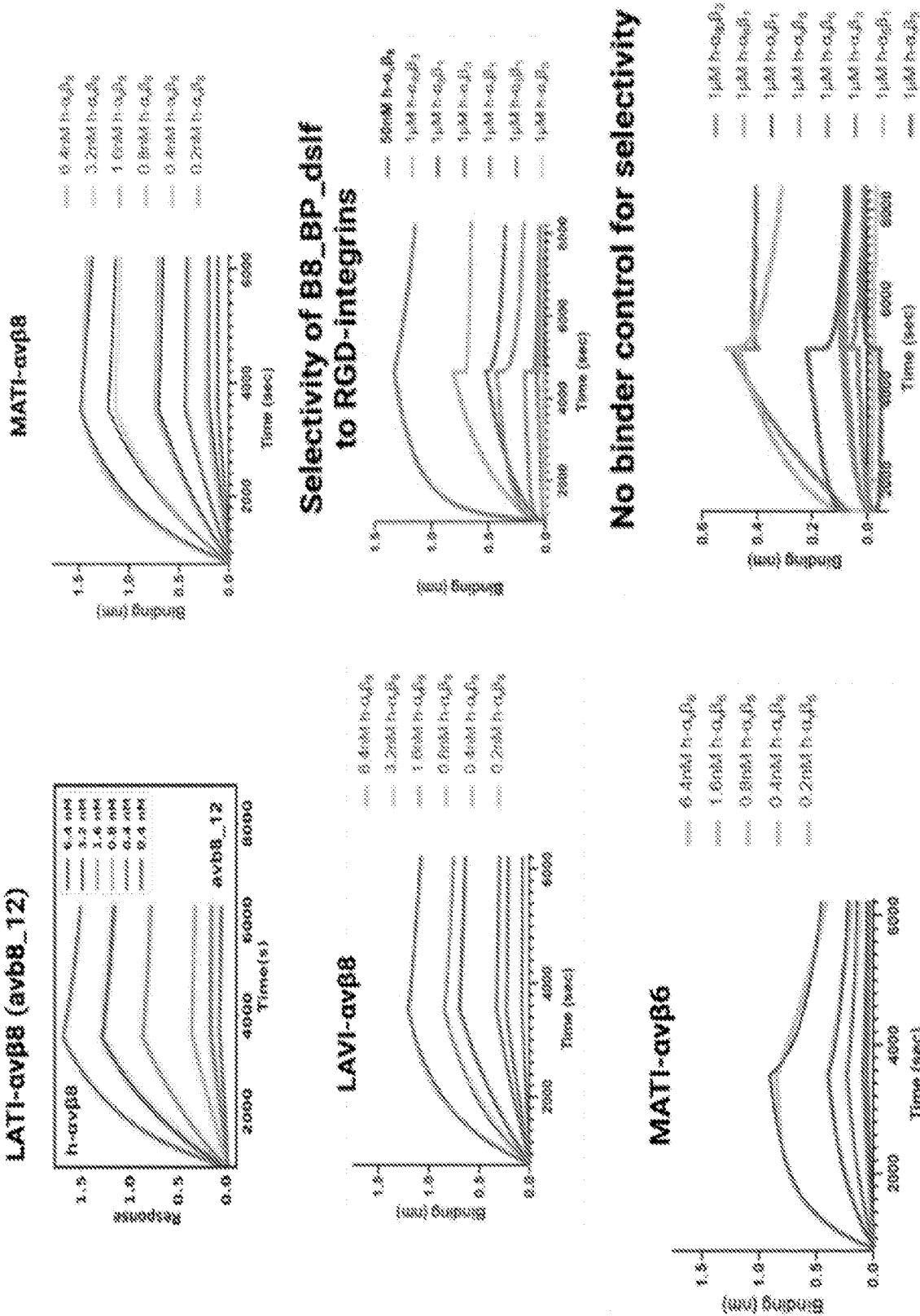
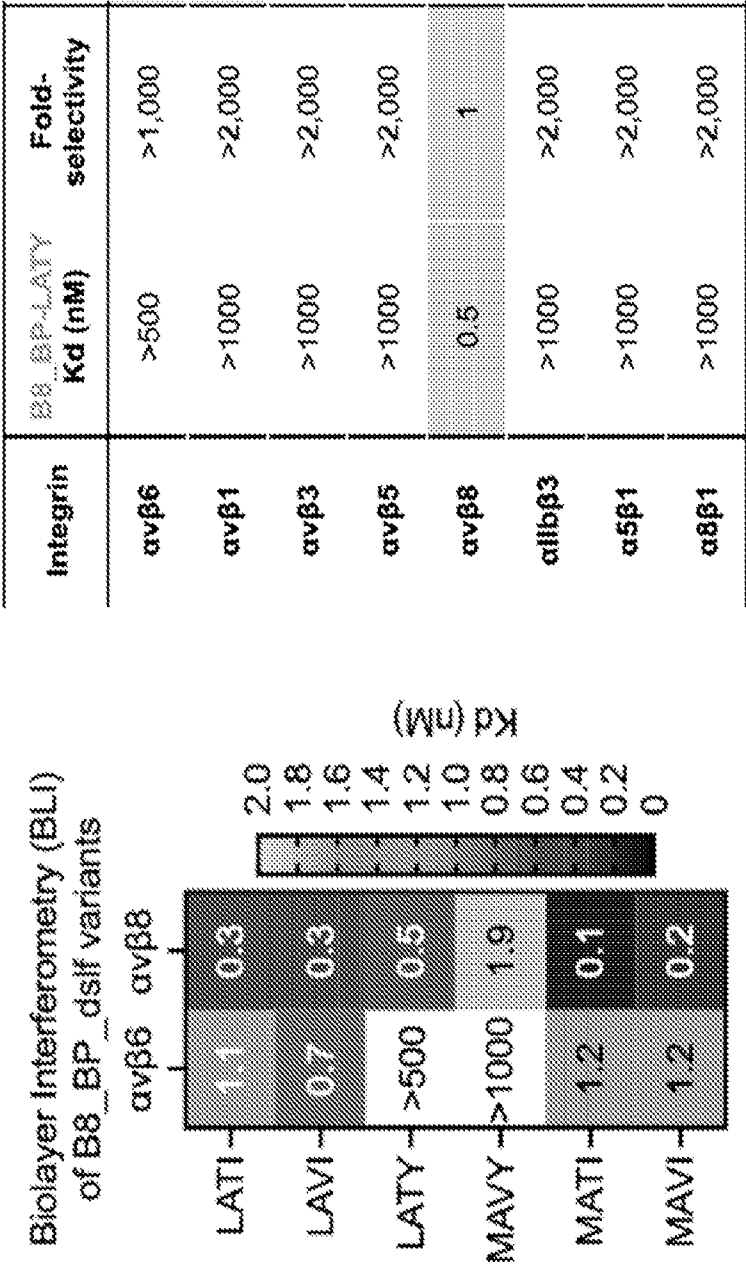


Figure 3 (Continued)

b.



Integrin	B8_Bp_dslf Kd (nM)	Fold-selectivity
$\alpha v\beta 6$	>500	>1,000
$\alpha v\beta 1$	>1000	>2,000
$\alpha v\beta 3$	>1000	>2,000
$\alpha v\beta 5$	>1000	>2,000
$\alpha v\beta 8$	0.5	1
$\alpha 11\beta 3$	>1000	>2,000
$\alpha 5\beta 1$	>1000	>2,000
$\alpha 8\beta 1$	>1000	>2,000

Figure 3 (Continued)

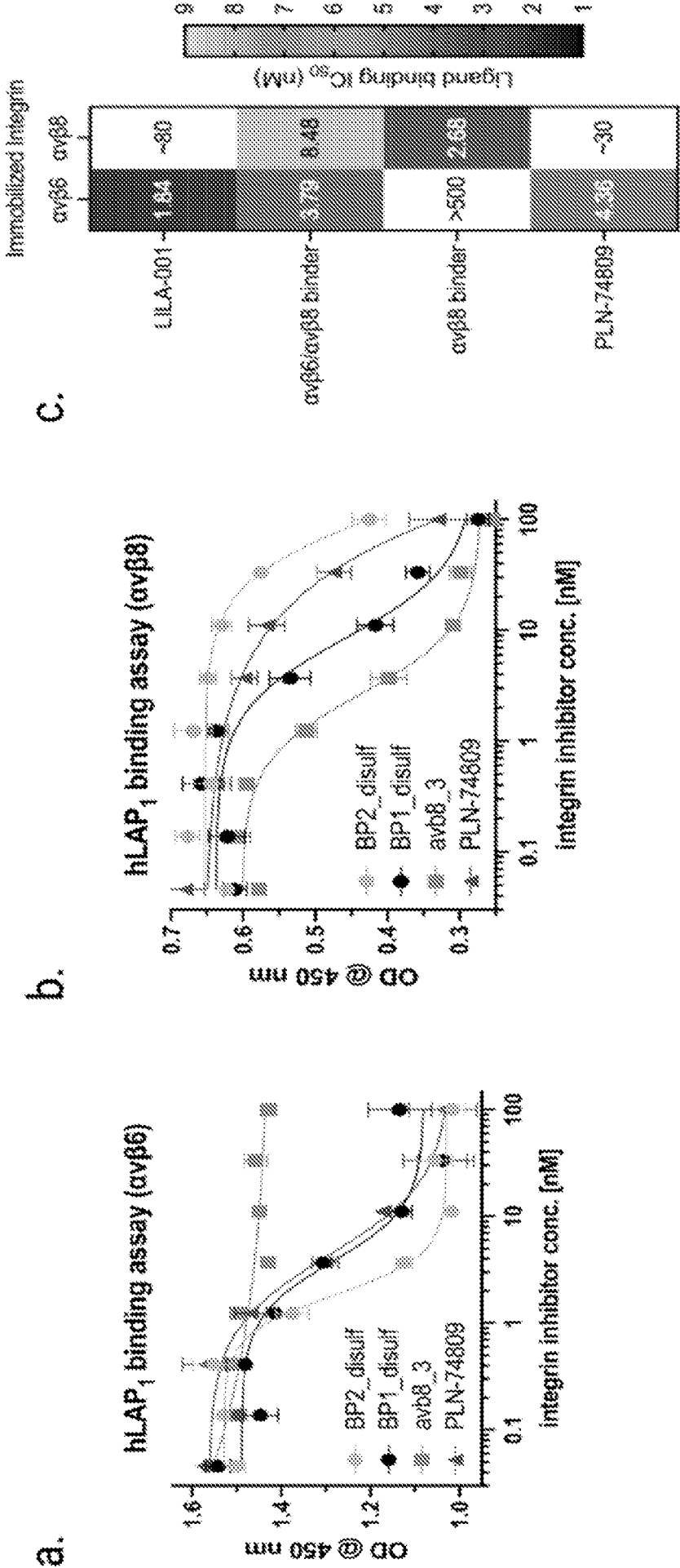


Figure 4

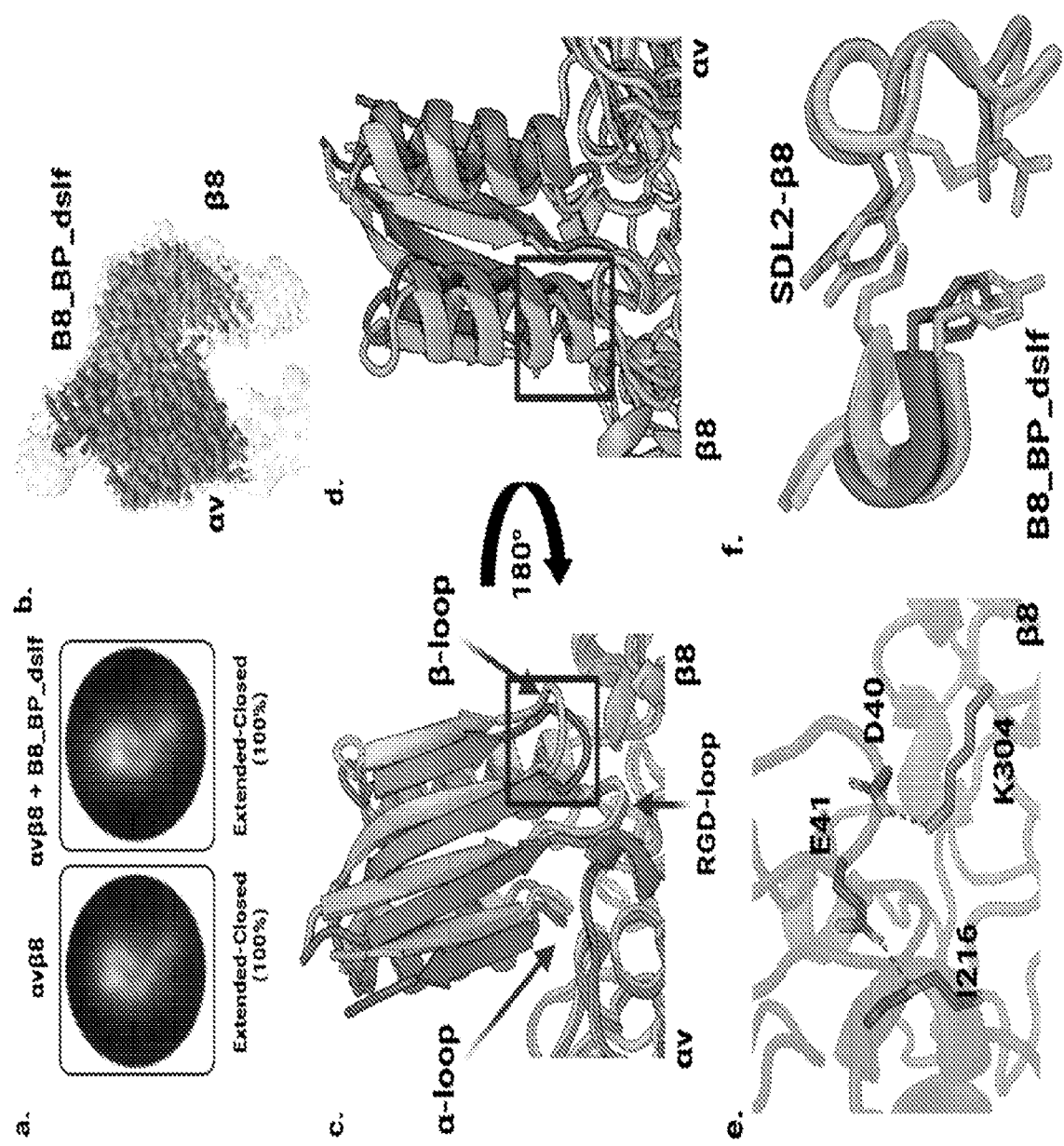


Figure 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/033514

A. CLASSIFICATION OF SUBJECT MATTERIPC: **C07K 14/705** (2024.01); **A61K 38/17** (2024.01); **A61P 35/00** (2024.01)CPC: **C07K 14/70546**; **A61K 38/1777**; **A61P 35/00**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2014/0271478 A1 (NISHIMURA et al.) 18 September 2014 (18.09.2014) entire document	1-3
A	WO 2021/081301 A1 (ROY et al.) 29 April 2021 (29.04.2021) entire document	1-3
A	US 2022/0348609 A1 (UNIVERSITY OF WASHINGTON et al.) 03 November 2022 (03.11.2022) entire document	1-3

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“D” document cited by the applicant in the international application

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

17 August 2024 (17.08.2024)

Date of mailing of the international search report

07 November 2024 (07.11.2024)

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/033514

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: **4-37**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-3 are drawn to alpha(v) beta (8) integrin ($\alpha\beta 8$)-selective binding polypeptides.

The first invention of Group I+ is restricted to an alpha(v) beta (8) integrin ($\alpha\beta 8$)-selective binding polypeptide comprising or consisting of an amino acid sequence selected to be SEQ ID NO: 1 wherein residues 10-12 are RGD, residue 13 is F, residue 16 is Y, and residue 40 is D. The first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. Specifically, the first named invention was selected based on the first listed element for each of the variables presented in the claims (claim 1). It is believed that claim 1-3 read on this first named invention and thus these claims will be searched without fee to the extent that they read on SEQ ID NO: 1 wherein residues 10-12 are RGD, residue 13 is F, residue 16 is Y, and residue 40 is D.

Applicant is invited to elect additional binding polypeptides, and their respective, corresponding, SEQ ID NOs to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be an alpha(v) beta (8) integrin ($\alpha\beta 8$)-selective binding polypeptide comprising or consisting of an amino acid sequence selected to be SEQ ID NO: 1 wherein residues 10-12 are RGD, residue 13 is M, residue 16 is V, and residue 40 is E. Additional antibody binding polypeptides, and their respective, corresponding, SEQ ID NOs will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element, requiring the selection of alternative binding polypeptides where "alpha(v) beta (8) integrin ($\alpha\beta 8$)-selective binding polypeptide comprising or consisting of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from SEQ

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

ID NO: 1-6, not including any amino acid insertions, wherein the polypeptide selectively binds to $\alpha v\beta 8$, and wherein: (a) residues 10-12 relative to the reference sequence are RGD (b) residue 13 relative to the reference sequence is F, M, or L; (c) residue 16 relative to the reference sequence is Y or V; and (d) residue 40 relative to the reference sequence is D, E, or P.”

Additionally, even if Groups I+ were considered to share the technical features of an alpha(v) beta (8) integrin ($\alpha v\beta 8$)-selective binding polypeptide comprising or consisting of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from SEQ ID NO: 1-6, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2014/0271478 A1 to Nishimura et al. (hereinafter, “Nishimura”) teaches an alpha(v) beta (8) integrin ($\alpha v\beta 8$)-selective binding polypeptide (Claim 1, isolated antibody that specifically binds $\alpha v\beta 8$).

Further, WO 2021/081301 A1 to Roy et al. (hereinafter, “Roy”) teaches a polypeptide comprising or consisting of an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from SEQ ID NO: 1-6 (Claim 19, polypeptide that is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence selected from the group consisting of SEQ ID NOS:4-30 and 36, wherein SEQ ID NO: 30 of Roy comprises 75.5% sequence identity to instant SEQ ID NO: 1).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-3**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.