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(54) Title: SYNERGISTIC TUMOR TREATMENT WITH IL-2 AND INTEGRIN-BINDING-FC-FUSION PROTEIN

(57) **Abrégé/Abstract:**

The present invention provides a method of treating cancer with a combination of IL- 2 and an integrin-binding-Fc fusion protein. The methods of the invention can be applied to a broad range of cancer types.



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- (54) Title: SYNERGISTIC TUMOR TREATMENT WITH IL-2 AND INTEGRIN-BINDING-FC-FUSION PROTEIN
- (57) Abstract: The present invention provides a method of treating cancer with a combination of IL- 2 and an integrin-binding-Fc fusion protein. The methods of the invention can be applied to a broad range of cancer types.

SYNERGISTIC TUMOR TREATMENT WITH IL-2 AND INTEGRIN-BINDING-FC FUSION PROTEIN

RELATED APPLICATIONS

This application claims the benefit of the priority date of U.S. Provisional Application No. 62/036554, which was filed on August 12, 2014. The content of this provisional application is hereby incorporated by reference in its entirety.

GOVERNMENT FUNDING

This invention was made with Government support under contract CA174795 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Interleukin-2 (IL-2) is a pleiotropic cytokine that activates and induces the proliferation of T cells and NK cells. Although IL-2 is an FDA approved therapy, systemic IL-2 treatment has significant toxicity and therefore the response rate of patients is less than 25%. Combining extended half-life IL-2 and an antibody against a tumor-specific antigen shows promising results for treatment. However, antibody-based therapies often suffer from the fact that many tumors lack known tumor-associated antigens.

Integrins are a family of extracellular matrix adhesion receptors that regulate a diverse array of cellular functions crucial to the initiation, progression and metastasis of solid tumors. The importance of integrins in tumor progression has made them an appealing target for cancer therapy and allows for the treatment of a variety of cancer types. The integrins present on cancerous cells include $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$. A variety of therapeutics have been developed to target individual integrins associated with cancer, including antibodies, linear peptides, cyclic peptides, and peptidomimetics. However, none have utilized small, structured peptide scaffolds or targeted more than two integrins simultaneously. Additionally, current integrin targeting drugs are given as a monotherapy. Novel combination therapies are needed to more effectively combat various cancers.

SUMMARY OF THE INVENTION

The present invention is based, in part, on the discovery that administration of IL-2 attached to a pharmacokinetic modifying group (hereafter referred to as “extended-pharmacokinetic (PK) IL-2”) and an integrin-binding-Fc fusion protein provides synergistic tumor control and prolongs survival relative to monotherapy of either agent alone. The integrin-binding-Fc fusion protein comprises (i) an integrin-binding polypeptide having an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain. An improved cancer therapy is provided that involves the combined administration of an effective amount of IL-2 and an integrin-binding-Fc fusion protein.

Accordingly, in one aspect, the invention provides a method for treating cancer in a subject comprising administering to the subject an effective amount of interleukin (IL)-2, and an integrin-binding-Fc fusion protein, wherein the integrin-binding-Fc fusion protein comprises (i) an integrin-binding polypeptide comprising an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain.

In one embodiment of the foregoing aspects, the IL-2 is in the form of an extended-PK IL-2, such as an IL-2 fusion protein. In one embodiment, the fusion protein comprises an IL-2 moiety and a moiety selected from the group consisting of an immunoglobulin fragment, human serum albumin, and Fn3. In another embodiment, the fusion protein comprises an IL-2 moiety operably linked to an immunoglobulin Fc domain. In another embodiment, the fusion protein comprises an IL-2 moiety operably linked to human serum albumin. In another embodiment, the extended-PK IL-2 comprises an IL-2 moiety conjugated to a non-protein polymer. In one embodiment, the non-protein polymer is polyethylene glycol.

In one embodiment of the foregoing aspects, the integrin-binding-Fc fusion protein includes an integrin-binding polypeptide that binds to a tumor associated integrin selected from the group consisting of $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$, or combination thereof. In one embodiment, the integrin-binding polypeptide binds to $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$.

In one embodiment of the foregoing aspects, the integrin-binding polypeptide includes an integrin-binding loop within a knottin polypeptide scaffold. In some embodiments, the knottin polypeptide scaffold comprises at least three cysteine disulfide linkages or crosslinked cysteine residues, and the integrin-binding loop is adjacent cysteine residues of the knottin polypeptide scaffold. In one embodiment, the integrin-binding loop

comprises an RGD peptide sequence. In another embodiment, the knottin polypeptide scaffold is derived from a knottin protein selected from the group consisting of EETI-II, AgRP, and agatoxin. In one embodiment, the knottin protein is EETI-II.

In one embodiment of the foregoing aspects, the integrin-binding polypeptide includes an integrin-binding loop comprising an RGD peptide sequence and the knottin polypeptide scaffold is derived from EETI-II.

In one embodiment of the foregoing aspects, the knottin polypeptide scaffold is derived from EETI-II and the integrin-binding loop comprises the sequence, $X_1X_2X_3RGDX_7X_8X_9X_{10}X_{11}$, wherein each X represents any amino acid, wherein the loop is inserted between 2 cysteine residues in the EETI-II sequence and replaces the native EETI-II sequence. In another embodiment, the integrin-binding loop is inserted after the first cysteine in the native EETI-II sequence.

In one embodiment of the foregoing aspects, the integrin-binding polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 42 or 43, wherein X_1 is selected from the group consisting of A, V, L, P, F, Y, S, H, D, and N; X_2 is selected from the group consisting of G, V, L, P, R, E, and Q; X_3 is selected from the group consisting of G, A, and P; X_7 is selected from the group consisting of W and N; X_8 is selected from the group consisting of A, P, and S; X_9 is selected from the group consisting of P and R; X_{10} is selected from the group consisting of A, V, L, P, S, T, and E; and X_{11} is selected from the group consisting of G, A, W, S, T, K, and E. In a further embodiment, the integrin-binding-Fc fusion comprises an integrin-binding polypeptide, as set forth in SEQ ID NOs: 42 or 43, operably linked to a human IgG Fc domain, as set forth in SEQ ID NOs: 2 or 3.

In one embodiment of the foregoing aspects, the integrin-binding polypeptide comprises an amino acid sequence selected from the amino acid sequences set forth in Table 1. In another embodiment, the integrin-binding polypeptide comprises an amino acid sequence from the group consisting of SEQ ID NOs: 59-125. In a further embodiment, an integrin-binding polypeptide, set forth in Table 1, is operably linked to a human IgG1 Fc domain, set forth in SEQ ID NOs: 2 or 3.

In one embodiment of the foregoing aspects, the integrin-binding polypeptide comprises the amino acid sequence of SEQ ID NO: 85, 86, 87 or 88.

In one embodiment of the foregoing aspects, the Fc domain is a human IgG1 Fc domain.

In one embodiment of the foregoing aspects, the integrin-binding polypeptide is operably linked with or without a linker to the Fc domain. In some embodiments, the

integrin-binding polypeptide is linked to the N-terminus or C-terminus of the Fc domain without a linker. In other embodiments, the integrin-binding polypeptide is linked to the N-terminus or C-terminus of the Fc donor with a linker such as a Gly-Ser linker.

In one embodiment of the foregoing aspects, the integrin-binding-Fc fusion protein comprises the amino acid sequence of SEQ ID NO: 48, 49, 50 or 51.

In any of the foregoing aspects, the methods comprise administering IL-2 and the integrin-binding-Fc fusion protein in the form of a pharmaceutical composition with a pharmaceutically acceptable carrier.

In some embodiments, the integrin-binding-Fc fusion protein is in the form of a dimer.

In any of the foregoing aspects, the methods comprise administering IL-2 and the integrin-binding-Fc fusion protein simultaneously or sequentially.

In any of the foregoing aspects, the methods further comprise administering an immune checkpoint blocker, such an antibody or antibody fragment targeting PD-1, PD-L1, CTLA-4, TIM3, LAG3, or a member of the B7 family. In one embodiment, the immune checkpoint blocker is an antibody or antibody fragment thereof targeting PD-1. In another embodiment, the immune checkpoint blocker is an antibody or antibody fragment targeting CTLA4.

In certain embodiments, an antagonist of VEGF is administered in place of an immune checkpoint blocker. In a further embodiment, the antagonist of VEGF is an antibody or antibody fragment thereof that binds VEGF, an antibody or antibody fragment thereof that binds VEGF receptor, a small molecule inhibitor of the VEGF receptor tyrosine kinases, a dominant negative VEGF, or a VEGF receptor.

In one embodiment of the foregoing aspects, the methods comprise administering an integrin-binding-Fc fusion protein and an immune checkpoint blocker, with or without IL-2.

In any of the foregoing aspects, the cancer is selected from the group consisting of melanoma, leukemia, lung cancer, breast cancer, prostate cancer, ovarian cancer, colon cancer, renal cell carcinoma, pancreatic cancer, cervical cancer, and brain cancer. In some embodiments, the cancer is melanoma. In other embodiments, the cancer is brain cancer, such as medulloblastoma. In other embodiments, the cancer is colon cancer.

In another aspect, the invention provides a method for inhibiting growth and/or proliferation of tumor cells in a subject comprising administering to the subject an effective amount of an extended-pharmacokinetic (PK) interleukin (IL)-2, and an integrin-binding-Fc fusion protein, wherein the integrin-binding-Fc fusion protein comprises (i) an integrin-

binding polypeptide comprising an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain.

In another aspect, the invention provides a method for inhibiting growth and/or proliferation of tumor cells in a subject comprising administering to the subject an effective amount of an integrin-binding-Fc fusion protein, wherein the integrin-binding-Fc fusion protein comprises (i) an integrin-binding polypeptide comprising an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain, and an immune checkpoint blocker. In a further embodiment, an effective amount of IL-2 is also administered.

In one embodiment of the foregoing aspect, the immune checkpoint blocker is an antibody or antibody fragment targeting PD-1. In another aspect, the immune checkpoint blocker is an antibody or antibody fragment targeting CTLA-4.

In another aspect, the invention provides a method for inhibiting growth and/or proliferation of tumor cells in a subject comprising administering to the subject an effective amount of an integrin-binding-Fc fusion protein, wherein the integrin-binding-Fc fusion protein comprises (i) an integrin-binding polypeptide comprising an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain, and an antagonist of VEGF. In certain embodiments, the antagonist of VEGF is an antibody or antibody fragment thereof that binds VEGF, an antibody or antibody fragment thereof that binds VEGF receptor, a small molecule inhibitor of the VEGF receptor tyrosine kinases, a dominant negative VEGF, or a VEGF receptor.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings.

Figure 1 shows the positions of the Cys-Cys disulfide linkages in the sequences of knottin proteins EETI-II (SEQ ID NO: 39), AgRP (SEQ ID NO: 40) and omega agatoxin 4B (SEQ ID NO: 41). Cysteine residues can be seen to be immediately flanking the RGD mimic loops, which, in the present engineered peptides, are between the brackets. For example, in AgRP, it can be seen that the cysteines flanking the RGD sequence will be linked to each other,

whereas in EETI they are not. The size of the grafted sequence will depend on the molecular framework structure, such that shorter loops will be preferred in cases where they are in the framework adjacent to linked cysteines. Disulfide linkages for other knottin proteins are set forth in the knottin database. FIG. 1 is adapted from *Biochemistry*, 40, 15520-15527 (2001) and *J. Biol. Chem.*, 2003, 278:6314-6322.

Figure 2 is a graph comparing tumor control with TA99 and integrin binding knottin-Fc (SEQ ID NO: 45) in subcutaneous B16F10 melanoma tumors. TA99 is an antibody against TYRP-1, an antigen that is overexpressed on melanoma cells. Tumors were established by injecting 2.5×10^5 B16F10 cells into the flanks of C57BL/6 mice. Starting on the day of tumor inoculation and every 2 days after, 80 μ g knottin-Fc, 80 μ g knottin-Fc D265A, or 200 μ g TA99 was administered. Error bars represent standard error of the mean (SEM).

Figures 3A and 3B depict synergistic tumor control in established B16F10 melanoma tumors. 1×10^6 B16F10 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2 and/or 500 μ g knottin-Fc was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. **Figure 3A)** Tumor area graphs for each treatment. **Figure 3B)** Kaplan-Meier survival plot.

Figures 4A and 4B depict synergistic tumor control in established MC38 colon tumors. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice and 30 μ g MSA/IL-2 and/or 500 μ g knottin-Fc was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. **Figure 4A)** Tumor area graphs for each treatment. **Figure 4B)** Kaplan-Meier survival plot.

Figures 5A and 5B depict synergistic tumor control in established Ag104A fibrosarcoma tumors. 1×10^6 Ag104A cells were injected into the flanks of C3H/HeN mice. 12.5 μ g MSA/IL-2 and/or 500 μ g knottin-Fc was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. **Figure 5A)** Tumor area graphs for each treatment. **Figure 5B)** Kaplan-Meier survival plot.

Figures 6A and 6B depict synergistic tumor control in established B16F10 melanoma tumors. 1×10^6 B16F10 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2 was and 500 μ g knottin-Fc or knottin-Fc D265A was administered on day 6 after tumor inoculation and every day after for a total of four treatments. **Figure 6A)** Tumor area graphs for each treatment. **Figure 6B)** Kaplan-Meier survival plot.

Figures 7A and 7B depict synergistic tumor control in established MC38 tumors. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice and 30 μ g MSA/IL-2 and 500 μ g

knottin-Fc or knottin-Fc D265A was administered on day 6 after tumor inoculation and every 6 days after for a total of four treatments. **Figure 7A)** Tumor area graphs for each treatment. **Figure 7B)** Kaplan-Meier survival plot.

Figures 8A and 8B depict synergistic tumor control with an antibody in established B16F10 tumors. 1×10^6 B16F10 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2 was administered every 6 days beginning on day 6 after tumor inoculation for a total of 5 treatments. 200 μ g knottin-Fc was administered daily from days 6-30 after tumor inoculation. Antibodies against TYRP-1 (TA99) were administered at 100 μ g per mouse every 6 days starting on day 6 after tumor inoculation. Antibodies against PD-1 (for immune checkpoint blockade) were administered at 200 μ g per mouse every 6 days starting on day 6 after tumor inoculation. **Figure 8A)** Tumor area graphs for each treatment. **Figure 8B)** Kaplan-Meier survival plot.

Figures 9A and 9B depict synergistic tumor control with an antibody in established MC38 tumors. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2, 500 μ g knottin-Fc, and/or 200 μ g anti-PD-1 antibody was administered on day 6 after tumor inoculation and every 6 days after for a total of four treatments. **Figure 9A)** Tumor area graphs for each treatment. **Figure 9B)** Kaplan-Meier survival plot.

Figure 10 is a line graph depicting tumor growth control comparing dosing schedules of knottin-Fc with MSA-IL-2 in the B16F10 tumor model. 30 μ g MSA/IL-2 was administered on days 6, 12, 18 and 24 after tumor inoculation. 200 μ g knottin-Fc was administered daily or every other day starting 6 days after tumor inoculation. 500 μ g knottin-Fc was administered for the weekly regiment on the same days as MSA/IL-2. Mean tumor area is plotted with 5 mice per group and error bars represent SEM.

Figure 11 is a line graph depicting tumor growth control comparing dosing schedules of knottin-Fc with MSA/IL-2 in the MC38 tumor model. 30 μ g MSA/IL-2 was administered on days 6, 12, 18 and 24 after tumor inoculation. 200 μ g knottin-Fc was administered daily or every other day starting 6 days after tumor inoculation. 500 μ g knottin-Fc was administered for the weekly regiment on the same days as MSA/IL-2. Mean tumor area is plotted with 5 mice per group and error bars represent SEM.

Figure 12 is a line graph depicting tumor control in subcutaneous B16F10 tumors. Fc was either fused to the N-terminus (knottin-Fc) or C-terminus (Fc-knottin) to compare efficacies of fusion placement. Additionally, knottin was fused to the heavy chain of an irrelevant IgG

on the C-terminus (IgG-knottin). Mice were treated with 80 µg knottin-Fc or Fc-knottin or 200 µg IgG-knottin every two days starting immediately after tumor inoculation.

Figure 13 is a line graph depicting tumor control in subcutaneous MC38 tumors. Fc was either fused to the N-terminus (knottin-Fc) or C-terminus (Fc-knottin) to compare efficacies of fusion placement. Additionally, knottin was fused to the heavy chain of an irrelevant IgG on the C-terminus (IgG-knottin). Mice were treated with 200 µg knottin-Fc or Fc-knottin every two days starting immediately after tumor inoculation.

Figures 14A and 14B depict tumor control after secondary tumor challenge with MC38 tumors cells. Previously cured mice (treated with MSA/IL-2 and knottin-Fc) and age-matched naïve mice were inoculated with 1×10^6 MC38 tumor cells in the opposite flank 16-20 weeks after the initial tumor inoculation. No further treatment was administered. **Figure 14A)** Tumor area graphs for previously cured mice and age-matched naïve mice following secondary tumor challenge. **Figure 14B)** Kaplan-Meier plot of mice subjected to secondary tumor challenge.

Figures 15A and 15B depict synergistic tumor control in established B16F10 melanoma tumors. 1×10^6 B16F10 cells were injected into the flanks of C57BL/6 mice. 30 µg MSA/IL-2, 500 µg knottin-Fc that targets 3 integrins ("2.5F_knottin-Fc"), and/or knottin-Fc that targets 2 integrins ("2.5D_knottin-Fc"), was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. **Figure 15A)** Tumor area graphs for each treatment. **Figure 15B)** Kaplan-Meier survival plot.

Figures 16A and 16B depict synergistic tumor control in established MC38 tumors. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice. 30 µg MSA/IL-2, 500 µg knottin-Fc that targets 3 integrins ("2.5F_knottin-Fc"), and/or knottin-Fc that targets 2 integrins ("2.5D_knottin-Fc"), was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. **Figure 16A)** Tumor area graphs for each treatment.

Figure 16B) Kaplan-Meier survival plot.

Figures 17A and 17B depict synergistic tumor control in established Ag104A angiosarcoma tumors. 1×10^6 Ag104A cells were injected into the flanks of C3H/HeN mice. 30 µg MSA/IL-2, 500 µg knottin-Fc that targets 3 integrins ("2.5F_knottin-Fc"), and/or knottin-Fc that targets 2 integrins ("2.5D_knottin-Fc") was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. **Figure 17A)** Tumor area graphs for each treatment. **Figure 17B)** Kaplan-Meier survival plot.

Figures 18A and 18B depict the effect of immune cell depletions on survival of mice with established MC38 tumors treated with knottin-Fc and MSA/IL-2. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2 and 500 μ g knottin-Fc was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. 400 μ g anti-CD8, anti-CD4, anti-NK1.1, anti-Ly6G or anti-CD19 antibodies were dosed every four days for a total of six treatments starting on day 4 after tumor inoculation. 300 μ g anti-CSF-1R was dosed every two days for a total of eleven treatments starting on day 5 of tumor inoculation. 30 μ g cobra venom factor (CVF) was administered every 6 days for a total of 4 treatments starting on day 5 after tumor inoculation. **Figure 18A)** Tumor area graphs for each treatment. **Figure 18B)** Kaplan-Meier survival plots.

Figures 19A and 19B depict the effect of dendritic cell depletion on survival of mice with established MC38 tumors. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice or Batf3^{-/-} mice. 30 μ g MSA/IL-2 and 500 μ g knottin-Fc was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. **Figure 19A)** Tumor area graphs for each treatment. **Figure 19B)** Kaplan-Meier survival plot.

Figures 20A and 20B depict the role of IFN γ in the efficacy of treatment with MSA/IL-2 and knottin-Fc in mice with established MC38 tumors. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2 and 500 μ g knottin-Fc was administered on day 6 after tumor inoculation, and every 6 days after for a total of four treatments. 200 μ g anti-IFN γ antibody was administered every two days for a total of eleven treatments starting on day 5 after tumor inoculation. **Figure 20A)** Tumor area graphs for each treatment. **Figure 20B)** Kaplan-Meier survival plot.

Figures 21A and 21B depict synergistic tumor control in established MC38 tumors. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2, 500 μ g knottin-Fc, and/or 200 μ g anti-VEGF antibody was administered on day 6 after tumor inoculation, and every 6 days after for a total for 4 treatments. **Figure 21A)** Tumor area graphs for each treatment. **Figure 21B)** Kaplan-Meier survival plot.

Figure 22 shows tumor area graphs depicting synergistic tumor control in established MC38 tumors. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2, 500 μ g knottin-Fc, and/or 200 μ g anti-CTLA-4 antibody was administered on day 6 after tumor inoculation, and every 6 days after for a total for 4 treatments.

Figures 23A and 23B depict the synergistic tumor control in established B16F10 tumors. 1×10^6 B16F10 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2, 500 μ g

knottin-Fc, 200 µg anti-VEGF antibody and/or 200 µg anti-CTLA-4 antibody was administered on day 6 after tumor inoculation, and every 6 days after for a total for 4 treatments. **Figure 23A)** Tumor area graphs for each treatment. **Figure 23B).** Kaplan-Meier survival plots

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Terms used in the claims and specification are defined as set forth below unless otherwise specified. In the case of direct conflict with a term used in a parent provisional patent application, the term used in the instant specification shall control.

"Amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α -carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid. Amino acids can be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, can be referred to by their commonly accepted single-letter codes.

An "amino acid substitution" refers to the replacement of at least one existing amino acid residue in a predetermined amino acid sequence (an amino acid sequence of a starting polypeptide) with a second, different "replacement" amino acid residue. An "amino acid insertion" refers to the incorporation of at least one additional amino acid into a predetermined amino acid sequence. While the insertion will usually consist of the insertion of one or two amino acid residues, the present larger "peptide insertions," can be made, e.g. insertion of about three to about five or even up to about ten, fifteen, or twenty amino acid

residues. The inserted residue(s) may be naturally occurring or non- naturally occurring as disclosed above. An "amino acid deletion" refers to the removal of at least one amino acid residue from a predetermined amino acid sequence.

"Polypeptide," "peptide", and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double- stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., *Nucleic Acid Res.* 19:5081, 1991; Ohtsuka et al. . *Biol. Chem.* 260:2605-2608, 1985; and Cassol et al, 1992; Rossolini et al, *Mol. Cell. Probes* 8:91-98, 1994). For arginine and leucine, modifications at the second base can also be conservative. The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene. Polynucleotides used herein can be composed of any polyribonucleotide or polydeoxribonucleotide, which can be unmodified RNA or DNA or modified RNA or DNA. For example, polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double- stranded regions, single- and double- stranded RNA, and RNA that is mixture of single- and double- stranded regions, hybrid molecules comprising DNA and RNA that can be single- stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, the polynucleotide can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. A polynucleotide can also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

As used herein, "interleukin (IL)-2," refers to a pleiotropic cytokine that activates and induces proliferation of T cells and natural killer (NK) cells. IL-2 signals by binding its receptor, IL-2R, which is comprised of alpha, beta, and gamma subunits. IL-2 signaling stimulates proliferation of antigen-activated T cells.

As used herein, the term "PK" is an acronym for "pharmacokinetic" and encompasses properties of a compound including, by way of example, absorption, distribution, metabolism, and elimination by a subject. As used herein, an "extended-PK group" refers to a protein, peptide, or moiety that increases the circulation half-life of a biologically active molecule when fused to or administered together with the biologically active molecule. Examples of an extended-PK group include PEG, human serum albumin (HSA) binders (as disclosed in U.S. Publication Nos. 2005/0287153 and 2007/0003549, PCT Publication Nos. WO 2009/083804 and WO 2009/133208, and SABA molecules as described in US2012/094909), human serum albumin, Fc or Fc fragments and variants thereof, and sugars (e.g., sialic acid). Other exemplary extended-PK groups are disclosed in Kontermann et al., *Current Opinion in Biotechnology* 2011;22:868-876, which is herein incorporated by reference in its entirety. As used herein, an "extended-PK IL-2" refers to an IL-2 moiety in combination with an extended-PK group. In one embodiment, the extended-PK IL-2 is a fusion protein in which an IL-2 moiety is linked or fused to an extended-PK group. An exemplary fusion protein is an HSA/IL-2 fusion in which one or more IL-2 moieties are linked to HSA.

The term "extended-PK IL-2" is also intended to encompass IL-2 mutants with mutations in one or more amino acid residues that enhance the affinity of IL-2 for one or more of its receptors, for example, CD25. In one embodiment, the IL-2 moiety of extended-PK IL-2 is wild-type IL-2. In another embodiment, the IL-2 moiety is a mutant IL-2 which exhibits greater affinity for CD25 than wild-type IL-2. When a particular type of extended-PK group is indicated, such as HSA-IL-2, it should be understood that this encompasses both HSA or MSA fused to a wild-type IL-2 moiety or HSA or MSA fused to a mutant IL-2 moiety.

In certain aspects, the extended-PK IL-2 or knottin-Fc described can employ one or more "linker domains," such as polypeptide linkers. As used herein, the term "linker" or "linker domain" refers to a sequence which connects two or more domains (e.g., the PK moiety and IL-2) in a linear sequence. As used herein, the term "polypeptide linker" refers to a peptide or polypeptide sequence (e.g., a synthetic peptide or polypeptide sequence) which connects two or more domains in a linear amino acid sequence of a polypeptide chain. For

example, polypeptide linkers may be used to connect an IL-2 moiety or an integrin binding polypeptide to an Fc domain or other PK-extender such as HSA. In some embodiments, such polypeptide linkers can provide flexibility to the polypeptide molecule. Exemplary linkers include Gly-Ser linkers.

As used herein, the terms "linked," "fused", or "fusion" are used interchangeably. These terms refer to the joining together of two or more elements or components or domains, by whatever means including chemical conjugation or recombinant means. Methods of chemical conjugation (e.g., using heterobifunctional crosslinking agents) are known in the art.

The term "integrin" means a transmembrane heterodimeric protein important for cell adhesion. Integrins comprise an α and β subunit. These proteins bind to extracellular matrix components (e.g., fibronectin, collagen, laminin, etc.) and respond by inducing signaling cascades. Integrins bind to extracellular matrix components by recognition of the RGD motif. Certain integrins are found on the surface of tumor cells and therefore make promising therapeutic targets. In certain embodiments, the integrins being targeted are $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$, individually or in combination.

The term "integrin-binding polypeptide" refers to a polypeptide which includes an integrin-binding domain or loop within a knottin polypeptide scaffold. The integrin binding domain or loop includes at least one RGD peptide. In certain embodiments, the RGD peptide is recognized by $\alpha_v\beta_3$, $\alpha_v\beta_5$, or $\alpha_5\beta_1$. In certain embodiments the RGD peptide binds to a combination of $\alpha_v\beta_3$, $\alpha_v\beta_5$, and/or $\alpha_5\beta_1$. These specific integrins are found on tumor cells and their vasculature and are therefore the targets of interest.

The term "loop domain" refers to an amino acid subsequence within a peptide chain that has no ordered secondary structure, and resides generally on the surface of the peptide. The term "loop" is understood in the art as referring to secondary structures that are not ordered as in the form of an alpha helix, beta sheet, etc.

The term "integrin-binding loop" refers to a primary sequence of about 9-13 amino acids which is typically created *ab initio* through experimental methods such as directed molecular evolution to bind to integrins. In certain embodiments, the integrin-binding loop includes an RGD peptide sequence, or the like, placed between amino acids which are particular to the scaffold and the binding specificity desired. The RGD-containing peptide or like (RYD, etc) is generally not simply taken from a natural binding sequence of a known protein. The integrin-binding loop is preferably inserted within a knottin polypeptide scaffold

between cysteine residues, and the length of the loop adjusted for optimal integrin-binding depending on the three-dimensional spacing between cysteine residues. For example, if the flanking cysteine residues in the knottin scaffold are linked to each other, the optimal loop may be shorter than if the flanking cysteine residues are linked to cysteine residues separated in primary sequence. Otherwise, particular amino acid substitutions can be introduced to constrain a longer RGD-containing loop into an optimal conformation for high affinity integrin binding. The knottin polypeptide scaffolds used herein may contain certain modifications made to truncate the native knottin, or to remove a loop or unnecessary cysteine residue or disulfide bond.

Incorporation of integrin-binding sequences into a molecular (e.g., knottin polypeptide) scaffold provides a framework for ligand presentation that is more rigid and stable than linear or cyclic peptide loops. In addition, the conformational flexibility of small peptides in solution is high, and results in large entropic penalties upon binding. Incorporation of an integrin-binding sequence into a knottin polypeptide scaffold provides conformational constraints that are required for high affinity integrin binding. Furthermore, the scaffold provides a platform to carry out protein engineering studies such as affinity or stability maturation.

As used herein, the term "knottin protein" refers to a structural family of small proteins, typically 25-40 amino acids, which bind to a range of molecular targets like proteins, sugars and lipids. Their three-dimensional structure is essentially defined by a peculiar arrangement of three to five disulfide bonds. A characteristic knotted topology with one disulfide bridge crossing the macro-cycle limited by the two other intra-chain disulfide bonds, which was found in several different microproteins with the same cystine network, lent its name to this class of biomolecules. Although their secondary structure content is generally low, the knottins share a small triple-stranded antiparallel β -sheet, which is stabilized by the disulfide bond framework. Biochemically well-defined members of the knottin family, also called cystine knot proteins, include the trypsin inhibitor EETI-II from *Ecballium elaterium* seeds, the neuronal N-type Ca^{2+} channel blocker ω -conotoxin from the venom of the predatory cone snail *Conus geographus*, agouti-related protein (AgRP, See Millhauser et al., "Loops and Links: Structural Insights into the Remarkable Function of the Agouti-Related Protein," *Ann. N.Y. Acad. Sci.*, Jun. 1, 2003; 994(1): 27-35), the omega agatoxin family, etc. A suitable agatoxin sequence [SEQ ID NO: 41] is given in US Patent 8,536,301, having a common inventor with the present application. Other agatoxin sequences suitable for use in the methods disclosed herein include, Omega-agatoxin-Aa4b (GenBank

Accession number P37045) and Omega-agatoxin-Aa3b (GenBank Accession number P81744). Other knottin sequences suitable for use in the methods disclosed herein include, knottin [*Bemisia tabaci*] (GenBank Accession number FJ601218.1), Omega-lycotoxin (Genbank Accession number P85079), mu-O conotoxin MrVIA=voltage-gated sodium channel blocker (Genbank Accession number AAB34917) and *Momordica cochinchinensis* Trypsin Inhibitor I (MCoTI-I) or II (McoTI-II) (Uniprot Accession numbers P82408 and P82409 respectively).

Knottin proteins have a characteristic disulfide linked structure. This structure is also illustrated in Gelly et al., "The KNOTTIN website and database: a new information system dedicated to the knottin scaffold," *Nucleic Acids Research*, 2004, Vol. 32, Database issue D156-D159. A triple-stranded β -sheet is present in many knottins. The spacing between cysteine residues is important, as is the molecular topology and conformation of the integrin-binding loop.

The term "molecular scaffold" means a polymer having a predefined three-dimensional structure, into which an integrin-binding loop is incorporated, such as an RGD peptide sequence as described herein. The term "molecular scaffold" has an art-recognized meaning (in other contexts), which is also intended here. For example, a review by Skerra, "Engineered protein scaffolds for molecular recognition," *J. Mol. Recognit.* 2000; 13:167-187 describes the following scaffolds: single domains of antibodies of the immunoglobulin superfamily, protease inhibitors, helix-bundle proteins, disulfide-knotted peptides and lipocalins. Guidance is given for the selection of an appropriate molecular scaffold.

The term "knottin polypeptide scaffold" refers to a knottin protein suitable for use as a molecular scaffold, as described herein. Characteristics of a desirable knottin polypeptide scaffold for engineering include 1) high stability *in vitro* and *in vivo*, 2) the ability to replace amino acid regions of the scaffold with other sequences without disrupting the overall fold, 3) the ability to create multifunctional or bispecific targeting by engineering separate regions of the molecule, and 4) a small size to allow for chemical synthesis and incorporation of non-natural amino acids if desired. Scaffolds derived from human proteins are favored for therapeutic applications to reduce toxicity or immunogenicity concerns, but are not always a strict requirement. Other scaffolds that have been used for protein design include fibronectin (Koide et al., 1998), lipocalin (Beste et al., 1999), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (Hufton et al., 2000), and tendamistat (McConnell and Hoess, 1995; Li et al., 2003). While these scaffolds have proved to be useful frameworks for protein engineering,

molecular scaffolds such as knottins have distinct advantages: their small size and high stability.

As used herein, the term "EETI" means Protein Data Bank Entry (PDB) 2ETI. Its entry in the Knottin database is EETI-II. It has the sequence:

GC PRILMRCKQDSCLAGCVCGPNGFCG (SEQ ID NO: 39).

As used herein, the term "AgRP" means PDB entry 1HYK. Its entry in the Knottin database is SwissProt AGRP_HUMAN, where the full-length sequence of 129 amino acids may be found. It comprises the sequence beginning at amino acid 87. An additional G is added to this construct. It also includes a C105A mutation described in Jackson, et al. 2002 Biochemistry, 41, 7565.

GCVRLHESCLGQQVPCCDPCATCYC **RFFNAF** CYCR-KLGTAMNPCSRT (SEQ ID NO: 40)

The bold and underlined portion, from loop 4, is replaced by the RGD sequences described herein. Loops 1 and 3 are shown between brackets below:

GC[VRLHES]CLGQQVPCC[DPCAT]CYCR**RFFNAF**CYCR-KLGTAMNPCSRT

As used herein, "integrin-binding-Fc fusion" is used interchangeably with "knottin-Fc" and refers to an integrin-binding polypeptide that includes an integrin-binding amino acid sequence within a knottin polypeptide scaffold and is operably linked to an Fc domain. In certain embodiments, the Fc domain is fused to the N-terminus of the integrin-binding polypeptide. In certain embodiments, the Fc domain is fused to the C-terminus of the integrin binding polypeptide. In some embodiments, the Fc domain is operably linked to the integrin-binding polypeptide via a linker.

As used herein, the term "Fc region" refers to the portion of a native immunoglobulin formed by the respective Fc domains (or Fc moieties) of its two heavy chains. As used herein, the term "Fc domain" refers to a portion of a single immunoglobulin (Ig) heavy chain wherein the Fc domain does not comprise an Fv domain. As such, an Fc domain can also be referred to as "Ig" or "IgG." In certain embodiments, an Fc domain begins in the hinge region just upstream of the papain cleavage site and ends at the C-terminus of the antibody. Accordingly, a complete Fc domain comprises at least a hinge domain, a CH2 domain, and a CH3 domain. In certain embodiments, an Fc domain comprises at least one of: a hinge (e.g., upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, a CH4 domain, or a variant, portion, or fragment thereof. In other embodiments, an Fc domain comprises a complete Fc domain (i.e., a hinge domain, a CH2 domain, and a CH3 domain). In one embodiment, an Fc domain comprises a hinge domain (or portion thereof) fused to a CH3

domain (or portion thereof). In another embodiment, an Fc domain comprises a CH2 domain (or portion thereof) fused to a CH3 domain (or portion thereof). In another embodiment, an Fc domain consists of a CH3 domain or portion thereof. In another embodiment, an Fc domain consists of a hinge domain (or portion thereof) and a CH3 domain (or portion thereof). In another embodiment, an Fc domain consists of a CH2 domain (or portion thereof) and a CH3 domain. In another embodiment, an Fc domain consists of a hinge domain (or portion thereof) and a CH2 domain (or portion thereof). In one embodiment, an Fc domain lacks at least a portion of a CH2 domain (e.g., all or part of a CH2 domain). An Fc domain herein generally refers to a polypeptide comprising all or part of the Fc domain of an immunoglobulin heavy-chain. This includes, but is not limited to, polypeptides comprising the entire CH1, hinge, CH2, and/or CH3 domains as well as fragments of such peptides comprising only, e.g., the hinge, CH2, and CH3 domain. The Fc domain may be derived from an immunoglobulin of any species and/or any subtype, including, but not limited to, a human IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM antibody. A human IgG1 constant region can be found at Uniprot P01857 and in Table 2 (i.e., SEQ ID NO: 1). The Fc domain of human IgG1 can be found in Table 2 (i.e., SEQ ID NO: 2). The Fc domain of human IgG1 with a deletion of the upper hinge region can be found in Table 2 (i.e., SEQ ID NO: 3). The Fc domain encompasses native Fc and Fc variant molecules. As with Fc variants and native Fc's, the term Fc domain includes molecules in monomeric or multimeric (e.g., dimeric) form, whether digested from whole antibody or produced by other means. The assignment of amino acid residue numbers to an Fc domain is in accordance with the definitions of Kabat. See, e.g., Sequences of Proteins of Immunological Interest (Table of Contents, Introduction and Constant Region Sequences sections), 5th edition, Bethesda, MD:NIH vol. 1:647-723 (1991); Kabat et al., "Introduction" Sequences of Proteins of Immunological Interest, US Dept of Health and Human Services, NIH, 5th edition, Bethesda, MD vol. 1:xiii-xcvi (1991); Chothia & Lesk, J. Mol. Biol. 196:901-917 (1987); Chothia et al., Nature 342:878-883 (1989), each of which is herein incorporated by reference for all purposes.

As set forth herein, it will be understood by one of ordinary skill in the art that any Fc domain may be modified such that it varies in amino acid sequence from the native Fc domain of a naturally occurring immunoglobulin molecule. In certain exemplary embodiments, the Fc domain has increased effector function (e.g., FcγR binding).

The Fc domains of a polypeptide of the invention may be derived from different immunoglobulin molecules. For example, an Fc domain of a polypeptide may comprise a

CH2 and/or CH3 domain derived from an IgG1 molecule and a hinge region derived from an IgG3 molecule. In another example, an Fc domain can comprise a chimeric hinge region derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. In another example, an Fc domain can comprise a chimeric hinge derived, in part, from an IgG1 molecule and, in part, from an IgG4 molecule.

A polypeptide or amino acid sequence "derived from" a designated polypeptide or protein refers to the origin of the polypeptide. Preferably, the polypeptide or amino acid sequence which is derived from a particular sequence has an amino acid sequence that is essentially identical to that sequence or a portion thereof, wherein the portion consists of at least 10-20 amino acids, preferably at least 20-30 amino acids, more preferably at least 30-50 amino acids, or which is otherwise identifiable to one of ordinary skill in the art as having its origin in the sequence. Polypeptides derived from another peptide may have one or more mutations relative to the starting polypeptide, e.g., one or more amino acid residues which have been substituted with another amino acid residue or which has one or more amino acid residue insertions or deletions.

A polypeptide can comprise an amino acid sequence which is not naturally occurring. Such variants, in the context of IL-2 or a knottin protein, necessarily have less than 100% sequence identity or similarity with the starting IL-2 or knottin protein. In a preferred embodiment, the variant will have an amino acid sequence from about 75% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of the starting polypeptide, more preferably from about 80% to less than 100%, more preferably from about 85% to less than 100%, more preferably from about 90% to less than 100% (e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) and most preferably from about 95% to less than 100%, e.g., over the length of the variant molecule.

In one embodiment, there is one amino acid difference between a starting polypeptide sequence and the sequence derived therefrom. Identity or similarity with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical (i.e., same residue) with the starting amino acid residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity.

In one embodiment, a polypeptide comprising IL-2 or a variant thereof, for use in extended-PK IL-2 consists of, consists essentially of, or comprises an amino acid sequence selected from SEQ ID Nos: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, and 35. In an embodiment, a polypeptide includes an amino acid sequence at least 80%, 81%, 82%, 83%,

84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from SEQ ID Nos: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, and 35. In an embodiment, a polypeptide includes a contiguous amino acid sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a contiguous amino acid sequence selected from SEQ ID Nos: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, and 35. In an embodiment, a polypeptide includes an amino acid sequence having at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, or 500 (or any integer within these numbers) contiguous amino acids of an amino acid sequence selected from SEQ ID Nos: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, and 35.

In an embodiment, the peptides are encoded by a nucleotide sequence. Nucleotide sequences can be useful for a number of applications, including: cloning, gene therapy, protein expression and purification, mutation introduction, DNA vaccination of a host in need thereof, antibody generation for, e.g., passive immunization, PCR, primer and probe generation, and the like. In an embodiment, the nucleotide sequence of the invention comprises, consists of, or consists essentially of, a nucleotide sequence of IL-2, or a variant thereof, selected from SEQ ID Nos: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34. In an embodiment, a nucleotide sequence includes a nucleotide sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a nucleotide sequence set forth in SEQ ID Nos: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34. In an embodiment, a nucleotide sequence includes a contiguous nucleotide sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a contiguous nucleotide sequence set forth in SEQ ID Nos: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34. In an embodiment, a nucleotide sequence includes a nucleotide sequence having at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, or 500 (or any integer within these numbers) contiguous nucleotides of a nucleotide sequence set forth in SEQ ID Nos: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34.

In one embodiment, a polypeptide comprising integrin-binding peptide or a variant thereof, consists of, consists essentially of, or comprises an amino acid sequence selected from SEQ ID Nos: 59-125. In an embodiment, a polypeptide includes an amino acid sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to an amino acid sequence selected from

SEQ ID Nos: 59-125. In an embodiment, a polypeptide includes a contiguous amino acid sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a contiguous amino acid sequence selected from SEQ ID Nos: 59-125. In an embodiment, a polypeptide includes an amino acid sequence having at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, or 500 (or any integer within these numbers) contiguous amino acids of an amino acid sequence selected from SEQ ID Nos: 59-125.

It will also be understood by one of ordinary skill in the art that the extended-PK IL-2 or a knottin-Fc fusion used herein may be altered such that they vary in sequence from the naturally occurring or native sequences from which they were derived, while retaining the desirable activity of the native sequences. For example, nucleotide or amino acid substitutions leading to conservative substitutions or changes at "non-essential" amino acid residues may be made. Mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

The polypeptides described herein (e.g., IL-2, extended-PK IL-2, PK moieties, knottin, Fc, knottin-Fc, and the like) may comprise conservative amino acid substitutions at one or more amino acid residues, e.g., at essential or non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagines, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in a binding polypeptide is preferably replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members. Alternatively, in another embodiment, mutations may be introduced randomly along all or part of a coding sequence, such as by saturation mutagenesis, and the resultant mutants can be incorporated into binding polypeptides of the invention and screened for their ability to bind to the desired target.

The "Programmed Death-1 (PD-1)" receptor refers to an immuno-inhibitory receptor belonging to the CD28 family. PD-1 is expressed predominantly on previously activated T

cells in vivo, and binds to two ligands, PD-L1 and PD-L2. The term "PD-1" as used herein includes human PD-1 (hPD-1), variants, isoforms, and species homologs of hPD-1, and analogs having at least one common epitope with hPD-1. The complete hPD-1 sequence can be found under GenBank Accession No. AAC51773 (SEQ ID NO: 52).

"Programmed Death Ligand-1 (PD-L1)" is one of two cell surface glycoprotein ligands for PD-1 (the other being PD-L2) that downregulates T cell activation and cytokine secretion upon binding to PD-1. The term "PD-L1" as used herein includes human PD-L1 (hPD-L1), variants, isoforms, and species homologs of hPD-L1, and analogs having at least one common epitope with hPD-L1. The complete hPD-L1 sequence can be found under GenBank Accession No. Q9NZQ7 (SEQ ID NO: 53).

"Cytotoxic T Lymphocyte Associated Antigen-4 (CTLA-4)" is a T cell surface molecule and is a member of the immunoglobulin superfamily. This protein downregulates the immune system by binding to CD80 and CD86. The term "CTLA-4" as used herein includes human CTLA-4 (hCTLA-4), variants, isoforms, and species homologs of hCTLA-4, and analogs having at least one common epitope with hCTLA-4. The complete hCTLA-4 sequence can be found under GenBank Accession No. P16410 (SEQ ID NO: 54):

"Lymphocyte Activation Gene-3 (LAG3)" is an inhibitory receptor associated with inhibition of lymphocyte activity by binding to MHC class II molecules. This receptor enhances the function of Treg cells and inhibits CD8+ effector T cell function. The term "LAG3" as used herein includes human LAG3 (hLAG3), variants, isoforms, and species homologs of hLAG3, and analogs having at least one common epitope. The complete hLAG3 sequence can be found under GenBank Accession No. P18627 (SEQ ID NO: 55).

"T Cell Membrane Protein-3 (TIM3)" is an inhibitory receptor involved in the inhibition of lymphocyte activity by inhibition of T_H1 cells responses. Its ligand is galectin 9, which is upregulated in various types of cancers. The term "TIM3" as used herein includes human TIM3 (hTIM3), variants, isoforms, and species homologs of hTIM3, and analogs having at least one common epitope. The complete hTIM3 sequence can be found under GenBank Accession No. Q8TDQo (SEQ ID NO: 56).

The "B7 family" refers to inhibitory ligands with undefined receptors. The B7 family encompasses B7-H3 and B7-H4, both upregulated on tumor cells and tumor infiltrating cells. The complete hB7-H3 and hB7-H4 sequence can be found under GenBank Accession Nos. Q5ZPR3 and AAZ17406 (SEQ ID Nos: 57 and 58) respectively.

"Vascular Endothelial Growth Factor (VEGF)" is a secreted disulfide-linked homodimer that selectively stimulates endothelial cells to proliferate, migrate, and produce

matrix-degrading enzymes, all of which are processes required for the formation of new vessels. In addition to being the only known endothelial cell specific mitogen, VEGF is unique among angiogenic growth factors in its ability to induce a transient increase in blood vessel permeability to macromolecules. The term "VEGF" or "VEGF-A" is used to refer to the 165-amino acid human vascular endothelial cell growth factor and related 121-, 145-, 189-, and 206-amino acid human vascular endothelial cell growth factors, as described by, e.g., Leung et al. *Science*, 246: 1306 (1989), and Houck et al. *Mol. Endocrin.*, 5: 1806 (1991), together with the naturally occurring allelic and processed forms thereof. VEGF-A is part of a gene family including VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF. VEGF-A primarily binds to two high affinity receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), the latter being the major transmitter of vascular endothelial cell mitogenic signals of VEGF-A.

As used herein, "immune checkpoint" refers to co-stimulatory and inhibitory signals that regulate the amplitude and quality of T cell receptor recognition of an antigen. In certain embodiments, the immune checkpoint is an inhibitory signal. In certain embodiments, the inhibitory signal is the interaction between PD-1 and PD-L1. In certain embodiments, the inhibitory signal is the interaction between CTLA-4 and CD80 or CD86 to displace CD28 binding. In certain embodiments the inhibitory signal is the interaction between LAG3 and MHC class II molecules. In certain embodiments, the inhibitory signal is the interaction between TIM3 and galectin 9.

As used herein, "immune checkpoint blocker" refers to a molecule that reduces, inhibits, interferes with or modulates one or more checkpoint proteins. In certain embodiments, the immune checkpoint blocker prevents inhibitory signals associated with the immune checkpoint. In certain embodiments, the immune checkpoint blocker is an antibody, or fragment thereof, that disrupts inhibitory signaling associated with the immune checkpoint. In certain embodiments, the immune checkpoint blocker is a small molecule that disrupts inhibitory signaling. In certain embodiments, the immune checkpoint blocker is an antibody, fragment thereof, or antibody mimic, that prevents the interaction between checkpoint blocker proteins, e.g., an antibody, or fragment thereof, that prevents the interaction between PD-1 and PD-L1. In certain embodiments, the immune checkpoint blocker is an antibody, or fragment thereof, that prevents the interaction between CTLA-4 and CD80 or CD86. In certain embodiments, the immune checkpoint blocker is an antibody, or fragment thereof, that prevents the interaction between LAG3 and MHC class II molecules. In certain embodiments the, the immune checkpoint blocker is an antibody, or fragment thereof, that

prevents the interaction between TIM3 and galectin9. The checkpoint blocker may also be in the form of the soluble form of the molecules (or mutation thereof) themselves, e.g., a soluble PD-L1 or PD-L1 fusion.

The term "ameliorating" refers to any therapeutically beneficial result in the treatment of a disease state, e.g., cancer, including prophylaxis, lessening in the severity or progression, remission, or cure thereof.

The term "*in vivo*" refers to processes that occur in a living organism.

The term "mammal" or "subject" or "patient" as used herein includes both humans and non-humans and include but is not limited to humans, non-human primates, canines, felines, murines, bovines, equines, and porcines.

The term "percent identity," in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described below (e.g., BLASTP and BLASTN or other algorithms available to persons of skill) or by visual inspection. Depending on the application, the "percent identity" can exist over a region of the sequence being compared, e.g., over a functional domain, or, alternatively, exist over the full length of the two sequences to be compared.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally Ausubel et al., *infra*).

One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et

al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website.

As used herein, the term "gly-ser polypeptide linker" refers to a peptide that consists of glycine and serine residues. An exemplary gly-ser polypeptide linker comprises the amino acid sequence Ser(Gly₄Ser)_n. In one embodiment, n=1. In one embodiment, n=2. In another embodiment, n=3, i.e., Ser(Gly₄Ser)₃. In another embodiment, n=4, i.e., Ser(Gly₄Ser)₄. In another embodiment, n=5. In yet another embodiment, n=6. In another embodiment, n=7. In yet another embodiment, n=8. In another embodiment, n=9. In yet another embodiment, n=10. Another exemplary gly-ser polypeptide linker comprises the amino acid sequence (Gly₄Ser)_n. In one embodiment, n=1. In one embodiment, n=2. In a preferred embodiment, n=3. In another embodiment, n=4. In another embodiment, n=5. In yet another embodiment, n=6. Another exemplary gly-ser polypeptide linker comprises the amino acid sequence (Gly₃Ser)_n. In one embodiment, n=1. In one embodiment, n=2. In a preferred embodiment, n=3. In another embodiment, n=4. In another embodiment, n=5. In yet another embodiment, n=6.

As used herein, "half-life" refers to the time taken for the serum or plasma concentration of a polypeptide to reduce by 50%, in vivo, for example due to degradation and/or clearance or sequestration by natural mechanisms. The extended-PK IL-2 used herein is stabilized in vivo and its half-life increased by, e.g., fusion to HSA, MSA or Fc, through PEGylation, or by binding to serum albumin molecules (e.g., human serum albumin) which resist degradation and/or clearance or sequestration. The half-life can be determined in any manner known per se, such as by pharmacokinetic analysis. Suitable techniques will be clear to the person skilled in the art, and may for example generally involve the steps of suitably administering a suitable dose of the amino acid sequence or compound of the invention to a subject; collecting blood samples or other samples from said subject at regular intervals; determining the level or concentration of the amino acid sequence or compound of the invention in said blood sample; and calculating, from (a plot of) the data thus obtained, the time until the level or concentration of the amino acid sequence or compound of the invention has been reduced by 50% compared to the initial level upon dosing. Further details are provided in, e.g., standard handbooks, such as Kenneth, A. et al., Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists and in Peters et al., Pharmacokinetic Analysis: A Practical Approach (1996). Reference is also made to Gibaldi, M. et al., Pharmacokinetics, 2nd Rev. Edition, Marcel Dekker (1982).

As used herein, a "small molecule" is a molecule with a molecular weight below about 500 Daltons.

As used herein, "therapeutic protein" refers to any polypeptide, protein, protein variant, fusion protein and/or fragment thereof which may be administered to a subject as a medicament. An exemplary therapeutic protein is an interleukin, e.g., IL-7.

As used herein, "synergy" or "synergistic effect" with regard to an effect produced by two or more individual components refers to a phenomenon in which the total effect produced by these components, when utilized in combination, is greater than the sum of the individual effects of each component acting alone.

The term "sufficient amount" or "amount sufficient to" means an amount sufficient to produce a desired effect, e.g., an amount sufficient to reduce the size of a tumor.

The term "therapeutically effective amount" is an amount that is effective to ameliorate a symptom of a disease. A therapeutically effective amount can be a "prophylactically effective amount" as prophylaxis can be considered therapy.

As used herein, "combination therapy" embraces administration of each agent or therapy in a sequential manner in a regimen that will provide beneficial effects of the combination and co-administration of these agents or therapies in a substantially simultaneous manner. Combination therapy also includes combinations where individual elements may be administered at different times and/or by different routes but which act in combination to provide a beneficial effect by co-action or pharmacokinetic and pharmacodynamics effect of each agent or tumor treatment approaches of the combination therapy.

As used herein, "about" will be understood by persons of ordinary skill and will vary to some extent depending on the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill given the context in which it is used, "about" will mean up to plus or minus 10% of the particular value.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

Various aspects described herein are described in further detail in the following subsections.

IL-2 and Extended-PK IL-2

Interleukin-2 (IL-2) is a cytokine that induces proliferation of antigen-activated T cells and stimulates natural killer (NK) cells. The biological activity of IL-2 is mediated through a multi-subunit IL-2 receptor complex (IL-2R) of three polypeptide subunits that span the cell membrane: p55 (IL-2R α , the alpha subunit, also known as CD25 in humans), p75 (IL-2R β , the beta subunit, also known as CD 122 in humans) and p64 (IL-2R γ , the gamma subunit, also known as CD 132 in humans). T cell response to IL-2 depends on a variety of factors, including: (1) the concentration of IL-2; (2) the number of IL-2R molecules on the cell surface; and (3) the number of IL-2R occupied by IL-2 (i.e., the affinity of the binding interaction between IL-2 and IL-2R (Smith, "Cell Growth Signal Transduction is Quantal" In Receptor Activation by Antigens, Cytokines, Hormones, and Growth Factors 766:263-271, 1995)). The IL-2:IL-2R complex is internalized upon ligand binding and the different components undergo differential sorting. IL-2R α is recycled to the cell surface, while IL-2 associated with the IL-2:IL-2R $\beta\gamma$ complex is routed to the lysosome and degraded. When administered as an intravenous (i.v.) bolus, IL-2 has a rapid systemic clearance (an initial clearance phase with a half-life of 12.9 minutes followed by a slower clearance phase with a half-life of 85 minutes) (Konrad et al., Cancer Res. 50:2009-2017, 1990).

Thus, in some embodiments, IL-2 therapy, such as systemic IL-2, is administered to a subject in an effective amount in combination with an integrin-binding-Fc fusion protein, and optionally an immune checkpoint blocker.

However, outcomes of systemic IL-2 administration in cancer patients are far from ideal. While 15 to 20 percent of patients respond objectively to high-dose IL-2, the great majority do not, and many suffer severe, life-threatening side effects, including nausea, confusion, hypotension, and septic shock. The severe toxicity associated with IL-2 treatment is largely attributable to the activity of natural killer (NK) cells. NK cells express the intermediate-affinity receptor, IL-2R $\beta\gamma$, and thus are stimulated at nanomolar concentrations of IL-2, which do in fact result in patient sera during high-dose IL-2 therapy. Attempts to reduce serum concentration, and hence selectively stimulate IL-2R $\alpha\beta\gamma$ -bearing cells, by reducing dose and adjusting dosing regimen have been attempted, and while less toxic, such treatments were also less efficacious. Given the toxicity issues associated with high dose IL-2 cancer therapy, numerous groups have attempted to improve anti-cancer efficacy of IL-2 by simultaneously administering therapeutic antibodies. Yet, such efforts have been largely

unsuccessful, yielding no additional or limited clinical benefit compared to IL-2 therapy alone. Accordingly, novel IL-2 therapies are needed to more effectively combat various cancers.

Applicants recently discovered that the ability of IL-2 to control tumors in various cancer models could be substantially increased by attaching IL-2 to a pharmacokinetic modifying group. The resulting molecule, hereafter referred to as "extended-pharmacokinetic (PK) IL-2," has a prolonged circulation half-life relative to free IL-2. The prolonged circulation half-life of extended-PK IL-2 permits in vivo serum IL-2 concentrations to be maintained within a therapeutic range, leading to the enhanced activation of many types of immune cells, including T cells. Because of its favorable pharmacokinetic profile, extended-PK IL-2 can be dosed less frequently and for longer periods of time when compared with unmodified IL-2. Extended-PK IL-2 is described in detail in International Patent Application NO. PCT/US2013/042057, filed May 21, 2013, and claiming the benefit of priority to US Provisional Patent Application NO. 61/650,277, filed May 22, 2012. The entire contents of the foregoing applications are incorporated by reference herein.

A. IL-2 and mutants thereof

In certain embodiments, an effective amount of human IL-2 is administered systemically. In some embodiments, an effective amount of an extended-PK IL-2 is administered systemically. In one embodiment, the IL-2 is a human recombinant IL-2 such as Proleukin® (aldesleukin). Proleukin® is a human recombinant interleukin-2 product produced in *E. coli*. Proleukin® differs from the native interleukin-2 in the following ways: a) it is not glycosylated; b) it has no N-terminal alanine; and c) it has serine substituted for cysteine at amino acid positions 125. Proleukin® exists as biologically active, non-covalently bound microaggregates with an average size of 27 recombinant interleukin-2 molecules. Proleukin® (aldesleukin) is administered by intravenous infusion. In some aspects, the IL-2 portion of the extended-PK IL-2 is wild-type IL-2 (e.g., human IL-2 in its precursor form (SEQ ID NO: 33) or mature IL-2 (SEQ ID NO: 35)).

In certain embodiments, the extended-PK IL-2 is mutated such that it has an altered affinity (e.g., a higher affinity) for the IL-2R alpha receptor compared with unmodified IL-2.

Site-directed mutagenesis can be used to isolate IL-2 mutants that exhibit high affinity binding to CD25, i.e., IL-2R α , as compared to wild-type IL-2. Increasing the affinity of IL-2 for IL-2R α at the cell surface will increase receptor occupancy within a limited range of IL-2 concentration, as well as raise the local concentration of IL-2 at the cell surface.

In certain embodiments, the invention features IL-2 mutants, which may be, but are not necessarily, substantially purified and which can function as high affinity CD25 binders. IL-2 is a T cell growth factor that induces proliferation of antigen- activated T cells and stimulation of NK cells. Exemplary IL-2 mutants which are high affinity binders include those described in WO2013/177187A2 (herein incorporated by reference in its entirety), such as those with amino acid sequences set forth in SEQ ID Nos: 7, 23, 25, 27, 29, and 31. Further exemplary IL- 2 mutants with increased affinity for CD25 are disclosed in US7,569,215, the contents of which are incorporated herein by reference. In one embodiment, the IL-2 mutant does not bind to CD25, e.g., those with amino acid sequences set forth in SEQ ID Nos: 9 and 11.

IL-2 mutants include an amino acid sequence that is at least 80% identical to SEQ ID NO: 33 that bind CD25. For example, an IL-2 mutant can have at least one mutation (e.g., a deletion, addition, or substitution of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more amino acid residues) that increases the affinity for the alpha subunit of the IL-2 receptor relative to wild-type IL-2. It should be understood that mutations identified in mouse IL-2 may be made at corresponding residues in full length human IL-2 (nucleic acid sequence (accession: NM000586) of SEQ ID NO: 32; amino acid sequence (accession: P60568) of SEQ ID NO: 33) or human IL-2 without the signal peptide (nucleic acid sequence of SEQ ID NO: 34; amino acid sequence of SEQ ID NO: 35). Accordingly, in certain embodiments, the IL-2 moiety of the extended-PK IL-2 is human IL-2. In other embodiments, the IL-2 moiety of the extended-PK IL-2 is a mutant human IL-2.

IL-2 mutants can be at least or about 50%, at least or about 65%, at least or about 70%, at least or about 80%, at least or about 85%, at least or about 87%, at least or about 90%, at least or about 95%, at least or about 97%, at least or about 98%, or at least or about 99% identical in amino acid sequence to wild-type IL-2 (in its precursor form or, preferably, the mature form). The mutation can consist of a change in the number or content of amino acid residues. For example, the IL-2 mutants can have a greater or a lesser number of amino acid residues than wild-type IL-2. Alternatively, or in addition, IL-2 mutants can contain a substitution of one or more amino acid residues that are present in the wild-type IL-2.

By way of illustration, a polypeptide that includes an amino acid sequence that is at least 95% identical to a reference amino acid sequence of SEQ ID NO: 33 is a polypeptide that includes a sequence that is identical to the reference sequence except for the inclusion of up to five alterations of the reference amino acid of SEQ ID NO: 33. For example, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with

another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino (N-) or carboxy (C-) terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

The substituted amino acid residue(s) can be, but are not necessarily, conservative substitutions, which typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagines, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. These mutations can be at amino acid residues that contact IL-2R α .

In general, the polypeptides used in the practice of the instant invention will be synthetic, or produced by expression of a recombinant nucleic acid molecule. In the event the polypeptide is an extended-PK IL-2 (e.g., a fusion protein containing at least IL-2 and a heterologous polypeptide, such as a hexa-histidine tag or hemagglutinin tag or an Fc region or human serum albumin), it can be encoded by a hybrid nucleic acid molecule containing one sequence that encodes IL-2 and a second sequence that encodes all or part of the heterologous polypeptide.

The techniques that are required to make IL-2 mutants are routine in the art, and can be performed without resort to undue experimentation by one of ordinary skill in the art. For example, a mutation that consists of a substitution of one or more of the amino acid residues in IL-2 can be created using a PCR-assisted mutagenesis technique (e.g., as known in the art and/or described herein for the creation of IL-2 mutants). Mutations that consist of deletions or additions of amino acid residues to an IL-2 polypeptide can also be made with standard recombinant techniques. In the event of a deletion or addition, the nucleic acid molecule encoding IL-2 is simply digested with an appropriate restriction endonuclease. The resulting fragment can either be expressed directly or manipulated further by, for example, ligating it to a second fragment. The ligation may be facilitated if the two ends of the nucleic acid molecules contain complementary nucleotides that overlap one another, but blunt-ended fragments can also be ligated. PCR-generated nucleic acids can also be used to generate various mutant sequences.

In addition to generating IL-2 mutants via expression of nucleic acid molecules that have been altered by recombinant molecular biological techniques, IL-2 mutants can be

chemically synthesized. Chemically synthesized polypeptides are routinely generated by those of skill in the art.

As noted above, IL-2 can also be prepared as fusion or chimeric polypeptides that include IL-2 and a heterologous polypeptide (i.e., a polypeptide that is not IL-2). The heterologous polypeptide can increase the circulating half-life of the chimeric polypeptide in vivo, and may, therefore, further enhance the properties of IL-2. As discussed in further detail *infra*, the polypeptide that increases the circulating half-life may be serum albumin, such as human or mouse serum albumin.

In other embodiments, the chimeric polypeptide can include IL-2 and a polypeptide that functions as an antigenic tag, such as a FLAG sequence. FLAG sequences are recognized by biotinylated, highly specific, anti-FLAG antibodies, as described herein (see also Blonar et al., Science 256: 1014, 1992; LeClair et al., Proc. Natl. Acad. Sci. USA 89:8145, 1992). In certain embodiments, the chimeric polypeptide further comprises a C-terminal c-myc epitope tag.

Chimeric polypeptides can be constructed using no more than conventional molecular biological techniques, which are well within the ability of those of ordinary skill in the art to perform.

a. Nucleic Acid Molecules Encoding IL-2

IL-2, either alone or as a part of a chimeric polypeptide, such as those described herein, can be obtained by expression of a nucleic acid molecule. Thus, nucleic acid molecules encoding polypeptides containing IL-2 or an IL-2 mutant are considered within the scope of the invention, such as those with nucleic acid sequences set forth in SEQ ID Nos: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34. Just as IL-2 mutants can be described in terms of their identity with wild-type IL-2, the nucleic acid molecules encoding them will necessarily have a certain identity with those that encode wild-type IL-2. For example, the nucleic acid molecule encoding an IL-2 mutant can be at least 50%, at least 65%, preferably at least 75%, more preferably at least 85%, and most preferably at least 95% (e.g., 99%) identical to the nucleic acid encoding full length wild-type IL-2 (e.g., SEQ ID NO: 32 or wild-type IL-2 without the signal peptide (e.g., SEQ ID NO: 34).

The nucleic acid molecules of the invention can contain naturally occurring sequences, or sequences that differ from those that occur naturally, but, due to the degeneracy of the genetic code, encode the same polypeptide. These nucleic acid molecules can consist of RNA or DNA (for example, genomic DNA, cDNA, or synthetic DNA, such as that

produced by phosphoramidite-based synthesis), or combinations or modifications of the nucleotides within these types of nucleic acids. In addition, the nucleic acid molecules can be double-stranded or single-stranded (i.e., either a sense or an antisense strand).

The nucleic acid molecules are not limited to sequences that encode polypeptides; some or all of the non-coding sequences that lie upstream or downstream from a coding sequence (e.g., the coding sequence of IL-2) can also be included. Those of ordinary skill in the art of molecular biology are familiar with routine procedures for isolating nucleic acid molecules. They can, for example, be generated by treatment of genomic DNA with restriction endonucleases, or by performance of the polymerase chain reaction (PCR). In the event the nucleic acid molecule is a ribonucleic acid (RNA), molecules can be produced, for example, by in vitro transcription.

The isolated nucleic acid molecules can include fragments not found as such in the natural state. Thus, the invention encompasses use of recombinant molecules, such as those in which a nucleic acid sequence (for example, a sequence encoding an IL-2 mutant) is incorporated into a vector (e.g., a plasmid or viral vector) or into the genome of a heterologous cell (or the genome of a homologous cell, at a position other than the natural chromosomal location).

As described above, IL-2 mutants of the invention may exist as a part of a chimeric polypeptide. In addition to, or in place of, the heterologous polypeptides described above, a nucleic acid molecule of the invention can contain sequences encoding a "marker" or "reporter." Examples of marker or reporter genes include β -lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r , $G418^r$), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding β -galactosidase), and xanthine guanine phosphoribosyl transferase (XGPRT). As with many of the standard procedures associated with the practice of the invention, skilled artisans will be aware of additional useful reagents, for example, of additional sequences that can serve the function of a marker or reporter.

The nucleic acid molecules of the invention can be obtained by introducing a mutation into IL-2-encoding DNA obtained from any biological cell, such as the cell of a mammal. Thus, the nucleic acids used herein (and the polypeptides they encode) can be those of a mouse, rat, guinea pig, cow, sheep, horse, pig, rabbit, monkey, baboon, dog, or cat. Typically, the nucleic acid molecules will be those of a human.

B. Extended-PK Groups

As described supra, IL-2 or mutant IL-2 is fused to an extended-PK group, which increases circulation half-life. Non-limiting examples of extended-PK groups are described infra. It should be understood that other PK groups that increase the circulation half-life of IL-2, or variants thereof, are also applicable to extended-PK IL-2.

In certain embodiments, the serum half-life of extended-PK IL-2 is increased relative to IL-2 alone (i.e., IL-2 not fused to an extended-PK group). In certain embodiments, the serum half-life of extended-PK IL-2 is at least 20, 40, 60, 80, 100, 120, 150, 180, 200, 400, 600, 800, or 1000% longer relative to the serum half-life of IL-2 alone. In other embodiments, the serum half-life of the extended-PK IL-2 is at least 1.5- fold, 2-fold, 2.5-fold, 3-fold, 3.5 fold, 4-fold, 4.5-fold, 5-fold, 6-fold, 7-fold, 8-fold, 10- fold, 12-fold, 13-fold, 15-fold, 17-fold, 20-fold, 22-fold, 25-fold, 27-fold, 30-fold, 35- fold, 40-fold, or 50-fold greater than the serum half-life of IL-2 alone. In certain embodiments, the serum half-life of the extended-PK IL-2 is at least 10 hours, 15 hours, 20 hours, 25 hours, 30 hours, 35 hours, 40 hours, 50 hours, 60 hours, 70 hours, 80 hours, 90 hours, 100 hours, 110 hours, 120 hours, 130 hours, 135 hours, 140 hours, 150 hours, 160 hours, or 200 hours.

a. Serum albumin and serum albumin binding proteins

In certain embodiments, the extended-PK group is a serum albumin, or fragments thereof. Methods of fusing serum albumin to proteins are disclosed in, e.g., US2010/0144599, US2007/0048282, and US2011/0020345, which are herein incorporated by reference in their entirety. In certain embodiments, the extended-PK group is HSA, or variants or fragments thereof, such as those disclosed in US 5,876,969, WO 2011/124718, WO 2013/075066, and WO 2011/0514789.

In certain embodiments, the extended-PK group is a serum albumin binding protein such as those described in US2005/0287153, US2007/0003549, US2007/0178082, US2007/0269422, US2010/0113339, WO2009/083804, and WO2009/133208, which are herein incorporated by reference in their entirety.

b. PEGylation

In certain embodiments, an extended-PK IL-2 used herein includes a polyethylene glycol (PEG) domain. PEGylation is well known in the art to confer increased circulation half-life to proteins. Methods of PEGylation are well known and disclosed in, e.g., US7,610,156, US7,847,062, all of which are hereby incorporated by reference.

PEG is a well-known, water soluble polymer that is commercially available or can be prepared by ring-opening polymerization of ethylene glycol according to methods well known in the art (Sandler and Karo, Polymer Synthesis, Academic Press, New York, Vol. 3, pages 138-161). The term "PEG" is used broadly to encompass any polyethylene glycol molecule, without regard to size or to modification at an end of the PEG, and can be represented by the formula: $X-0(CH_2CH_2O)_n-1CH_2CH_2OH$, where n is 20 to 2300 and X is H or a terminal modification, e.g., a C_{1-4} alkyl. In one embodiment, the PEG of the invention terminates on one end with hydroxy or methoxy, i.e., X is H or CH_3 ("methoxy PEG"). PEG can contain further chemical groups which are necessary for binding reactions; which results from the chemical synthesis of the molecule; or which is a spacer for optimal distance of parts of the molecule. In addition, such a PEG can consist of one or more PEG side-chains which are linked together. PEGs with more than one PEG chain are called multiarmed or branched PEGs. Branched PEGs can be prepared, for example, by the addition of polyethylene oxide to various polyols, including glycerol, pentaerythriol, and sorbitol. For example, a four-armed branched PEG can be prepared from pentaerythriol and ethylene oxide. Branched PEG are described in, for example, EP- A 0 473 084 and US5, 932,462, both of which are hereby incorporated by reference. One form of PEGs includes two PEG side-chains (PEG2) linked via the primary amino groups of a lysine (Monfardini et al., Bioconjugate Chem 1995;6:62-9).

In one embodiment, pegylated IL-2 is produced by site-directed pegylation, particularly by conjugation of PEG to a cysteine moiety at the N- or C-terminus. A PEG moiety may also be attached by other chemistry, including by conjugation to amines.

PEG conjugation to peptides or proteins generally involves the activation of PEG and coupling of the activated PEG-intermediates directly to target proteins/peptides or to a linker, which is subsequently activated and coupled to target proteins/peptides (see Abuchowski et al., JBC 1977;252:3571 and JBC 1977;252:3582, and Harris et. al., in: Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications; (J. M. Harris ed.) Plenum Press: New York, 1992; Chap. 21 and 22).

A variety of molecular mass forms of PEG can be selected, e.g., from about 1,000 Daltons (Da) to 100,000 Da (n is 20 to 2300), for conjugating to IL-2. The number of repeating units "n" in the PEG is approximated for the molecular mass described in Daltons. It is preferred that the combined molecular mass of PEG on an activated linker is suitable for pharmaceutical use. Thus, in one embodiment, the molecular mass of the PEG molecules does not exceed 100,000 Da. For example, if three PEG molecules are attached to a linker,

where each PEG molecule has the same molecular mass of 12,000 Da (each n is about 270), then the total molecular mass of PEG on the linker is about 36,000 Da (total n is about 820). The molecular masses of the PEG attached to the linker can also be different, e.g., of three molecules on a linker two PEG molecules can be 5,000 Da each (each n is about 110) and one PEG molecule can be 12,000 Da (n is about 270).

One skilled in the art can select a suitable molecular mass for PEG, e.g., based on how the pegylated IL-2 will be used therapeutically, the desired dosage, circulation time, resistance to proteolysis, immunogenicity, and other considerations. For a discussion of PEG and its use to enhance the properties of proteins, see N. V. Katre, *Advanced Drug Delivery Reviews* 1993;10:91-114.

In one embodiment of the invention, PEG molecules may be activated to react with amino groups on IL-2 such as with lysines (Bencham C. O. et al., *Anal. Biochem.*, 131, 25 (1983); Veronese, F. M. et al., *Appl. Biochem.*, 11, 141 (1985); Zalipsky, S. et al., *Polymeric Drugs and Drug Delivery Systems*, adrs 9-110 ACS Symposium Series 469 (1999); Zalipsky, S. et al., *Europ. Polym. J.*, 19, 1177-1183 (1983); Delgado, C. et al., *Biotechnology and Applied Biochemistry*, 12, 119-128 (1990)).

In one embodiment, carbonate esters of PEG are used to form the PEG-IL-2 conjugates. N,N'-disuccinimidylcarbonate (DSC) may be used in the reaction with PEG to form active mixed PEG-succinimidyl carbonate that may be subsequently reacted with a nucleophilic group of a linker or an amino group of IL-2 (see U.S. Pat. No. 5,281,698 and U.S. Pat. No. 5,932,462). In a similar type of reaction, 1,1'-(dibenzotriazolyl)carbonate and di-(2-pyridyl)carbonate may be reacted with PEG to form PEG-benzotriazolyl and PEG-pyridyl mixed carbonate (U.S. Pat. No. 5,382,657), respectively.

Pegylation of IL-2 can be performed according to the methods of the state of the art, for example by reaction of IL-2 with electrophilically active PEGs (Shearwater Corp., USA, www.shearwatercorp.com). Preferred PEG reagents are, e.g., N-hydroxysuccinimidyl propionates (PEG-SPA), butanoates (PEG-SBA), PEG-succinimidyl propionate or branched N-hydroxysuccinimides such as mPEG2-NHS (Monfardini, C, et al., *Bioconjugate Chem.* 6 (1995) 62-69).

In another embodiment, PEG molecules may be coupled to sulfhydryl groups on IL-2 (Sartore, L., et al., *Appl. Biochem. Biotechnol.*, 27, 45 (1991); Morpurgo et al., *Biocon. Chem.*, 7, 363-368 (1996); Goodson et al., *Bio/Technology* (1990) 8, 343; US5,766,897). US6,610,281 and US5,766,897 describe exemplary reactive PEG species that may be coupled to sulfhydryl groups.

In certain embodiments where PEG molecules are conjugated to cysteine residues on IL-2 the cysteine residues are native to IL-2 whereas in other embodiments, one or more cysteine residues are engineered into IL-2. Mutations may be introduced into the coding sequence of IL-2 to generate cysteine residues. This might be achieved, for example, by mutating one or more amino acid residues to cysteine. Preferred amino acids for mutating to a cysteine residue include serine, threonine, alanine and other hydrophilic residues. Preferably, the residue to be mutated to cysteine is a surface-exposed residue. Algorithms are well-known in the art for predicting surface accessibility of residues based on primary sequence or a protein.

In another embodiment, pegylated IL-2 comprise one or more PEG molecules covalently attached to a linker.

In one embodiment, IL-2 is pegylated at the C-terminus. In a specific embodiment, a protein is pegylated at the C-terminus by the introduction of C-terminal azido-methionine and the subsequent conjugation of a methyl-PEG-triarylphosphine compound via the Staudinger reaction. This C-terminal conjugation method is described in Cazalis et al., C-Terminal Site-Specific PEGylation of a Truncated Thrombomodulin Mutant with Retention of Full Bioactivity, *Bioconjug Chem.* 2004; 15(5): 1005- 1009.

Monopegylation of IL-2 can also be achieved according to the general methods described in WO 94/01451. WO 94/01451 describes a method for preparing a recombinant polypeptide with a modified terminal amino acid alpha-carbon reactive group. The steps of the method involve forming the recombinant polypeptide and protecting it with one or more biologically added protecting groups at the N-terminal alpha-amine and C-terminal alpha-carboxyl. The polypeptide can then be reacted with chemical protecting agents to selectively protect reactive side chain groups and thereby prevent side chain groups from being modified. The polypeptide is then cleaved with a cleavage reagent specific for the biological protecting group to form an unprotected terminal amino acid alpha-carbon reactive group. The unprotected terminal amino acid alpha-carbon reactive group is modified with a chemical modifying agent. The side chain protected terminally modified single copy polypeptide is then deprotected at the side chain groups to form a terminally modified recombinant single copy polypeptide. The number and sequence of steps in the method can be varied to achieve selective modification at the N- and/or C-terminal amino acid of the polypeptide.

The ratio of IL-2 to activated PEG in the conjugation reaction can be from about 1:0.5 to 1:50, between from about 1: 1 to 1:30, or from about 1:5 to 1: 15. Various aqueous buffers can be used to catalyze the covalent addition of PEG to IL-2, or variants thereof. In one

embodiment, the pH of a buffer used is from about 7.0 to 9.0. In another embodiment, the pH is in a slightly basic range, e.g., from about 7.5 to 8.5. Buffers having a pKa close to neutral pH range may be used, e.g., phosphate buffer.

Conventional separation and purification techniques known in the art can be used to purify PEGylated IL-2, such as size exclusion (e.g. gel filtration) and ion exchange chromatography. Products may also be separated using SDS-PAGE. Products that may be separated include mono-, di-, tri- poly- and un-pegylated IL-2 as well as free PEG. The percentage of mono-PEG conjugates can be controlled by pooling broader fractions around the elution peak to increase the percentage of mono-PEG in the composition.

In one embodiment, PEGylated IL-2 of the invention contains one, two or more PEG moieties. In one embodiment, the PEG moiety(ies) are bound to an amino acid residue which is on the surface of the protein and/or away from the surface that contacts CD25. In one embodiment, the combined or total molecular mass of PEG in PEG-IL-2 is from about 3,000 Da to 60,000 Da, optionally from about 10,000 Da to 36,000 Da. In one embodiment, PEG in pegylated IL-2 is a substantially linear, straight-chain PEG.

In one embodiment, PEGylated IL-2 of the invention will preferably retain at least 25%, 50%, 60%, 70%, 80%, 85%, 90%, 95% or 100% of the biological activity associated with the unmodified protein. In one embodiment, biological activity refers to the ability to bind CD25. The serum clearance rate of PEG-modified IL-2 may be decreased by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or even 90%, relative to the clearance rate of the unmodified IL-2. PEG-modified IL-2 may have a circulation half-life which is enhanced relative to the half-life of unmodified IL-2. The half-life of PEG-IL-2, or variants thereof, may be enhanced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, 200%, 250%, 300%, 400% or 500%, or even by 1000% relative to the half-life of unmodified IL-2. In certain embodiments, the protein half-life is determined in vitro, such as in a buffered saline solution or in serum. In other embodiments, the protein half-life is an in vivo circulation half-life, such as the half-life of the protein in the serum or other bodily fluid of an animal.

c. Other extended-PK groups

In certain embodiments, the extended-PK group is transferrin, as disclosed in US 7,176,278 and US 8,158,579, which are herein incorporated by reference in their entirety.

In certain embodiments, the extended-PK group is a serum immunoglobulin binding protein such as those disclosed in US2007/0178082, which is herein incorporated by reference in its entirety.

In certain embodiments, the extended-PK group is a fibronectin (Fn)-based scaffold domain protein that binds to serum albumin, such as those disclosed in US2012/0094909, which is herein incorporated by reference in its entirety. Methods of making fibronectin-based scaffold domain proteins are also disclosed in US2012/0094909. A non-limiting example of a Fn3-based extended-PK group is Fn3(HSA), i.e., a Fn3 protein that binds to human serum albumin.

d. Fc Domains

In certain embodiments, an extended-PK IL-2 includes an Fc domain, as described in WO2013177187. The Fc domain does not contain a variable region that binds to antigen. Fc domains useful for producing the extended-PK IL-2 described herein may be obtained from a number of different sources. In certain embodiments, an Fc domain of the extended-PK IL-2 is derived from a human immunoglobulin. In a certain embodiment, the Fc domain is from a human IgG1 constant region (SEQ ID NO: 1). The Fc domain of human IgG1 is set forth in SEQ ID NO: 2. In certain embodiments, the Fc domain of human IgG1 does not have the upper hinge region (SEQ ID NO: 3). It is understood, however, that the Fc domain may be derived from an immunoglobulin of another mammalian species, including for example, a rodent (e.g. a mouse, rat, rabbit, guinea pig) or non-human primate (e.g. chimpanzee, macaque) species. Moreover, the Fc domain or portion thereof may be derived from any immunoglobulin class, including IgM, IgG, IgD, IgA, and IgE, and any immunoglobulin isotype, including IgG1, IgG2, IgG3, and IgG4.

In some aspects, an extended-PK IL-2 includes a mutant Fc domain. In some aspects, an extended-PK IL-2 includes a mutant, IgG1 Fc domain. In some aspects, a mutant Fc domain comprises one or more mutations in the hinge, CH2, and/or CH3 domains. In some aspects, a mutant Fc domain includes a D265A mutation. .

In one embodiment, the extended-PK IL-2 of the invention lacks one or more constant region domains of a complete Fc region, i.e., they are partially or entirely deleted. In certain embodiments, the extended-PK IL-2 of the invention will lack an entire CH2 domain. In certain embodiments, the extended-PK IL-2 of the invention comprise CH2 domain-deleted Fc regions derived from a vector (e.g., from IDEC Pharmaceuticals, San Diego) encoding an IgG1 human constant region domain (see, e.g., WO02/060955A2 and WO02/096948A2).

This exemplary vector is engineered to delete the CH2 domain and provide a synthetic vector expressing a domain-deleted IgG1 constant region. It will be noted that these exemplary constructs are preferably engineered to fuse a binding CH3 domain directly to a hinge region of the respective Fc domain.

Integrin-Binding-Fc Fusion Proteins

Integrins are a family of extracellular matrix adhesion receptors that regulate a diverse array of cellular functions crucial to the initiation, progression and metastasis of solid tumors. The importance of integrins in tumor progression has made them an appealing target for cancer therapy and allows for the treatment of a variety of cancer types. The integrins present on cancerous cells include $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$.

Knottin proteins are small compact peptides that have high thermal and proteolytic stability and are tolerant to mutagenesis, making them good molecular scaffolds. These peptides contain at least 3 disulfide bonds that form a "knot" core. They also contain several loops exposed to the surface, allowing these loops to bind targets. These loops can be engineered to bind specific targets with high affinity, making them a useful tool for therapy.

The present invention involves the use of a knottin polypeptide scaffold engineered with an RGD sequence capable of binding integrins, fused to an Fc donor, which confers a therapeutic benefit (also referred to as "knottin-Fc"). As described *supra*, Fc fragments have been added to proteins and/or therapeutics to extend half-life. In the context of knottin-Fc as used herein, the effector function of Fc contributes to the treatment of a variety of cancers when used in conjunction with systemic IL-2, such as extended-PK IL-2. In certain embodiments, a knottin-Fc that binds two integrins simultaneously is used (2.5D, SEQ ID NO: 85 or 87). In certain embodiments, a knottin-Fc that binds three integrins simultaneously, reflected in Table 1, is used (2.5F, SEQ ID NO: 86 or 88).

A. Methods of Engineering Knottin Polypeptide Scaffolds

Knottin polypeptide scaffolds are used to insert an integrin-binding sequence, preferably in the form of a loop, to confer specific integrin binding. Integrin-binding is preferably engineered into a knottin polypeptide scaffold by inserting an integrin-binding peptide sequence, such as an RGD peptide. In some embodiments, insertion of an integrin-binding peptide sequence results in replacement of portion of the native knottin protein. For example, in one embodiment an RGD peptide sequence is inserted into a native solvent exposed loop by replacing all or a portion of the loop with an RGD-containing peptide sequence (*e.g.*, 5-12

amino acid sequence) that has been selected for binding to one or more integrins. The solvent-exposed loop (*i.e.*, on the surface) will generally be anchored by disulfide-linked cysteine residues in the native knottin protein sequence. The integrin-binding replacement amino acid sequence can be obtained by randomizing codons in the loop portion, expressing the engineered peptide, and selecting the mutants with the highest binding to the predetermined ligand. This selection step may be repeated several times, taking the tightest binding proteins from the previous step and re-randomizing the loops.

Integrin-binding polypeptides may be modified in a number of ways. For example, the polypeptide may be further cross-linked internally, or may be cross-linked to each other, or the RGD loops may be grafted onto other cross linked molecular scaffolds. There are a number of commercially available crosslinking reagents for preparing protein or peptide bioconjugates. Many of these crosslinkers allow dimeric homo- or heteroconjugation of biological molecules through free amine or sulfhydryl groups in protein side chains. More recently, other crosslinking methods involving coupling through carbohydrate groups with hydrazide moieties have been developed. These reagents have offered convenient, facile, crosslinking strategies for researchers with little or no chemistry experience in preparing bioconjugates.

The EETI-II knottin protein (SEQ ID NO: 39) contains a disulfide knotted topology and possesses multiple solvent-exposed loops that are amenable to mutagenesis. Preferred embodiments use EETI-II as the molecular scaffold.

Another example of a knottin protein which can be used as a molecular scaffold is AgRP or agatoxin. The amino acid sequences of AgRP (SEQ ID NO: 40) and agatoxin (SEQ ID NO: 41) differ but their structure is identical. Exemplary AgRP knottins are found in Table 1.

Additional AgRP engineered knottins can be made as described in the above-referenced US 2009/0257952 to Cochran et al. (the contents of which are incorporated herein by reference). AgRP knottin fusions can be prepared using AgRP loops 1, 2 and 3, as well as loop 4 as exemplified above.

The present polypeptides may be produced by recombinant DNA or may be synthesized in solid phase using a peptide synthesizer, which has been done for the peptides of all three scaffolds described herein. They may further be capped at their N-termini by reaction with fluorescein isothiocyanate (FITC) or other labels, and, still further, may be synthesized with amino acid residues selected for additional crosslinking reactions. TentaGel S RAM Fmoc resin (Advanced ChemTech) may be used to give a C-terminal amide upon

cleavage. B-alanine is used as the N-terminal amino acid to prevent thiazolidone formation and release of fluorescein during peptide deprotection (Hermanson, 1996). Peptides are cleaved from the resin and side-chains are deprotected with 8% trifluoroacetic acid, 2% triisopropylsilane, 5% dithiothreitol, and the final product is recovered by ether precipitation. Peptides are purified by reverse phase HPLC using an acetonitrile gradient in 0.1% trifluoroacetic acid and a C4 or C18 column (Vydac) and verified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) or electrospray ionization-mass spectrometry (ESI-MS).

When the present peptides are produced by recombinant DNA, expression vectors encoding the selected peptide are transformed into a suitable host. The host should be selected to ensure proper peptide folding and disulfide bond formation as described above. Certain peptides, such as EETI-II, can fold properly when expressed in prokaryotic hosts such as bacteria.

Dimeric, trimeric, and tetrameric complexes of the present peptides can be formed through genetic engineering of the above sequences or by reaction of the synthetic cross-linkers with engineered peptides carrying an introduced cysteine residue, for example on the C-terminus of the peptide. These oligomeric peptide complexes can be purified by gel filtration. Oligomers of the present peptides can be prepared by preparing vectors encoding multiple peptide sequences end-to-end. Also, multimers may be prepared by complexing the peptides, such as, e.g., described in U.S. Pat. No. 6,265,539. There, an active HIV peptide is prepared in multimer form by altering the amino-terminal residue of the peptide so that it is peptide-bonded to a spacer peptide that contains an amino-terminal lysyl residue and one to about five amino acid residues such as glycyl residues to form a composite polypeptide. Alternatively, each peptide is synthesized to contain a cysteine (Cys) residue at each of its amino- and carboxy-termini. The resulting di-cysteine-terminated (di-Cys) peptide is then oxidized to polymerize the di-Cys peptide monomers into a polymer or cyclic peptide multimer. Multimers may also be prepared by solid phase peptide synthesis utilizing a lysine core matrix. The present peptides may also be prepared as nanoparticles. See, "Multivalent Effects of RGD Peptides Obtained by Nanoparticle Display," Montet, et al., J. Med. Chem.; 2006; 49(20) pp 6087-6093. EETI dimerization may be carried out with the present EETI-II peptides according to the EETI-II dimerization paper: "Grafting of thrombopoietin-mimetic peptides into cystine knot miniproteins yields high-affinity thrombopoietin antagonist and agonists," Krause, et al., FEBS Journal; 2006; 274 pp 86-95. This is further described in PCT application No. PCT/US2013/065610, herein incorporated by reference.

Synergistic sites on fibronectin and other adhesion proteins have been identified for enhanced integrin binding (Ruoslahti, 1996; Koivunen et al., 1994; Aota et al., 1994; Healy et al., 1995). The ability to incorporate different integrin-specific motifs into one soluble molecule would have an important impact on therapeutic development. Crosslinkers with heterofunctional specificity may be used for creating integrin-binding proteins with synergistic binding effects. In addition, these same crosslinkers could easily be used to create bispecific targeting molecules, or as vehicles for delivery of radionuclides or toxic agents for therapeutic applications.

B. Integrin-Binding Peptides

The integrin-binding polypeptides for use in Fc fusions include an integrin-binding loop (e.g., RGD peptide sequence) and a knottin polypeptide scaffold. Such integrin-binding polypeptides are described in US Patent 8,536,301, the contents of which are incorporated herein by reference. As described in US Patent 8,536,301, integrin-binding polypeptides may be varied in the non-RGD residues to a certain degree without affecting binding specificity and potency. For example, if three of the eleven residues were varied, one would have about 70% identity to 2.5D. Table 1 shows exemplary integrin-binding polypeptides within the scope of the invention, and their specific knottin polypeptide scaffold (e.g., EETI-II or AgRP). Preferred integrin-binding polypeptides for use in Fc fusions are peptides 2.5D and 2.5F.

In certain embodiments, the integrin-binding polypeptide binds to $\alpha_v\beta_3$, $\alpha_v\beta_5$, or $\alpha_5\beta_1$ separately.

In certain embodiments, the integrin-binding polypeptide binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ simultaneously.

In certain embodiments, the integrin-binding polypeptide binds to $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ simultaneously.

In certain embodiments, the integrin-binding loop is within an engineered EETI-II scaffold. In certain embodiments, the lysine in position 15 of the EETI-II scaffold is replaced with a serine. In certain embodiments, the integrin-binding polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 42 or 43, wherein X_1 is selected from the group consisting of A, V, L, P, F, Y, S, H, D, and N; X_2 is selected from the group consisting of G, V, L, P, R, E, and Q; X_3 is selected from the group consisting of G, A, and P; X_7 is selected from the group consisting of W and N; X_8 is selected from the group consisting of A, P, and

S; X₉ is selected from the group consisting of P and R; X₁₀ is selected from the group consisting of A, V, L, P, S, T, and E; and X₁₁ is selected from the group consisting of G, A, W, S, T, K, and E. In a further embodiment, the integrin-binding-Fc fusion comprises an integrin-binding polypeptide, as set forth in SEQ ID Nos: 42 or 43, operably linked to a human IgG Fc domain, as set forth in SEQ ID Nos: 2 or 3.

In certain embodiments, the integrin-binding loop is within an engineered AgRP or agatoxin scaffold.

In certain embodiments, the integrin-binding polypeptide is 2.5D and 2.5F, disclosed in Table 1. Any of the integrin-binding polypeptides in Table 1 can be used in Fc fusion as described herein.

Table 1: Integrin Binding Knottin Sequences

SEQ ID NO	Peptide Identifier	Scaffold	Sequence (RGD motif is underlined with flanking residues)
59	1.4A	EETI-II	GC <u>AEPRGDMPTW</u> CKQSDCLAGCVCGPNGFCG
60	1.4B	EETI-II	GC <u>VGGRGDWSPKW</u> CKQSDCPAGCVCGPNGFCG
61	1.4C	EETI-II	GC <u>AELRGDRSYPE</u> CKQSDCLAGCVCGPNGFCG
62	1.4E	EETI-II	GC <u>RLPRGDVPRPH</u> CKQSDCQAGCVCGPNGFCG
63	1.4H	EETI-II	GC <u>YPLRGDNPYAA</u> CKQSDCRAGCVCGPNGFCG
64	1.5B	EETI-II	GC <u>TIGRGDWAPSE</u> CKQSDCLAGCVCGPNGFCG
65	1.5F	EETI-II	GC <u>HPPRGDNPPVT</u> CKQSDCLAGCVCGPNGFCG
66	2.3A	EETI-II	GC <u>PEPRGDNPPPS</u> CKQSDCRAGCVCGPNGFCG
67	2.3B	EETI-II	GC <u>LPPRGDNPPPS</u> CKQSDCQAGCVCGPNGFCG
68	2.3C	EETI-II	GCH <u>LGRGDWAPVG</u> CKQSDCPAGCVCGPNGFCG
69	2.3D	EETI-II	GC <u>NVGRGDWAPSE</u> CKQSDCPAGCVCGPNGFCG
70	2.3E	EETI-II	GC <u>FPGRGDWAPSS</u> CKQSDCRAGCVCGPNGFCG
71	2.3F	EETI-II	GC <u>PLPRGDNPTE</u> CKQSDCQAGCVCGPNGFCG
72	2.3G	EETI-II	GC <u>SEARGDNPRLS</u> CKQSDCRAGCVCGPNGFCG
73	2.3H	EETI-II	GCL <u>LGRGDWAP</u> EACKQSDCRAGCVCPNGFCG
74	2.3I	EETI-II	GCH <u>VGRGDWAPLK</u> CKQSDCQAGCVCGPNGFCG
75	2.3J	EETI-II	GC <u>VRGRGDWAPPS</u> CKQSDCPAGCVCGPNGFCG
76	2.4A	EETI-II	GCL <u>GGRGDWAPP</u> ACKQSDCRAGCVCGPNGFCG
77	2.4C	EETI-II	GC <u>FVGRGDWAPLT</u> CKQSDCQAGCVCGPNGFCG

78	2.4D	EETI-II	GC <u>PVGRGDWSPAS</u> CKQDSDCRAGCVCGPNGFCG
79	2.4E	EETI-II	GC <u>PRPRGDNPLT</u> CKQDSDCLAGCVCGPNGFCG
80	2.4F	EETI-II	GC <u>YQGRGDWSPSS</u> CKQDSDCPAGCVCGPNGFCG
81	2.4G	EETI-II	GC <u>APGRGDWAPSE</u> CKQDSDCQAGCVCGPNGFCG
82	2.4J	EETI-II	GC <u>VQGRGDWSPPS</u> CKQDSDCPAGCVCGPNGFCG
83	2.5A	EETI-II	GC <u>HVGRGDWAPEE</u> CKQDSDCQAGCVCGPNGFCG
84	2.5C	EETI-II	GC <u>DGGRGDWAPPA</u> CKQDSDCRAGCVCGPNGFCG
85	2.5D	EETI-II	GC <u>PQGRGDWAPTS</u> CKQDSDCRAGCVCGPNGFCG
86	2.5F	EETI-II	GC <u>PRPRGDNPLT</u> CKQDSDCLAGCVCGPNGFCG
87	2.5D K15S Mutant	EETI-II	GC <u>PQGRGDWAPTS</u> CSQDSDCLAGCVCGPNGFCG
88	2.5F K15S Mutant	EETI-II	GC <u>PRPRGDNPLT</u> CSQDSDCLAGCVCGPNGFCG
89	2.5H	EETI-II	GC <u>PQGRGDWAPew</u> CKQDSDCPAGCVCGPNGFCG
90	2.5J	EETI-II	GC <u>PRGRGDWSPPA</u> CKQDSDCQAGCVCGPNGFCG
91	3A	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>VVRGDWRKR</u> CYCR
92	3B	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>EERGDMLEK</u> CYCR
93	3C	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>ETRGDGKEK</u> CYCR
94	3D	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>QWRGDGDVK</u> CYCR
95	3E	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>SRRGDMRER</u> CYCR
96	3F	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>QYRGDMKH</u> CYCR
97	3G	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>TGRGDTKVL</u> CYCR
98	3H	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>VERGDMKRR</u> CYCR
99	3I	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>TGRGDVRMN</u> CYCR
100	3J	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>VERGDGMSK</u> CYCR
101	4A	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>RGRGDMRRE</u> CYCR
102	4B	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>EGRGDVKVN</u> CYCR
103	4C	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>VGRGDEKMS</u> CYCR
104	4D	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>VSRGDMRKR</u> CYCR
105	4E	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>ERRGDSVKK</u> CYCR
106	4F	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>EGRGDTRRR</u> CYCR
107	4G	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>EGRGDVVR</u> CYCR
108	4H	AgRp	GCVRLHESCLGQQVPCCDPAATCYC <u>KGRGDNKRK</u> CYCR
109	4I	AgRp	GCVRLHESCLGQQVPCCDPAXTCYC <u>KGRGDVRRV</u> CYCR

110	4J	AgRp	GCVRLHESCLGQQVPCCDPAATCYC VGRGDNKVK CYCR
111	5A	AgRp	GCVRLHESCLGQQVPCCDPAATCYC VGRGDNRLK CYCR
112	5B	AgRp	GCVRLHESCLGQQVPCCDPAATCYC VERGDGMKK CYCR
113	5C	AgRp	GCVRLHESCLGQQVPCCDPAATCYC EGRGDMRRR CYCR
114	5D	AgRp	GCVRLHESCLGQQVPCCDPAATCYC QGRGDGDVK CYCR
115	5E	AgRp	GCVRLHESCLGQQVPCCDPAATCYC SGRGDNDLV CYCR
116	5F	AgRp	GCVRLHESCLGQQVPCCDPAATCYC VERGDGMIR CYCR
117	5G	AgRp	GCVRLHESCLGQQVPCCDPAATCYC SGRGDNDLV CYCR
118	5H	AgRp	GCVRLHESCLGQQVPCCDPAATCYC EGRGDMKMK CYCR
119	5I	AgRp	GCVRLHESCLGQQVPCCDPAATCYC IGRGDVRRR CYCR
120	5J	AgRp	GCVRLHESCLGQQVPCCDPAATCYC EERGDGRKK CYCR
121	6B	AgRp	GCVRLHESCLGQQVPCCDPAATCYC EGRGDRDMK CYCR
122	6C	AgRp	GCVRLHESCLGQQVPCCDPAATCYC TGRGDEKLRC CYCR
123	6E	AgRp	GCVRLHESCLGQQVPCCDPAATCYC VERGDGNRR CYCR
124	6F	AgRp	GCVRLHESCLGQQVPCCDPAATCYC ESRGDVVRK CYCR
125	7C	AgRp	GCVRLHESCLGQQVPCCDPAATCYC YGRGDNDLRC CYCR

The present polypeptides target $\alpha_v\beta_3$, $\alpha_v\beta_5$, and in some cases $\alpha_5\beta_1$ integrin receptors. They do not bind to other integrins tested, such as $\alpha_{iib}\beta_3$, where there was little to no affinity. Thus, these engineered integrin-binding polypeptides have broad diagnostic and therapeutic applications in a variety of human cancers that specifically overexpress the above named integrins. As described below, these polypeptides bind with high affinity to both detergent-solubilized and tumor cell surface integrin receptors.

The $\alpha_v\beta_3$ (and $\alpha_v\beta_5$) integrins are also highly expressed on many tumor cells including osteosarcomas, neuroblastomas, carcinomas of the lung, breast, prostate, and bladder, glioblastomas, and invasive melanomas. The $\alpha_v\beta_3$ integrin has been shown to be expressed on tumor cells and/or the vasculature of breast, ovarian, prostate, and colon carcinomas, but not on normal adult tissues or blood vessels. Also, the $\alpha_5\beta_1$ integrin has been shown to be expressed on tumor cells and/or the vasculature of breast, ovarian, prostate, and colon carcinomas, but not on normal adult tissue or blood vessels. The present, small, conformationally-constrained polypeptides (about 33 amino acids) are so constrained by intramolecular bonds. For example, EETI-II has three disulfide linkages. This will make it more stable in vivo. These peptides target α_v integrins alone, or both α_v and $\alpha_5\beta_1$ integrins.

Until now, it is believed that the development of a single agent that can bind $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins with high affinity and specificity has not been achieved. Since all three of these integrins are expressed on tumors and are involved in mediating angiogenesis and metastasis, a broad spectrum targeting agent (i.e., $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$) will likely be more effective for diagnostic and therapeutic applications.

The present engineered knottin-Fc fusions have several advantages over previously identified integrin-targeting compounds. They possess a compact, disulfide-bonded core that confers proteolytic resistance and exceptional in vivo stability.

Our studies indicate the half-life of integrin-binding-Fc fusion protein in mouse serum to be greater than 90 hours. Their larger size (~3-4 kDa) and enhanced affinity compared to RGD-based cyclic peptides confer enhanced pharmacokinetics and biodistribution for molecular imaging and therapeutic applications. These knottin-Fc proteins are small enough to allow for chemical synthesis and site-specific conjugation of imaging probes, radioisotopes, or chemotherapeutic agents. Furthermore, they can easily be chemically modified to further improve in vivo properties if necessary.

C. Knottin-Fc Fusion

The knottin-Fc fusions described herein and in U.S. Patent Application No. 2014/0073518, herein incorporated by reference in its entirety, combine an engineered integrin-binding polypeptide (within a knottin scaffold) and an Fc domain or antibody like construct capable of binding Fc γ R and inducing ADCC.

The Fc portion of an antibody is formed by the two carboxy terminal domains of the two heavy chains that make up an immunoglobulin molecule. The IgG molecule contains 2 heavy chains (~50 kDa each) and 2 light chains (~25 kDa each). The general structure of all antibodies is very similar, a small region at the tip of the protein is extremely variable, allowing millions of antibodies with slightly different tip structures to exist. This region is known as the hypervariable region (Fab). The other fragment contains no antigen-binding activity but was originally observed to crystallize readily, and for this reason was named the Fc fragment, for Fragment crystallizable. This fragment corresponds to the paired CH₂ and CH₃ domains and is the part of the antibody molecule that interacts with effector molecules and cells. The functional differences between heavy-chain isotypes lie mainly in the Fc fragment. The hinge region that links the Fc and Fab portions of the antibody molecule is in reality a flexible tether, allowing independent movement of the two Fab arms, rather than a

rigid hinge. This has been demonstrated by electron microscopy of antibodies bound to haptens. Thus the present fusion proteins can be made to contain two knottin peptides, one on each arm of the antibody fragment.

The Fc portion varies between antibody classes (and subclasses) but is identical within that class. The C-terminal end of the heavy chain forms the Fc region. The Fc region plays an important role as a receptor binding portion. The Fc portion of antibodies will bind to Fc receptors in two different ways. For example, after IgG and IgM bind to a pathogen by their Fab portion their Fc portions can bind to receptors on phagocytic cells (like macrophages) inducing phagocytosis.

The present knottin-Fc fusions can be implemented such that the Fc portion is used to provide dual binding capability, and/or for half-life extension, for improving expression levels, etc. The Fc fragment in the knottin-Fc can be, for example, from murine IgG2a or human IgG1. Linkers can be optionally used to connect the knottin to the Fc portion. Preferably, the linkers do not affect the binding affinity of the knottin-Fc to integrins or Fc receptors. A variety of Fc domain gene sequences (e.g., mouse and human constant region gene sequences) are available in the form of publicly accessible deposits.

a. Fc-Domains

A variety of Fc domain gene sequences (e.g., mouse and human constant region gene sequences) are available in the form of publicly accessible deposits. Constant region domains comprising an Fc domain sequence can be selected lacking a particular effector function and/or with a particular modification to reduce immunogenicity. Many sequences of antibodies and antibody-encoding genes have been published and suitable Fc domain sequences (e.g., hinge, CH2, and/or CH3 sequences, or portions thereof) can be derived from these sequences using art recognized techniques. The genetic material obtained using any of the foregoing methods may then be altered or synthesized to obtain polypeptides used herein. It will further be appreciated that alleles, variants and mutations of constant region DNA sequences are suitable for use in the methods disclosed herein.

Knottin-Fc suitable for use in the methods disclosed herein may comprise one or more Fc domains (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more Fc domains). In one embodiment, the Fc domains may be of different types. In one embodiment, at least one Fc domain present in a knottin-Fc comprises a hinge domain or portion thereof. In another embodiment, a knottin-Fc comprises at least one Fc domain which comprises at least one CH2 domain or portion thereof. In another embodiment, a knottin-Fc comprises at least one Fc domain which

comprises at least one CH3 domain or portion thereof. In another embodiment, a knottin-Fc comprises at least one Fc domain which comprises at least one CH4 domain or portion thereof. In another embodiment, a knottin-Fc comprises at least one Fc domain which comprises at least one hinge domain or portion thereof and at least one CH2 domain or portion thereof (e.g. in the hinge-CH2 orientation). In another embodiment, a knottin-Fc comprises at least one Fc domain which comprises at least one CH2 domain or portion thereof and at least one CH3 domain or portion thereof (e.g. in the CH2-CH3 orientation). In another embodiment, a knottin-Fc comprises at least one Fc domain comprising at least one hinge domain or portion thereof, at least one CH2 domain or portion thereof, and least one CH3 domain or portion thereof, for example in the orientation hinge-CH2-CH3, hinge-CH3-CH2, or CH2-CH3-hinge.

In certain embodiments, a knottin-Fc comprises at least one complete Fc region derived from one or more immunoglobulin heavy chains (e.g., an Fc domain including hinge, CH2, and CH3 domains, although these need not be derived from the same antibody). In other embodiments a knottin-Fc comprises at least two complete Fc domains derived from one or more immunoglobulin heavy chains. In certain embodiments, the complete Fc domain is derived from a human IgG immunoglobulin heavy chain (e.g., human IgG1).

In another embodiment, a knottin-Fc comprises at least one Fc domain comprising a complete CH3 domain. In another embodiment, a knottin-Fc comprises at least one Fc domain comprising a complete CH2 domain. In another embodiment, a knottin-Fc comprises at least one Fc domain comprising at least a CH3 domain, and at least one of a hinge region, and a CH2 domain. In one embodiment, a knottin-Fc comprises at least one Fc domain comprising a hinge and a CH3 domain. In another embodiment, a knottin-Fc comprises at least one Fc domain comprising a hinge, a CH2, and a CH3 domain. In certain embodiments, the Fc domain is derived from a human IgG immunoglobulin heavy chain (e.g., human IgG1). In certain embodiments, a human IgG1 Fc domain is used with a hinge region mutation, substitution, or deletion to remove or substitute one or more hinge region cysteine residues.

The constant region domains or portions thereof making up an Fc domain of a knottin-Fc may be derived from different immunoglobulin molecules. For example, a polypeptide used in the invention may comprise a CH2 domain or portion thereof derived from an IgG1 molecule and a CH3 region or portion thereof derived from an IgG3 molecule. In another example, a knottin-Fc can comprise an Fc domain comprising a hinge domain derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. As set forth herein, it will be understood by one of ordinary skill in the art that an Fc domain may be

altered such that it varies in amino acid sequence from a naturally occurring antibody molecule.

In other constructs it may be desirable to provide a peptide spacer between one or more constituent Fc domains. For example, a peptide spacer may be placed between a hinge region and a CH2 domain and/or between a CH2 and a CH3 domain. For example, compatible constructs could be expressed wherein the CH2 domain has been deleted and the remaining CH3 domain (synthetic or unsynthetic) is joined to the hinge region with a 1-20, 1-10, or 1-5 amino acid peptide spacer. Such a peptide spacer may be added, for instance, to ensure that the regulatory elements of the constant region domain remain free and accessible or that the hinge region remains flexible. Preferably, any linker peptide compatible with the instant invention will be relatively non-immunogenic and not prevent proper folding of the Fc.

b. Changes to Fc Amino Acids

In certain embodiments, an Fc domain is altered or modified, e.g., by amino acid mutation (e.g., addition, deletion, or substitution). As used herein, the term "Fc domain variant" refers to an Fc domain having at least one amino acid modification, such as an amino acid substitution, as compared to the wild-type Fc from which the Fc domain is derived. For example, wherein the Fc domain is derived from a human IgG1 antibody, a variant comprises at least one amino acid mutation (e.g., substitution) as compared to a wild type amino acid at the corresponding position of the human IgG1 Fc region.

In certain embodiments, the hinge region of human IgG1 Fc domain is altered by an amino acid substitution or deletion to mutate or remove one or more of three hinge region cysteine residues (located at residues 220, 226, and 229 by EU numbering). In some aspects, the upper hinge region is deleted to remove a cysteine that pairs with the light chain. For example, amino acids "EPKSC" in the upper hinge region are deleted, as set forth in SEQ ID NO: 3. In other aspects, one or more of three hinge region cysteines is mutated (e.g., to serine). In certain embodiments, cysteine 220 is mutated to serine.

In certain embodiments, the Fc variant comprises a substitution at an amino acid position located in a hinge domain or portion thereof. In another embodiment, the Fc variant comprises a substitution at an amino acid position located in a CH2 domain or portion thereof. In another embodiment, the Fc variant comprises a substitution at an amino acid position located in a CH3 domain or portion thereof. In another embodiment, the Fc variant

comprises a substitution at an amino acid position located in a CH4 domain or portion thereof.

In certain embodiments, a knottin-Fc fusion comprises an Fc variant comprising more than one amino acid substitution. The knottin-Fc fusion used in the methods described herein may comprise, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid substitutions. Preferably, the amino acid substitutions are spatially positioned from each other by an interval of at least 1 amino acid position or more, for example, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid positions or more. More preferably, the engineered amino acids are spatially positioned apart from each other by an interval of at least 5, 10, 15, 20, or 25 amino acid positions or more.

In certain embodiments, a knottin-Fc fusion comprises an amino acid substitution to an Fc domain which alters the antigen-independent effector functions of the polypeptide, in particular the circulating half-life of the polypeptide.

In one embodiment, the knottin-Fc exhibits enhanced binding to an activating FcγR (e.g. Fcγ1, Fcγ1a, or FcγRIIIa). Exemplary amino acid substitutions which altered FcR or complement binding activity are disclosed in International PCT Publication No. WO 2005/063815 which is incorporated by reference herein. In certain embodiments the Fc region contains at least one of the following mutations: S239D, S239E, L261A, H268D, S298A, A330H, A330L, I332D, I332E, I332Q, K334V, A378F, A378K, A378W, A378Y, H435S, or H435G. In certain embodiments, the Fc region contains at least one of the following mutations: S239D, S239E, I332D or I332E or H268D. In certain embodiments, the Fc region contains at least one of the following mutations: I332D or I332E or H268D.

The knottin-Fc used herein may also comprise an amino acid substitution which alters the glycosylation of the knottin-Fc. For example, the Fc domain of the knottin-Fc may comprise an Fc domain having a mutation leading to reduced glycosylation (e.g., N- or O-linked glycosylation) or may comprise an altered glycoform of the wild-type Fc domain (e.g., a low fucose or fucose-free glycan). In another embodiment, the knottin-Fc has an amino acid substitution near or within a glycosylation motif, for example, an N-linked glycosylation motif that contains the amino acid sequence NXT or NXS. Exemplary amino acid substitutions which reduce or alter glycosylation are disclosed in WO05/018572 and US2007/0111281, which are incorporated by reference herein. In other embodiments, the knottin-Fc used herein comprises at least one Fc domain having engineered cysteine residue or analog thereof which is located at the solvent-exposed surface. In certain embodiments, the

knottin-Fc used herein comprises an Fc domain comprising at least one engineered free cysteine residue or analog thereof that is substantially free of disulfide bonding with a second cysteine residue. Any of the above engineered cysteine residues or analogs thereof may subsequently be conjugated to a functional domain using art-recognized techniques (e.g., conjugated with a thiol-reactive heterobifunctional linker).

In one embodiment, the knottin-Fc used herein may comprise a genetically fused Fc domain having two or more of its constituent Fc domains independently selected from the Fc domains described herein. In one embodiment, the Fc domains are the same. In another embodiment, at least two of the Fc domains are different. For example, the Fc domains of the knottin-Fc used herein comprise the same number of amino acid residues or they may differ in length by one or more amino acid residues (e.g., by about 5 amino acid residues (e.g., 1, 2, 3, 4, or 5 amino acid residues), about 10 residues, about 15 residues, about 20 residues, about 30 residues, about 40 residues, or about 50 residues). In yet other embodiments, the Fc domains of the knottin-Fc used herein may differ in sequence at one or more amino acid positions. For example, at least two of the Fc domains may differ at about 5 amino acid positions (e.g., 1, 2, 3, 4, or 5 amino acid positions), about 10 positions, about 15 positions, about 20 positions, about 30 positions, about 40 positions, or about 50 positions).

Immune Checkpoint Blocker

In certain embodiments, immune checkpoint blockers are used in combination with other therapeutic agents described herein (e.g., extended-PK IL-2 and integrin-binding-Fc fusion protein). T cell activation and effector functions are balanced by co-stimulatory and inhibitory signals, referred to as "immune checkpoints." Inhibitory ligands and receptors that regulate T cell effector functions are overexpressed on tumor cells. Subsequently, agonists of co-stimulatory receptors or antagonists of inhibitory signals, result in the amplification of antigen-specific T cell responses. In contrast to therapeutic antibodies which target tumor cells directly, immune checkpoint blockers enhance endogenous anti-tumor activity. In certain embodiments, the immune checkpoint blocker suitable for use in the methods disclosed herein, is an antagonist of inhibitory signals, e.g., an antibody which targets, for example, PD-1, PD-L1, CTLA-4, LAG3, B7-H3, B7-H4, and TIM3. These ligands and receptors are reviewed in Pardoll, D., *Nature*. 12: 252-264, 2012.

Disclosed herein are methods for treating a subject afflicted with diseases such as cancer, which methods comprise administering to the subject a composition comprising a therapeutically effective amount of a molecule which blocks the immune checkpoint, and an

integrin-binding-Fc fusion protein. In certain embodiments, the methods for treating a subject afflicted with diseases such as cancer, which methods comprise administering to the subject a composition comprising a therapeutically effective amount of a molecule which blocks the immune checkpoint, an integrin-binding-Fc fusion protein, and IL-2 (e.g., extended-PK IL-2). In certain embodiments, the immune checkpoint blocker is an antibody or an antigen-binding portion thereof, that disrupts or inhibits signaling from an inhibitory immunoregulator. In certain embodiments, the immune checkpoint blocker is a small molecule that disrupts or inhibits signaling from an inhibitory immunoregulator.

In certain embodiments, the inhibitory immunoregulator (immune checkpoint blocker) is a component of the PD-1/PD-L1 signaling pathway. Accordingly, certain embodiments provide methods for immunotherapy of a subject afflicted with cancer, which methods comprise administering to the subject a therapeutically effective amount of an antibody or an antigen-binding portion thereof that disrupts the interaction between the PD-1 receptor and its ligand, PD-L1. Antibodies known in the art which bind to PD-1 and disrupt the interaction between the PD-1 and its ligand, PD-L1, and stimulates an anti-tumor immune response, are suitable for use in the methods disclosed herein. In certain embodiments, the antibody or antigen-binding portion thereof binds specifically to PD-1. For example, antibodies that target PD-1 include, e.g., nivolumab (BMS-936558, Bristol-Myers Squibb) and pembrolizumab (lambrolizumab, MK03475, Merck). Other suitable antibodies for use in the methods disclosed herein are anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, herein incorporated by reference. In certain embodiments, the antibody or antigen-binding portion thereof binds specifically to PD-L1 and inhibits its interaction with PD-1, thereby increasing immune activity. Antibodies known in the art which bind to PD-L1 and disrupt the interaction between the PD-1 and PD-L1, and stimulates an anti-tumor immune response, are suitable for use in the methods disclosed herein. For example, antibodies that target PD-L1 and are in clinical trials, include BMS-936559 (Bristol-Myers Squibb) and MPDL3280A (Genetech). Other suitable antibodies that target PD-L1 are disclosed in U.S. Patent No. 7,943,743, herein incorporated by reference. It will be understood by one of ordinary skill that any antibody which binds to PD-1 or PD-L1, disrupts the PD-1/PD-L1 interaction, and stimulates an anti-tumor immune response, are suitable for use in the methods disclosed herein.

In certain embodiments, the inhibitory immunoregulator is a component of the CTLA-4 signaling pathway. Accordingly, certain embodiments provide methods for immunotherapy of a subject afflicted with cancer, which methods comprise administering to the subject a

therapeutically effective amount of an antibody or an antigen-binding portion thereof that targets CTLA-4 and disrupts its interaction with CD80 and CD86. Exemplary antibodies that target CTLA-4 include ipilimumab (MDX-010, MDX-101, Bristol-Myers Squibb), which is FDA approved, and tremelimumab (ticilimumab, CP-675, 206, Pfizer), currently undergoing human trials. Other suitable antibodies that target CTLA-4 are disclosed in WO 2012/120125, U.S. Patents No. 6,984,720, No. 6,682,7368, and U.S. Patent Applications 2002/0039581, 2002/0086014, and 2005/0201994, herein incorporated by reference. It will be understood by one of ordinary skill that any antibody which binds to CTLA-4, disrupts its interaction with CD80 and CD86, and stimulates an anti-tumor immune response, are suitable for use in the methods disclosed herein.

In certain embodiments, the inhibitory immunoregulator is a component of the LAG3 (lymphocyte activation gene 3) signaling pathway. Accordingly, certain embodiments provide methods for immunotherapy of a subject afflicted with cancer, which methods comprise administering to the subject a therapeutically effective amount of an antibody or an antigen-binding portion thereof that targets LAG3 and disrupts its interaction with MHC class II molecules. An exemplary antibody that targets LAG3 is IMP321 (Immutep), currently undergoing human trials. Other suitable antibodies that target LAG3 are disclosed in U.S. Patent Application 2011/0150892, herein incorporated by reference. It will be understood by one of ordinary skill that any antibody which binds to LAG3, disrupts its interaction with MHC class II molecules, and stimulates an anti-tumor immune response, are suitable for use in the methods disclosed herein.

In certain embodiments, the inhibitory immunoregulator is a component of the B7 family signaling pathway. In certain embodiments, the B7 family members are B7-H3 and B7-H4. Accordingly, certain embodiments provide methods for immunotherapy of a subject afflicted with cancer, which methods comprise administering to the subject a therapeutically effective amount of an antibody or an antigen-binding portion thereof that targets B7-H3 or -H4. The B7 family does not have any defined receptors but these ligands are upregulated on tumor cells or tumor-infiltrating cells. Preclinical mouse models have shown that blockade of these ligands can enhance anti-tumor immunity. An exemplary antibody that targets B7-H3 is MGA271 (MacroGenics), currently undergoing human trials. Other suitable antibodies that target B7 family members are disclosed in U.S. Patent Application 2013/0149236, herein incorporated by reference. It will be understood by one of ordinary skill that any antibody which binds to B7-H3 or H4, and stimulates an anti-tumor immune response, are suitable for use in the methods disclosed herein.

In certain embodiments, the inhibitory immunoregulator is a component of the TIM3 (T cell membrane protein 3) signaling pathway. Accordingly, certain embodiments provide methods for immunotherapy of a subject afflicted with cancer, which methods comprise administering to the subject a therapeutically effective amount of an antibody or an antigen-binding portion thereof that targets TIM3 and disrupts its interaction with galectin 9. Suitable antibodies that target TIM3 are disclosed in U.S. Patent Application 2013/0022623, herein incorporated by reference. It will be understood by one of ordinary skill that any antibody which binds to TIM3, disrupts its interaction with galectin 9, and stimulates an anti-tumor immune response, are suitable for use in the methods disclosed herein.

It should be understood that antibodies targeting immune checkpoints suitable for use in the methods disclosed herein are not limited to those described *supra*. Moreover, it will be understood by one of ordinary skill in the art that other immune checkpoint targets can also be targeted by antagonists or antibodies in the methods described herein, provided that the targeting results in the stimulation of an anti-tumor immune response as reflected in, e.g., an increase in T cell proliferation, enhanced T cell activation, and/or increased cytokine production (e.g., IFN- γ , IL-2).

Alternatives to Immune Checkpoint Blockers

In certain embodiments, an antagonist of vascular endothelial growth factor (VEGF) is used in place of an immune checkpoint blocker. VEGF has recently been demonstrated to play a role in immune suppression (Liang, W.-C. et al. *J. Biol. Chem.* (2006) Vol 281: 951–961; Voron, T. et al. *Front Oncol* (2014) Vol. 4: Article 70; Terme, M. et al., *Clin Dev Immunol* (2012) Vol. 2012: Article ID 492920; Kandalaft, E. et al., *Curr Top Microbiol Immunol* (2011) Vol 344: 129-48), therefore blocking its activity would enhance the immune response, similar to that of an immune checkpoint blocker. A "VEGF antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with VEGF activities including its binding to one or more VEGF receptors. Non-limiting examples of VEGF antagonists include anti-VEGF antibodies and antigen-binding fragments thereof, receptor molecules and derivatives which bind specifically to VEGF thereby sequestering its binding to one or more receptors (e.g., a VEGF receptor), anti-VEGF receptor antibodies, VEGF receptor antagonists such as small molecule inhibitors of the VEGFR tyrosine kinases, or a dominant negative VEGF.

In certain embodiments, the VEGF antagonist is an antibody. An "anti-VEGF antibody" is an antibody that binds to VEGF with sufficient affinity and specificity. Non-

limiting examples of anti-VEGF antibodies are described in U.S. Patent Nos. 6,884,879, 7,060,269, 6,582,959, 6,703,030, 6,054,297, US Patent Application Nos. 2006009360, 20050186208, 20030206899, 20030190317, 20030203409, 20050112126, and PCT Publication Nos. WO 98/45332, 96/30046, 94/10202, 05/044853, 13/181452. The contents of these patents and patent applications are herein incorporated by reference. In certain embodiments the VEGF antibody is bevacizumab (Avastin ® Genentech/Roche) or ranibizumab (Lucentis ® Genentech/Roche).

In certain embodiments, the VEGF antagonist binds to the VEGF receptor. VEGF receptors, or fragments thereof, that specifically bind to VEGF can be used to bind to and sequester the VEGF protein, thereby preventing it from activating downstream signaling. In certain embodiments, the VEGF receptor, or VEGF binding fragment thereof, is a soluble VEGF receptor, such as sFlt-1. The soluble form of the receptor exerts an inhibitory effect on the biological activity of VEGF by binding to VEGF, thereby preventing it from binding to its natural receptors present on the surface of target cells. Non-limiting examples of VEGF antagonists which bind the VEGF receptor are disclosed in PCT Application Nos. 97/44453, 05/000895 and U.S. Patent Application No. 20140057851. In certain embodiments the VEGF antagonist is a polypeptide with a bifunctional single-chain antagonistic human VEGF variant comprising a modified VEGF wherein the modified VEGF comprises a loop with an integrin-recognition RGD sequence, as described in U.S. Patent Nos. 8,741,839, herein incorporated by reference.

Linkers

In certain embodiments, the extended-PK group is optionally fused to IL-2 via a linker. In certain embodiments, an integrin-binding polypeptide is fused to an Fc fragment via a linker. Suitable linkers are well known in the art, such as those disclosed in, e.g., US2010/0210511 US2010/0179094, and US2012/0094909, which are herein incorporated by reference in its entirety. Exemplary linkers include gly-ser polypeptide linkers, glycine-proline polypeptide linkers, and proline-alanine polypeptide linkers. In a certain embodiment, the linker is a gly-ser polypeptide linker, i.e., a peptide that consists of glycine and serine residues.

Exemplary gly-ser polypeptide linkers comprise the amino acid sequence Ser(Gly₄Ser)_n. In one embodiment, n=1. In one embodiment, n=2. In another embodiment, n=3, i.e., Ser(Gly₄Ser)₃. In another embodiment, n=4, i.e., Ser(Gly₄Ser)₄. In another embodiment, n=5. In yet another embodiment, n=6. In another embodiment, n=7. In yet

another embodiment, $n=8$. In another embodiment, $n=9$. In yet another embodiment, $n=10$. Another exemplary gly-ser polypeptide linker comprises the amino acid sequence $\text{Ser}(\text{Gly}_4\text{Ser})_n$. In one embodiment, $n=1$. In one embodiment, $n=2$. In a certain embodiment, $n=3$. In another embodiment, $n=4$. In another embodiment, $n=5$. In yet another embodiment, $n=6$. Another exemplary gly-ser polypeptide linker comprises $(\text{Gly}_4\text{Ser})_n$. In one embodiment, $n=1$. In one embodiment, $n=2$. In a certain embodiment, $n=3$. In another embodiment, $n=4$. In another embodiment, $n=5$. In yet another embodiment, $n=6$. Another exemplary gly-ser polypeptide linker comprises $(\text{Gly}_3\text{Ser})_n$. In one embodiment, $n=1$. In one embodiment, $n=2$. In a certain embodiment, $n=3$. In another embodiment, $n=4$. In another embodiment, $n=5$. In yet another embodiment, $n=6$.

Other Therapeutic Agents

The integrin-binding-Fc fusion protein suitable for use in the methods disclosed herein, can be used in conjunction with one or more therapeutic agents. In one embodiment, the therapeutic agent is a therapeutic antibody. In another embodiment, the therapeutic agent is a therapeutic protein. In another embodiment, the therapeutic agent is a small molecule. In another embodiment, the therapeutic agent is an antigen. In another embodiment, the therapeutic agent is a population of cells.

Engineered Fusion Molecules

Also provided herein are engineered molecules that comprise two or more of IL-2, and an antibody (e.g., a therapeutic antibody, an immune checkpoint blocker, or an antibody that antagonizes VEGF) or antibody fragment described herein. Such engineered molecules can effectively reduce the number of components to be administered to a subject (e.g., a cancer patient) in the methods described herein. In some embodiments, the antibody or antibody fragment serves as the scaffold for conjugation with other components (e.g., IL-2).

Accordingly, in certain embodiments, the engineered molecule comprises IL-2 and an antibody or antibody fragment. In a particular embodiment, the antibody for use in the engineered protein is a bispecific antibody, wherein one component is a therapeutic antibody and the other component is an antibody that binds to an immune checkpoint blocker or an antibody that antagonizes VEGF activity. Methods for generating bispecific antibodies are known in the art.

Accordingly, in certain embodiments, the engineered molecule comprises IL-2 and a bispecific antibody which binds to a therapeutic target and an immune checkpoint blocker or an antibody that antagonizes VEGF.

In certain embodiments, the IL-2 component for use in the engineered protein is an IL-2 lacking a pharmacokinetic moiety (i.e., a non-extended-PK IL-2). In other embodiments, the IL-2 comprises a pharmacokinetic moiety (an extended-PK IL-2).

In certain embodiments, the components of the engineered molecule are conjugated to the antibody or bispecific antibody with or without a linker. Suitable linkers for conjugation are described herein and extensively described in the art.

Regions to which polypeptide-based components (e.g., IL-2) of the engineered molecule can be fused, with or without a linker, to the antibody are generally known in the art, and include, for example, the C-terminus of the antibody heavy chain, and the C-terminus of the antibody light chain.

In certain embodiments, components of the engineered molecule do not interfere with the function of the other components. By way of example, when the engineered protein comprises a therapeutic antibody and IL-2, the IL-2 will be fused to the therapeutic antibody in a manner such that the antibody retains its antigen-binding function, and IL-2 retains the ability to interact with its receptor. The methods described herein, e.g., in the Examples, can be used to determine whether components of the engineered protein retain their respective functions.

Methods of Making Polypeptides

In some aspects, the polypeptides described herein (e.g., IL-2, such as extended-PK IL-2, and knottin-Fc) are made in transformed host cells using recombinant DNA techniques. To do so, a recombinant DNA molecule coding for the peptide is prepared. Methods of preparing such DNA molecules are well known in the art. For instance, sequences coding for the peptides could be excised from DNA using suitable restriction enzymes. Alternatively, the DNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidate method. Also, a combination of these techniques could be used.

The methods of making polypeptides also include a vector capable of expressing the peptides in an appropriate host. The vector comprises the DNA molecule that codes for the peptides operatively linked to appropriate expression control sequences. Methods of affecting this operative linking, either before or after the DNA molecule is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers,

operators, ribosomal nuclease domains, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation.

The resulting vector having the DNA molecule thereon is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial hosts include bacteria (such as *E. coli* sp.), yeast (such as *Saccharomyces* sp.) and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

Next, the transformed host is cultured and purified. Host cells may be cultured under conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art. Finally, the peptides are purified from culture by methods well known in the art.

The compounds may also be made by synthetic methods. For example, solid phase synthesis techniques may be used. Suitable techniques are well known in the art, and include those described in Merrifield (1973), *Chem. Polypeptides*, pp. 335-61 (Katsoyannis and Panayotis eds.); Merrifield (1963), *J. Am. Chem. Soc.* 85: 2149; Davis et al. (1985), *Biochem. Intl.* 10: 394-414; Stewart and Young (1969), *Solid Phase Peptide Synthesis*; U.S. Pat. No. 3,941,763; Finn et al. (1976), *The Proteins* (3rd ed.) 2: 105-253; and Erickson et al. (1976), *The Proteins* (3rd ed.) 2: 257-527. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides. Compounds that contain derivatized peptides or which contain non-peptide groups may be synthesized by well-known organic chemistry techniques.

Other methods of molecule expression/synthesis are generally known in the art to one of ordinary skill.

Expression of Polypeptides

The nucleic acid molecules described above can be contained within a vector that is capable of directing their expression in, for example, a cell that has been transduced with the vector. Accordingly, in addition to extended-PK IL-2 and knottin-Fc mutants, expression vectors containing a nucleic acid molecule encoding an extended-PK IL-2 or knottin-Fc mutant and cells transfected with these vectors are among the certain embodiments.

Vectors suitable for use include T7-based vectors for use in bacteria (see, for example, Rosenberg et al., *Gene* 56: 125, 1987), the pMSXND expression vector for use in mammalian cells (Lee and Nathans, *J. Biol. Chem.* 263:3521, 1988), and baculovirus-derived vectors (for example the expression vector pBacPAKS from Clontech, Palo Alto, Calif.) for use in insect cells. The nucleic acid inserts, which encode the polypeptide of interest in such vectors, can be operably linked to a promoter, which is selected based on, for example, the cell type in which expression is sought. For example, a T7 promoter can be used in bacteria, a polyhedrin promoter can be used in insect cells, and a cytomegalovirus or metallothionein promoter can be used in mammalian cells. Also, in the case of higher eukaryotes, tissue-specific and cell type-specific promoters are widely available. These promoters are so named for their ability to direct expression of a nucleic acid molecule in a given tissue or cell type within the body. Skilled artisans are well aware of numerous promoters and other regulatory elements which can be used to direct expression of nucleic acids.

In addition to sequences that facilitate transcription of the inserted nucleic acid molecule, vectors can contain origins of replication, and other genes that encode a selectable marker. For example, the neomycin-resistance (*neo^r*) gene imparts G418 resistance to cells in which it is expressed, and thus permits phenotypic selection of the transfected cells. Those of skill in the art can readily determine whether a given regulatory element or selectable marker is suitable for use in a particular experimental context.

Viral vectors that can be used in the invention include, for example, retroviral, adenoviral, and adeno-associated vectors, herpes virus, simian virus 40 (SV40), and bovine papilloma virus vectors (see, for example, Gluzman (Ed.), *Eukaryotic Viral Vectors*, CSH Laboratory Press, Cold Spring Harbor, N.Y.).

Prokaryotic or eukaryotic cells that contain and express a nucleic acid molecule that encodes an extended-PK IL-2 or knottin-Fc mutant are also features of the invention. A cell of the invention is a transfected cell, i.e., a cell into which a nucleic acid molecule, for example a nucleic acid molecule encoding an extended-PK IL-2 mutant or knottin-Fc, has

been introduced by means of recombinant DNA techniques. The progeny of such a cell are also considered within the scope of the invention.

The precise components of the expression system are not critical. For example, an extended-PK IL-2 or knottin-Fc mutant can be produced in a prokaryotic host, such as the bacterium *E. coli*, or in a eukaryotic host, such as an insect cell (e.g., an Sf21 cell), or mammalian cells (e.g., COS cells, NIH 3T3 cells, or HeLa cells). These cells are available from many sources, including the American Type Culture Collection (Manassas, Va.). In selecting an expression system, it matters only that the components are compatible with one another. Artisans of ordinary skill are able to make such a determination. Furthermore, if guidance is required in selecting an expression system, skilled artisans may consult Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley and Sons, New York, N.Y., 1993) and Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, 1985 Suppl. 1987).

The expressed polypeptides can be purified from the expression system using routine biochemical procedures, and can be used, e.g., as therapeutic agents, as described herein.

Pharmaceutical Compositions and Modes of Administration

In certain embodiments, IL-2 is administered together (simultaneously or sequentially) with a knottin-Fc. In certain embodiments, IL-2 is administered prior to the administration of a knottin-Fc. In certain embodiments, IL-2 is administered concurrently with the administration of a knottin-Fc. In certain embodiments, IL-2 is administered subsequent to the administration of a knottin-Fc. In certain embodiments, the IL-2 and a knottin-Fc are administered simultaneously. In other embodiments, the IL-2 and a knottin-Fc are administered sequentially. In yet other embodiments, the IL-2 and a knottin-Fc are administered within one, two, or three days of each other.

In certain embodiments, IL-2 and knottin-Fc are administered with an immune checkpoint blocker. In certain embodiments the immune checkpoint blocker is an anti-PD-1 antibody. In certain embodiments, the immune checkpoint blocker is an anti-CTLA-4 antibody. In certain embodiments, an antagonist of VEGF is used in place of an immune checkpoint blocker.

Pharmaceutical compositions of the invention can be administered in combination therapy, i.e., combined with other agents. Agents include, but are not limited to, in vitro synthetically prepared chemical compositions, antibodies, antigen binding regions, and

combinations and conjugates thereof. In certain embodiments, an agent can act as an agonist, antagonist, allosteric modulator, or toxin.

In certain embodiments, the invention provides for separate pharmaceutical compositions comprising extended-PK IL-2 with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant, and another pharmaceutical composition comprising a knottin-Fc with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. In certain embodiments, the invention further provides for a separate pharmaceutical composition comprising an immune checkpoint blocker (or an antagonist of VEGF) with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. In certain embodiments, the pharmaceutical compositions comprise both extended-PK IL-2 and knottin-Fc with a pharmaceutically acceptable diluents, carrier, solubilizer, emulsifier, preservative and/or adjuvant. In certain embodiments, the pharmaceutical composition comprises extended-PK IL-2, knottin-Fc, and an immune checkpoint blocker (or an antagonist of VEGF) with a pharmaceutically acceptable diluents, carrier, solubilizer, emulsifier, preservative and/or adjuvant.

In certain embodiments, the invention provides for pharmaceutical compositions comprising extended-PK IL-2, together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant, and another pharmaceutical composition comprises a knottin-Fc, together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. In certain embodiments, the invention provides for pharmaceutical compositions comprising an immune checkpoint blocker, together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. In certain embodiments, each of the agents, e.g., extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), can be formulated as separate compositions. In certain embodiments, acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed. In certain embodiments, the formulation material(s) are for s.c. and/or I.V. administration. In certain embodiments, the pharmaceutical composition can contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolality, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. In certain embodiments, suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); antimicrobials; antioxidants (such as ascorbic acid, sodium sulfite or sodium

hydrogen- sulfite); buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates or other organic acids); bulking agents (such as mannitol or glycine); chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta- cyclodextrin); fillers; monosaccharides; disaccharides; and other carbohydrates (such as glucose, mannose or dextrans); proteins (such as serum albumin, gelatin or immunoglobulins); coloring, flavoring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such as glycerin, propylene glycol or polyethylene glycol); sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20, polysorbate 80, triton, tromethamine, lecithin, cholesterol, tyloxapal); stability enhancing agents (such as sucrose or sorbitol); tonicity enhancing agents (such as alkali metal halides, preferably sodium or potassium chloride, mannitol sorbitol); delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. (Remington's Pharmaceutical Sciences, 18th Edition, A. R. Gennaro, ed., Mack Publishing Company (1995)). In certain embodiments, the formulation comprises PBS; 20 mM NaOAC, pH 5.2, 50 mM NaCl; and/or 10 mM NAOAC, pH 5.2, 9% Sucrose. In certain embodiments, the optimal pharmaceutical composition will be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format and desired dosage. See, for example, Remington's Pharmaceutical Sciences, *supra*. In certain embodiments, such compositions may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF).

In certain embodiments, the primary vehicle or 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 extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), can be 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 extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), can be formulated as a lyophilizate using appropriate excipients such as sucrose.

In certain embodiments, the pharmaceutical composition can be selected for parenteral delivery. In certain embodiments, the compositions can be selected for inhalation or for delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the ability of one skilled in the art.

In certain embodiments, the formulation components are present in concentrations that are acceptable to the site of administration. In certain embodiments, buffers are used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

In certain embodiments, when parenteral administration is contemplated, a therapeutic composition can be in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising a desired extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), in a pharmaceutically acceptable vehicle. In certain embodiments, a vehicle for parenteral injection is sterile distilled water in which extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), are formulated as a sterile, isotonic solution, properly preserved. In certain embodiments, the preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid or polyglycolic acid), beads or liposomes, that can provide for the controlled or sustained release of the product which can then be delivered via a depot injection. In certain embodiments, hyaluronic acid can also be used, and can have the effect of promoting sustained duration in the circulation. In certain embodiments, implantable drug delivery devices can be used to introduce the desired molecule.

In certain embodiments, a pharmaceutical composition can be formulated for inhalation. In certain embodiments, extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), can be formulated as a dry powder for inhalation. In certain embodiments, an inhalation solution comprising extended-PK IL-2,

a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), can be formulated with a propellant for aerosol delivery. In certain embodiments, solutions can be nebulized. Pulmonary administration is further described in PCT application No. PCT/US94/001875, which describes pulmonary delivery of chemically modified proteins.

In certain embodiments, it is contemplated that formulations can be administered orally. In certain embodiments, extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), that is administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. In certain embodiments, a capsule can be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. In certain embodiments, at least one additional agent can be included to facilitate absorption of extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF). In certain embodiments, diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

In certain embodiments, a pharmaceutical composition can involve an effective quantity of extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), in a mixture with non-toxic excipients which are suitable for the manufacture of tablets. In certain embodiments, by dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit-dose form. In certain embodiments, suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional pharmaceutical compositions will be evident to those skilled in the art, including formulations involving extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), in sustained- or controlled-delivery formulations. In certain embodiments, techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. See for example, PCT Application No. PCT/US93/00829 which describes the controlled release of porous polymeric microparticles for the delivery of pharmaceutical compositions. In certain embodiments, sustained-release preparations can include semipermeable polymer

matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices can include polyesters, hydrogels, polylactides (U.S. Pat. No. 3,773,919 and EP 058,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., *Biopolymers*, 22:547-556 (1983)), poly (2-hydroxyethyl-methacrylate) (Langer et al., *J. Biomed. Mater. Res.*, 15: 167-277 (1981) and Langer, *Chem. Tech.*, 12:98- 105 (1982)), ethylene vinyl acetate (Langer et al., *supra*) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). In certain embodiments, sustained release compositions can also include liposomes, which can be prepared by any of several methods known in the art. See, e.g., Eppstein et al, *Proc. Natl. Acad. Sci. USA*, 82:3688-3692 (1985); EP 036,676; EP 088,046 and EP 143,949.

The pharmaceutical composition to be used for in vivo administration typically is sterile. In certain embodiments, this can be accomplished by filtration through sterile filtration membranes. In certain embodiments, where the composition is lyophilized, sterilization using this method can be conducted either prior to or following lyophilization and reconstitution. In certain embodiments, the composition for parenteral administration can be stored in lyophilized form or in a solution. In certain embodiments, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

In certain embodiments, once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. In certain embodiments, such formulations can be stored either in a ready-to-use form or in a form (e.g., lyophilized) that is reconstituted prior to administration.

In certain embodiments, kits are provided for producing a single-dose administration unit. In certain embodiments, the kit can contain both a first container having a dried protein and a second container having an aqueous formulation. In certain embodiments, kits containing single and multi-chambered pre-filled syringes (e.g., liquid syringes and lyosyringes) are included.

In certain embodiments, the effective amount of a pharmaceutical composition comprising extended-PK IL-2 and/or one or more pharmaceutical compositions comprising a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment, according to certain embodiments, will thus vary depending, in part, upon the molecule delivered, the indication for which extended-PK IL-2, a knottin-Fc, and optionally

an immune checkpoint blocker (or an antagonist of VEGF), are being used, the route of administration, and the size (body weight, body surface or organ size) and/or condition (the age and general health) of the patient. In certain embodiments, the clinician can titer the dosage and modify the route of administration to obtain the optimal therapeutic effect. In certain embodiments, a typical dosage of extended-PK IL-2 and a knottin-Fc can each range from about 0.1 µg/kg to up to about 100 mg/kg or more, depending on the factors mentioned above. In certain embodiments, the dosage can range from 0.1 µg/kg up to about 100 mg/kg; or 1 µg/kg up to about 100 mg/kg; or 5 µg/kg up to about 100 mg/kg.

In certain embodiments, a typical dosage for an immune checkpoint blocker can range from about 0.1 mg/kg to up to about 300 mg/kg or more, depending on the factors mentioned above. In certain embodiments, the dosage can range from 1 mg/kg up to about 300 mg/kg; or 5 mg/kg up to about 300 mg/kg; or 10 mg/kg up to about 300 mg/kg.

In certain embodiments, the frequency of dosing will take into account the pharmacokinetic parameters of extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), in the formulation used. In certain embodiments, a clinician will administer the composition until a dosage is reached that achieves the desired effect. In certain embodiments, the composition can therefore be administered as a single dose, or as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. In certain embodiments, appropriate dosages can be ascertained through use of appropriate dose-response data. As described in the Examples below, dosing can occur daily, every other day, or weekly, all with similar anti-tumor responses.

In certain embodiments, the route of administration of the pharmaceutical composition is in accord with known methods, e.g. orally, through injection by intravenous, intraperitoneal, intracerebral (intra-parenchymal), intracerebroventricular, intramuscular, subcutaneously, intra-ocular, intraarterial, intraportal, or intralesional routes; by sustained release systems or by implantation devices. In certain embodiments, the compositions can be administered by bolus injection or continuously by infusion, or by implantation device. In certain embodiments, individual elements of the combination therapy may be administered by different routes.

In certain embodiments, the composition can be administered locally via implantation of a membrane, sponge or another appropriate material onto which the desired molecule has

been absorbed or encapsulated. In certain embodiments, where an implantation device is used, the device can be implanted into any suitable tissue or organ, and delivery of the desired molecule can be via diffusion, timed-release bolus, or continuous administration. In certain embodiments, it can be desirable to use a pharmaceutical composition comprising extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), in an ex vivo manner. In such instances, cells, tissues and/or organs that have been removed from the patient are exposed to a pharmaceutical composition comprising extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), after which the cells, tissues and/or organs are subsequently implanted back into the patient.

In certain embodiments, extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), can be delivered by implanting certain cells that have been genetically engineered, using methods such as those described herein, to express and secrete the polypeptides. In certain embodiments, such cells can be animal or human cells, and can be autologous, heterologous, or xenogeneic. In certain embodiments, the cells can be immortalized. In certain embodiments, in order to decrease the chance of an immunological response, the cells can be encapsulated to avoid infiltration of surrounding tissues. In certain embodiments, the encapsulation materials are typically biocompatible, semi-permeable polymeric enclosures or membranes that allow the release of the protein product(s) but prevent the destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissues.

Methods of Treatment

The extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), and/or nucleic acids expressing them, described herein, are useful for treating a disorder associated with abnormal apoptosis or a differentiative process (e.g., cellular proliferative disorders or cellular differentiative disorders, such as cancer). Non-limiting examples of cancers that are amenable to treatment with the methods of the present invention are described below.

Examples of cellular proliferative and/or differentiative disorders include cancer (e.g., carcinoma, sarcoma, metastatic disorders or hematopoietic neoplastic disorders, e.g., leukemias). A metastatic tumor can arise from a multitude of primary tumor types, including but not limited to those of prostate, colon, lung, breast and liver. Accordingly, the compositions used herein, comprising, e.g., extended-PK IL-2, a knottin-Fc, and optionally

an immune checkpoint blocker (or an antagonist of VEGF), can be administered to a patient who has cancer.

As used herein, we may use the terms "cancer" (or "cancerous"), "hyperproliferative," and "neoplastic" to refer to cells having the capacity for autonomous growth (i.e., an abnormal state or condition characterized by rapidly proliferating cell growth).

Hyperproliferative and neoplastic disease states may be categorized as pathologic (i.e., characterizing or constituting a disease state), or they may be categorized as non-pathologic (i.e., as a deviation from normal but not associated with a disease state). The terms are meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. "Pathologic hyperproliferative" cells occur in disease states characterized by malignant tumor growth. Examples of non-pathologic hyperproliferative cells include proliferation of cells associated with wound repair.

The terms "cancer" or "neoplasm" are used to refer to malignancies of the various organ systems, including those affecting the lung, breast, thyroid, lymph glands and lymphoid tissue, gastrointestinal organs, and the genitourinary tract, as well as to adenocarcinomas which are generally considered to include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus.

The term "carcinoma" is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. The mutant IL-2 polypeptides can be used to treat patients who have, who are suspected of having, or who may be at high risk for developing any type of cancer, including renal carcinoma or melanoma, or any viral disease. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures.

Additional examples of proliferative disorders include hematopoietic neoplastic disorders. As used herein, the term "hematopoietic neoplastic disorders" includes diseases involving hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. Preferably, the diseases arise from

poorly differentiated acute leukemias (e.g., erythroblastic leukemia and acute megakaryoblastic leukemia). Additional exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus, L. (1991) Crit. Rev. in Oncol./Hematol. 11:267-97); lymphoid malignancies include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macro globulinemia (WM). Additional forms of malignant lymphomas include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL), cutaneous T cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF), Hodgkin's disease and Reed-Sternberg disease.

It will be appreciated by those skilled in the art that amounts for each of the extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), that are sufficient to reduce tumor growth and size, or a therapeutically effective amount, will vary not only on the particular compounds or compositions selected, but also with the route of administration, the nature of the condition being treated, and the age and condition of the patient, and will ultimately be at the discretion of the patient's physician or pharmacist. The length of time during which the compounds used in the instant method will be given varies on an individual basis.

It will be appreciated by those skilled in the art that the B16 melanoma model used herein is a generalized model for solid tumors. That is, efficacy of treatments in this model is also predictive of efficacy of the treatments in other non-melanoma solid tumors. For example, as described in Baird et al. (J Immunology 2013; 190:469-78; Epub Dec 7, 2012), efficacy of *cps*, a parasite strain that induces an adaptive immune response, in mediating anti-tumor immunity against B16F10 tumors was found to be generalizable to other solid tumors, including models of lung carcinoma and ovarian cancer. In another example, results from a line of research into VEGF targeting lymphocytes also shows that results in B16F10 tumors were generalizable to the other tumor types studied (Chinnasamy et al., *JCI* 2010;120:3953-68; Chinnasamy et al., *Clin Cancer Res* 2012;18:1672-83). In yet another example, immunotherapy involving LAG-3 and PD-1 led to reduced tumor burden, with generalizable results in a fibrosarcoma and colon adenocarcinoma cell lines (Woo et al., *Cancer Res* 2012;72:917-27).

In certain embodiments, the extended-PK IL-2, knottin-Fc, and optional immune checkpoint blocker (or an antagonist of VEGF), are used to treat cancer.

In certain embodiments, the extended-PK IL-2, knottin-Fc, and optional immune checkpoint blocker (or an antagonist of VEGF) are used to treat melanoma, leukemia, lung cancer, breast cancer, prostate cancer, ovarian cancer, colon cancer, renal carcinoma, and brain cancer.

In certain embodiments, the extended-PK IL-2, knottin-Fc and optional immune checkpoint blocker (or an antagonist of VEGF) inhibit growth and/or proliferation of tumor cells.

In certain embodiments, the extended-PK IL-2, knottin-Fc, and optional immune checkpoint blocker (or an antagonist of VEGF) reduce tumor size.

In certain embodiments, the extended-PK IL-2, knottin-Fc, and optional immune checkpoint blocker (or an antagonist of VEGF) inhibit metastases of a primary tumor.

In certain embodiments, knottin-Fc and an immune checkpoint blocker (or an antagonist of VEGF), with or without IL-2, inhibit growth and/or proliferation of tumor cells. In certain embodiments, knottin-Fc and an immune checkpoint blocker (or an antagonist of VEGF), with or without IL-2, reduce tumor size. In certain embodiments, knottin-Fc and an immune checkpoint blocker, with or without IL-2, inhibit metastases of a primary tumor.

It will be appreciated by those skilled in the art that reference herein to treatment extends to prophylaxis as well as the treatment of the noted cancers and symptoms.

Kits

A kit can include extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF), as disclosed herein, and instructions for use. The kits may comprise, in a suitable container, extended-PK IL-2, an integrin binding knottin-Fc, an optional immune checkpoint blocker (or an antagonist of VEGF), one or more controls, and various buffers, reagents, enzymes and other standard ingredients well known in the art. Certain embodiments include a kit with extended-PK IL-2, knottin-Fc, and optional immune checkpoint blocker (or an antagonist of VEGF) in the same vial. In certain embodiments, a kit includes extended-PK IL-2, knottin-Fc, and optional immune checkpoint blocker (or an antagonist of VEGF) in separate vials.

The container can include at least one vial, well, test tube, flask, bottle, syringe, or other container means, into which extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF) may be placed, and in some instances, suitably aliquoted. Where an additional component is provided, the kit can contain additional containers into which this component may be placed. The kits can also include a means for

containing extended-PK IL-2, a knottin-Fc, and optionally an immune checkpoint blocker (or an antagonist of VEGF) and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained. Containers and/or kits can include labeling with instructions for use and/or warnings.

The present disclosure is further illustrated by the following examples, which should not be construed as further limiting. The contents of all figures and all references, Genbank sequences, patents and published patent applications cited throughout this application are expressly incorporated herein by reference. In particular, the disclosures of PCT publication WO 13/177187, U.S. Patent No. 8,536,301, and U.S. Patent Publication No. 2014/0073518 are expressly incorporated herein by reference.

Examples

Below are examples of specific embodiments for carrying out the methods described herein. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for. The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A.L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry 3rd Ed.* (Plenum Press) Vols A and B(1992).

Moreover, while the examples below employ extended-PK IL-2 of mouse origin (i.e., both the extended-PK group (mouse serum albumin) and IL-2 are of mouse origin) and mouse Fc fused to an integrin-binding knottin, it should be understood that corresponding human extended-PK IL-2 (i.e., human serum albumin (HSA) and human IL-2, and variants thereof) and integrin-binding-human-Fc (i.e., Fc from human IgG1 fused to knottin) can be

readily generated by those of ordinary skill in the art using methods described *supra*, and used in the methods disclosed herein.

Example 1

Knottin-Fc treatment is as effective as TA99 treatment in B16F10 tumors

To address the lack of general tumor-associated antigens, a knottin-Fc protein was engineered. The knottin-Fc protein comprises two parts: 1) an engineered cystine knot (knottin) peptide that binds with high affinity to tumor-associated $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ integrin receptors (specifically 2.5F, SEQ ID NO: 86 or 88), and 2) an antibody Fc domain that mediates immune effector functions *in vivo*. The knottin-Fc used is 2.5F with a K15S substitution, fused to a mouse IgG2a Fc domain, SEQ ID NO: 45, unless stated otherwise.

To determine the effects of the knottin-Fc on tumor growth, 2.5×10^5 B16F10 murine melanoma cells were injected into the flanks of C57BL/6 mice subcutaneously. Prophylactic treatment was done with 80 μ g knottin-Fc, 80 μ g knottin-D265A (The D265A mutation in the murine IgG2a Fc domain eliminates binding to Fc γ R and complement), or 200 μ g TA99, administered intraperitoneally every two days, starting on the day of tumor inoculation, for a total of ten treatments. TA99 is an antibody which binds to TYRP-1, (an antigen found on melanoma cells) and inhibits melanoma tumor growth.

Tumor area was measured and plotted (**Figure 2**). Tumor area was measured using calipers. The longest dimension of the tumor in any direction was measured first, followed by the measurement of the longest perpendicular dimension. The two values were then multiplied to quantify tumor area in square millimeters.

Both the TA99 treatment and the knottin-Fc treatment controlled tumor growth to a similar extent. The failure of knottin-D265A to prevent tumor growth indicates that tumor control by knottin-Fc is mediated by the effector function of Fc.

Example 2

Knottin-Fc and extended-PK IL-2 synergistically control tumor growth of B16F10 tumors

A therapeutic study was conducted to examine the effect of adding MSA/IL-2 to knottin-Fc treatment. Tumors were established by injecting C57BL/6 mice subcutaneously with 1×10^6 B16F10 murine melanoma cells. 30 μ g MSA/IL-2 and/or 500 μ g knottin-Fc was administered intraperitoneally on day 6 after tumor inoculation and every 6 days after that for 4 treatments total.

Tumor area was measured and plotted (**Figure 3A**). While MSA/IL-2 and knottin-Fc alone had no effect on tumor growth, the combination of MSA/IL-2 and knottin-Fc effectively controlled tumor growth. A Kaplan-Meier survival plot revealed that the combination of MSA/IL-2 and knottin-Fc extended the survival of these mice, whereas monotherapies had no significant effect (**Figure 3B**).

Example 3

Knottin-Fc and extended-PK IL-2 synergistically control tumor growth of MC38 tumors

A separate therapeutic study was conducted to examine the effect of MSA/IL-2 on knottin-Fc efficacy in a tumor type that has no reported targetable antigens. 1×10^6 MC38 murine colon carcinoma cells were injected into the flanks of C57BL/6 mice. 6 days after tumor inoculation and every 6 days after for a total of 4 treatments, 30 μ g MSA-IL-2 and/or 500 μ g knottin-Fc was administered intraperitoneally.

Tumor growth was controlled with the combination of MSA/IL-2 and knottin-Fc, but not when each component was administered as a monotherapy (**Figure 4A**). A Kaplan-Meier survival plot shows an increase in survival in the mice treated with the combination of MSA/IL-2 and knottin-Fc compared to when each component was administered as a monotherapy (**Figure 4B**).

Example 4

Knottin-Fc and extended-PK IL-2 synergistically control tumor growth of Ag104A tumors

An additional study was conducted to examine the effects of MSA/IL-2 on knottin-Fc efficacy in a different tumor type. Ag104A fibrosarcoma tumors were established by injecting 1.0×10^6 Ag104A cells into the flanks of C3H/HeN mice. Starting six days after tumor inoculation and every 6 days after, 30 μ g MSA/IL-2 and/or 500 μ g knottin-Fc was administered intraperitoneally.

Tumor growth was controlled with the combination of MSA/IL-2 and knottin-Fc, but not when each component was administered as a monotherapy (**Figure 5A**). A Kaplan-Meier survival plot shows an increase in survival in mice treated with the combination of MSA/IL-2 and knottin-Fc compared to when each component was administered alone (**Figure 5B**).

Example 5

Effector Function is Required for Synergistic Tumor Control

The results of Example 1 indicate that effector function is required for tumor control by knottin-Fc. To determine the role of effector function in tumor control by synergistic treatment with MSA/IL-2, a further study was conducted using both B16F10 and MC38 cells. 1×10^6 B16F10 cells (**Figures 6A and 6B**) or MC38 cells (**Figures 7A and 7B**) were injected into the flanks of C57BL/6 mice. 30 μ g MSA-IL-2 was administered intraperitoneally on day 6 after tumor inoculation and every 6 days after that for 4 treatments total. 500 μ g knottin-Fc or knottin-D265AFc (the D265A mutation in the murine IgG2a Fc domain eliminates binding to Fc γ R and complement) was administered intraperitoneally on day 6 after tumor inoculation and every six days thereafter for a total of 4 treatments.

Individual tumor size measurements (**Figures 6A and 7A**) and survival plots (**Figures 6B and 7B**) indicate that tumor control by knottin-Fc requires the effector function of Fc.

Example 6

Therapeutic antibody and immune checkpoint blocker enhance the efficacy of knottin-Fc and extended-PK IL-2 in B16F10 tumors

To determine the effect of antibodies on knottin-Fc and extended-PK IL-2 synergistic tumor control, an additional study was carried out using B16F10 cells. 1×10^6 B16F10 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2 was administered every 6 days beginning on day 6 after tumor inoculation for a total of 5 treatments and 200 μ g knottin-Fc was administered daily from days 6-30 after tumor inoculation. Antibodies against TYRP-1 (TA99) were administered at 100 μ g per mouse every 6 days starting on day 6 after B16F10 tumor inoculation. Antibodies against PD-1 were administered at 200 μ g per mouse every 6 days starting on day 6 after tumor inoculation.

Individual tumor size measurements (**Figures 8A**) and a survival plot (**Figures 8B**) indicate that antibodies enhanced tumor control via knottin-Fc and MSA/IL-2. However, this is only effective if the antibody targets an antigen present on the tumor cells. Combining knottin-Fc and MSA/IL-2 with a therapeutic antibody or immune checkpoint blocker increased the efficacy of tumor control and survival improvement.

Example 7

Immune checkpoint blocker enhances the efficacy of knottin-Fc and extended-PK IL-2 in MC38 tumors

To determine the effect of antibodies on knottin-Fc and extended-PK IL-2 synergistic tumor control, an additional study was carried out using MC38 cells. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2 and 500 μ g knottin-Fc were administered every 6 days beginning on day 6 after tumor inoculation for a total of 4 treatments. Antibodies against PD-1 were administered at 200 μ g per mouse every 6 days starting on day 6 after tumor inoculation.

Individual tumor size measurements (**Figures 9A**) and survival plots (**Figures 9B**) indicate that antibodies enhanced tumor control via knottin-Fc and MSA/IL-2. However, this is only effective if the antibody targets an antigen present on the tumor cells. The most effective treatment was to combine knottin-Fc and MSA/IL-2 with an immune checkpoint blockade (anti-PD-1) antibody.

Example 8

Knottin-Fc is effective when administered every other day or weekly

To determine the minimum dosing requirement for an anti-tumor response, the B16F10 melanoma and MC38 colon carcinoma tumor models were used and knottin-Fc was administered daily, every other day, or weekly. 1×10^6 B16F10 cells (**Figure 10**) or 1×10^6 MC38 cells (**Figure 11**) were injected into the flanks of C57BL/6 mice. 30 μ g of MSA/IL-2 was administered on days 6, 12, 18, and 24 after tumor inoculation. 200 μ g of knottin-Fc was administered daily or every other day starting on day 6 and ending on day 28. 500 μ g of knottin-Fc was administered on days 6, 12, 18, and 24 for the weekly regimen. In both models, administration of knottin-Fc in conjunction with MSA/IL-2 every other day or every 6 days was equivalent to daily administration.

Example 9

Knottin-Fc and Fc-Knottin constructs control tumor growth

To determine the optimal placement of Fc fused to knottin for an anti-tumor response, Fc was fused to either the N-terminus (Knottin-Fc) or the C-terminus (Fc-Knottin). 2.5×10^5 B16F10 melanoma cells were injected into the flanks of C57BL/6 mice. 80 μ g of knottin-Fc, 80 μ g of Fc-knottin, or 200 μ g of IgG-knottin was administered immediately after tumor inoculation and every two days after, for a total of 10 treatments. Tumor area was measured (**Figure 12**). There was essentially no difference in efficacy between the different knottin-Fc fusion proteins.

To validate this finding, another mouse tumor model was utilized. 1×10^6 MC38 colon carcinoma cells were injected into the flanks of albino C57BL/6 mice. 200 μ g of knottin-Fc or Fc-knottin were administered immediately after tumor inoculation and every two days after, for a total of 10 treatments. Tumor area was measured (**Figure 13**). There was essentially no difference in efficacy between knottin-Fc and Fc-knottin. The results from both of these mouse tumor models indicate that the placement of Fc in the knottin-Fc fusion protein does not affect efficacy for an anti-tumor response.

To determine optimal integrin-binding-knottin-Fc constructs, a fusion protein was made containing a (Gly4Ser)₃ linker in between the knottin and the Fc domain. No difference in integrin binding affinity to U87MG glioblastoma cells were observed compared to integrin-binding-knottin-Fc containing no linker (data not shown).

Example 10

Knottin-Fc and IL-2 Combination Protects Against Secondary Tumor Challenge

To determine if the combination of knottin-Fc and MSA/IL-2 could protect treated mice from a secondary challenge, the MC38 tumor model was used. 1×10^6 MC38 cells were injected into the flanks of C57BL/6 mice and both 30 μ g MSA/IL-2 and 500 μ g knottin-Fc were administered every 6 days beginning on day 6 after tumor inoculation for a total of 4 treatments. 16-20 weeks after the initial tumor inoculation, previously cured mice or age-matched naïve mice were inoculated with 1×10^6 MC38 cells in the opposite flank. No further treatment was administered.

Individual tumor size measurements (**Figure 14A**) and survival plot (**Figure 14B**) indicate that knottin-Fc and MSA/IL-2 protect previously treated mice against secondary tumor challenge, demonstrating a potent and sustained immune response against the tumors.

Example 11

Knottin-Fc Targeting All Three Integrins Most Effectively Controls Tumor Growth

To assess the efficacy of a knottin-Fc that targets all three integrins (i.e., α v β 3, α v β 5, and α 5 β 1, "2.5F_knottin-Fc" SEQ ID NO: 45) compared to a knottin-Fc that targets only two integrins (i.e., α v β 3 and α v β 5, "2.5D_knottin-Fc" SEQ ID NO: 47) in controlling tumor growth, an experiment was conducted using three different tumors models (i.e., B16F10, MC38, and Ag104A). 1×10^6 B16F10 (**Figures 15A and 15B**) or MC38 cells (**Figures 16A and 16B**) were injected into the flanks of C57BL/6 mice. 1.0×10^6 Ag104A cells (**Figures**

17A and 17B) were injected into the flanks of C3H/HeN mice. 30 µg MSA/IL-2, 500 µg 2.5F_knottin-Fc and/or 2.5D_knottin-Fc were administered every 6 days beginning on day 6 after tumor inoculation for a total of 4 treatments.

Individual tumor size measurements (**Figures 15A, 16A and 17A**) and survival plots (**Figures 15B, 16B and 17B**) indicate that a knottin-Fc that targets all three integrins more effectively controls tumor growth than a knottin-Fc that only targets two integrins.

Example 12

Immune Cell Populations Important for Survival in Tumor-bearing Mice

To assess the role of various immune cell populations in the improved survival of mice with MC38 tumors treated with knottin-Fc and MSA/IL-2, depletion experiments were conducted. 400 µg anti-CD8, anti-CD4, anti-NK1.1, anti-Ly6G or anti-CD19 antibodies were administered every four days for a total of six treatments starting on day 4 after tumor inoculation. 300 µg anti-CSF-1R antibody was administered every two days for a total of eleven treatments starting on day 4 of tumor inoculation. 30 µg cobra venom factor (CVF) was administered every 6 days for a total of 4 treatments starting on day 5 after tumor inoculation. Anti-CD8 depletes CD8+ T cells, anti-CD4 depletes CD4+ T cells, anti-NK1.1 depletes natural killer cells, anti-Ly6G depletes neutrophils, anti-CD19 depletes B cells, and anti-CSF-1R depletes tissue-resident/tumor-resident macrophages. CVF inhibits complement activity.

Individual tumor size measurements (**Figure 18A**) and survival plots (**Figure 18B**) indicate that CD8+ T cells and macrophages are particularly important for the synergistic effect of knottin-Fc and MSA/IL-2 on tumor control.

In addition to the immune cell depletions, Batf3^{-/-} mice were used to evaluate the role of cross-presenting CD8+ dendritic cells. Batf3^{-/-} mice lack the function of the basic leucine zipper transcription factor, ATF-like 3. Deletion of Batf3 has been shown to prevent the development of CD8+ dendritic cells, which are important for the cross-presentation of exogenous antigen on MHC Class I.

Tumor areas of treated C57BL/6 (i.e. "control") and Batf3^{-/-} mice were measured and plotted (**Figure 19A**), and demonstrated less tumor control in Batf3^{-/-} mice treated with MSA/IL-2 and knottin-Fc. A Kaplan-Meier survival plot was also generated, and revealed a significant decrease in survival in Batf3^{-/-} mice treated with knottin-Fc and MSA/IL-2 compared to C57BL/6 mice (**Figure 19B**). This indicated that cross-presentation by dendritic

cells may be a factor that contributes to the efficacy of the combination therapy on controlling the growth of MC38 tumors.

Since CD8⁺ T cells were found to be critical for efficacy of the knottin-Fc and MSA-IL-2 combination, and IFN γ was previously reported to be important in the efficacy of MSA-IL-2 and therapeutic antibody combinations (Zhu et al., *Cancer Cell* (2015) Vol 27: 489-501), the role of IFN γ was investigated in the MC38 tumor model. 1×10^6 MC38 were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2 and 500 μ g knottin-Fc were administered every 6 days beginning on day 6 after tumor inoculation for a total of 4 treatments. 200 μ g of anti-IFN γ antibody was administered every two days for a total of eleven treatments starting on day 5 after tumor inoculation.

As shown in **Figures 20A and 20B**, IFN γ depletion did not significantly affect tumor control or survival of mice with MC38 tumors.

Example 13

Antagonizing Immune Suppression Improves Survival of Tumor-bearing Mice

To determine whether the efficacy of tumor control could be enhanced by inhibiting suppressors of the immune system, an antagonist of vascular endothelial growth factor (VEGF) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) was administered in mice with MC38 or B16F10 tumors. 1×10^6 MC38 cells (**Figures 21A, 21B and 22**) or B16F10 (**Figures 23A and 23B**) were injected into the flanks of C57BL/6 mice. 30 μ g MSA/IL-2, 500 μ g knottin-Fc, and 200 μ g anti-VEGF antibody or anti-CTLA-4 antibody were administered every 6 days beginning on day 6 after tumor inoculation for a total of 4 treatments.

Synthetic genes, encoding the heavy (VH) and light (VL) chain variable regions of anti-VEGF (clone B20-4.1.1) (Bagri et al., *Clin. Cancer Res.* (2010) Vol 16: 3887-900) and anti-CTLA-4 (9D9) (Selby et al., *Cancer Immunol. Res.* (2013) Vol 1:32-42) antibodies, were codon-optimized for expression in mammalian cells (GeneArt, Life Technologies). B20-4.1.1 antibody constructs were generated by PCR-amplification of DNA inserts encoding for VH and VL regions and cloned via Gibson assembly into separate gWiz expression vectors (Genlantis) containing a CMV promoter, a kanamycin antibiotic resistance gene and the DNA sequence encoding for either murine IgG2a heavy-chain (CH1, CH2 and CH3) or light-chain (CL) constant regions (see below Sequence 1 and 2 for B20-4.1.1). The 9D9 antibody construct was generated by PCR-amplification of DNA inserts encoding the VH and VL

regions and sub-cloned into a double cassette p2MPT expression vector (EPFL Protein expression core facility, <http://pecf.epfl.ch>), containing a CMV promoter, an ampicillin antibiotic resistance gene and both murine IgG2a heavy-chain (CH1, CH2 and CH3) or light-chain (CL) constant regions, via the restriction sites *NotI/BamHI* and *EcoRI/XbaI*, respectively. All constructs were verified by DNA sequencing (Macrogen).

Anti-VEGF antibody B20-4.1.1 was expressed in transiently transfected human embryonic kidney (HEK293-F) cells using the Free-Style 293 Expression System (Life Technologies) as described previously (Zhu et al., *Cancer Cell* (2015) Vol 27: 489-501). Antibody 9D9 was expressed in transiently transfected Chinese hamster ovary (CHO) cells and provided by EPFL Protein expression core facility (<http://pecf.epfl.ch>). After 7 days of expression, cells were removed by centrifugation ($15,000 \times g$ for 30 min at 4 °C) and filtration (0.22 μ m PES membranes filter) and the proteins in the supernatant purified by protein A chromatography according to the manufacturer's instructions (GE Healthcare). Eluted antibodies were further desalted and purified on a HiLoad Superdex 200 10/600 size exclusion column (GE Healthcare) connected to an AKTApurifier system and equilibrated with buffer PBS 1X pH 7.4. The purified antibodies were concentrated using a 30000 NMWL Amicon Ultra centrifugal filter device (Millipore) at 4000 g and 4 °C and quantified by measuring absorbance at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Molecular weights were confirmed by reducing and non-reducing SDS/PAGE. Purity was evaluated by FPLC. Protein samples were analyzed by SDS-PAGE under denaturing and reducing conditions using NuPAGE 4–12% Bis-Tris Gels (Life Technologies) in MOPS buffer followed by Coomassie staining. Native size and oligomerization state of antibodies after concentration were also analyzed by size-exclusion chromatography with a Superdex 200 10/300 GL column (GE Healthcare) connected to an AKTApurifier system and equilibrated with buffer PBS 1X pH 7.4.

Tumor area was measured and plotted (**Figures 21A, 22 and 23A**) and survival plots were generated (**Figure 21B and 23B**). The triple combinations (i.e., MSA/IL-2+knottin-Fc+anti-VEGF and MSA/IL-2+knottin-Fc+anti-CTLA-4) significantly controlled tumor growth and improved survival compared to the double combinations (i.e., MSA/IL-2+anti-VEGF and knottin-Fc+anti-VEGF or MSA/IL-2+anti-CTLA-4 and knottin-Fc+anti-CTLA-4). This is similar to the results observed when adding an anti-PD-1 antibody to the combination of MSA/IL-2 and knottin-Fc, which indicated that other suppressors of the immune system could be inhibited to improve the efficacy of the combination of MSA/IL-2+knottin-Fc. The improved therapeutic efficacy by addition of an anti-VEGF antibody was

also observed in RENCA (renal adenocarcinoma) and LLC (Lewis lung carcinoma) tumor models (data not shown).

Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments described herein described herein. Such equivalents are intended to be encompassed by the following claims.

Table 2: Sequence Summary

SEQ ID NO	Description	Sequence
1	Human IgG1 constant region (amino acid sequence)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSLSLSPGK
2	Human IgG1 Fc domain (amino acid sequence)	EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT LPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS LSLSPGK
3	Human IgG1 Fc domain (amino acid sequence) Deletion (ΔEPKSC) Upper Hinge	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDV SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP GK
4	Mouse IL-2 (nucleic acid sequence)	GCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGG AGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGCAGGATGGA GAATTACAGGAACCTGAAACTCCCCAGGATGCTCACCTTCAA TTTTACTTGCCCAAGCAGGCCACAGAATTGAAAGATCTTCAGTG CCTAGAAGATGAACTTGACCTCTGCGGCATGTTCTGGATTGGA CTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTCA T CAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCTCTGAC AACACATTTGAGTGCCAATTCGATGATGAGTCAGCAACTGTGG TGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCATC TCAACAAGCCCTCAA
5	Mouse IL-2 (amino acid sequence)	APTSSSTSSSTA EAQQQQQQQQQQQHLEQLLMDLQELLSRMEN YRNLKLPRMLTFKFYLPKQATELKDLCLEDELGPLRHVLDLTQS KSFQLEDAENFISNIRVTVVKLKGS DNTFECQFDDESATVVD FLRR WIAFCQSIISTSPQ
6	QQ6210 (nucleic acid sequence)	GCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGG AGCAGCTGTTGATGGACCTACAGGAACTCCTGAGTAGGATGGA GGATCACAGGAACCTGAGACTCCCCAGGATGCTCACCTTCAA TTTTACTTGCCCGAGCAGGCCACAGAATTGGAAGATCTTCAGTG CCTAGAAGATGAACTTGAACCACTGCGGCAAGTTCTGGATTG ACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTCA

		TCAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCTCTGA CAACACATTTGAGTGCCAATTCGACGATGAGCCAGCAACTGTG GTGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCAT CTCAACAAGCCCTCAA
7	QQ6210 (amino acid sequence)	APTSSSTSSSTAEAQQQQQQQQQQQHLEQLLMDLQELLSRMED HRNLRRLPRMLTFKFYLPQATELEDLQCLEDELEPLRQVLDLTQSK SFQLEDAENFISNIRVTVVKLKGSNDTFECQFDDEPATVVDFLRRW IAFCQSIISTSPQ
8	E76A (nucleic acid sequence)	GCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGG AGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGCAGGATGGA GAATTACAGGAACCTGAAACTCCCCAGGATGCTCACCTTCAA TTTTACTTGCCCAAGCAGGCCACAGAATTGAAAGATCTTCAGTG CCTAGAAGATGCTCTTGGACCTCTGCGGCATGTTCTGGATTGGA CTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTTCAT CAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCTCTGAC AACACATTTGAGTGCCAATTCGATGATGAGTCAGCAACTGTGG TGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCATC TCAACAAGCCCTCAA
9	E76A (amino acid sequence)	APTSSSTSSSTAEAQQQQQQQQQQQHLEQLLMDLQELLSRMEN YRNLKLPRMLTFKFYLPKQATELKDLCLEDALGPLRHVLDLTQS KSFQLEDAENFISNIRVTVVKLKGSNDTFECQFDDESATVVDFLRR WIAFCQSIISTSPQ
10	E76G (nucleic acid sequence)	GCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGG AGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGCAGGATGGA GAATTACAGGAACCTGAAACTCCCCAGGATGCTCACCTTCAA TTTTACTTGCCCAAGCAGGCCACAGAATTGAAAGATCTTCAGTG CCTAGAAGATGGTCTTGGACCTCTGCGGCATGTTCTGGATTGGA CTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTTCAT CAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCTCTGAC AACACATTTGAGTGCCAATTCGATGATGAGTCAGCAACTGTGG TGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCATC TCAACAAGCCCTCAA
11	E76G (amino acid sequence)	APTSSSTSSSTAEAQQQQQQQQQQQHLEQLLMDLQELLSRMEN YRNLKLPRMLTFKFYLPKQATELKDLCLEDGLGPLRHVLDLTQS KSFQLEDAENFISNIRVTVVKLKGSNDTFECQFDDESATVVDFLRR WIAFCQSIISTSPQ
12	D265A Fc/Flag (nucleic acid sequence) (C-terminal flag tag is underlined)	ATGAGGGTCCCCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGCT CCCAGGTGCACGATGTGAGCCCAGAGTGCCCATAACACAGAAC CCCTGTCCTCCACTCAAAGAGTGTCCCCCATGCGCAGCTCCAGA CCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCA AGGATGTACTCATGATCTCCCTGAGCCCCATGGTCACATGTGTG GTGGTGGCCGTGAGCGAGGATGACCCAGACGTCCAGATCAGCT GGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAAC CCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCC CTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCA AATGCAAGGTCAACAACAGAGCCCTCCCATCCCCCATCGAGAA

		AACCATCTCAAAACCCAGAGGGCCAGTAAGAGCTCCACAGGTA TATGTCTTGCCTCCACCAGCAGAAGAGATGACTAAGAAAGAGT TCAGTCTGACCTGCATGATCACAGGCTTCTTACCTGCCGAAATT GCTGTGGACTGGACCAGCAATGGGCGTACAGAGCAAAACTACA AGAACACCGCAACAGTCCTGGACTCTGATGGTTCTTACTTCATG TACAGCAAGCTCAGAGTACAAAAGAGCACTTGGGAAAGAGGA AGTCTTTTCGCCTGCTCAGTGGTCCACGAGGGTCTGCACAATCA CCTTACGACTAAGACCATCTCCCGGTCTCTGGGTAAAGGTGGC GGATCTGACTACAAGGACGACGATGACAAGTGATAA
13	D265A Fc/Flag (amino acid sequence) (C-terminal flag tag is underlined)	MRVPAQLLGLLLLWLPGARCEPRVPITQNPCPPLKECPPCAAPDLL GGPSVFIFPPKIKDVLMSLSPMVTCVVVAVSEDDPDVQISWVNN VEVHTAQQTTHREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNN RALPSPIEKTISKPRGPVRAPQVYVLPPPAEEMTKKEFSLTCMITGF LPAEIAVDWTSNGRTEQNYKNTATVLDSDGSYFMYSKLRVQKST WERGSLFACSVVHEGLHNHLTTKTISRSLGKGGGSDYKDDDDK
14	D265A Fc/wt mIL-2 (nucleic acid sequence) (C-terminal 6x his tag is underlined)	ATGAGGGTCCCCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGCT CCCAGGTGCACGATGTGAGCCCAGAGTGCCCATAACACAGAAC CCCTGTCTCTCCACTCAAAGAGTGTCCCCCATGCGCAGCTCCAGA CCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCA AGGATGTACTCATGATCTCCCTGAGCCCCATGGTCACATGTGTG GTGGTGGCCGTGAGCGAGGATGACCCAGACGTCCAGATCAGCT GGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAAC CCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCC CTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCA AATGCAAGGTCAACAACAGAGCCCTCCCATCCCCCATCGAGAA AACCATCTCAAAACCCAGAGGGCCAGTAAGAGCTCCACAGGTA TATGTCTTGCCTCCACCAGCAGAAGAGATGACTAAGAAAGAGT TCAGTCTGACCTGCATGATCACAGGCTTCTTACCTGCCGAAATT GCTGTGGACTGGACCAGCAATGGGCGTACAGAGCAAAACTACA AGAACACCGCAACAGTCCTGGACTCTGATGGTTCTTACTTCATG TACAGCAAGCTCAGAGTACAAAAGAGCACTTGGGAAAGAGGA AGTCTTTTCGCCTGCTCAGTGGTCCACGAGGGTCTGCACAATCA CCTTACGACTAAGACCATCTCCCGGTCTCTGGGTAAAGGAGGG GGCTCCGCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGA AGCACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA CCTGGAGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGCAGG ATGGAGAATTACAGGAACCTGAAACTCCCCAGGATGCTCACCT TCAAATTTTACTTGCCCAAGCAGGCCACAGAATTGAAAGATCTT CAGTGCCTAGAAAGATGAACTTGGACCTCTGCGGCATGTTCTGG ATTTGACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAA TTTCATCAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGC TCTGACAACACATTTGAGTGCCAATTTCGATGATGAGTCAGCAA CTGTGGTGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGC ATCATCTCAACAAGCCCTCAACACCATCACCACCATCACTGATA A
15	D265A Fc/wt mIL-2 (amino acid sequence)	MRVPAQLLGLLLLWLPGARCEPRVPITQNPCPPLKECPPCAAPDLL GGPSVFIFPPKIKDVLMSLSPMVTCVVVAVSEDDPDVQISWVNN VEVHTAQQTTHREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNN RALPSPIEKTISKPRGPVRAPQVYVLPPPAEEMTKKEFSLTCMITGF

	(C-terminal 6x his tag is underlined)	LPAEIAVDWTSNGRTEQNYKNTATVLDSDGSYFMYSKLRVQKST WERGSLFACSVVHEGLHNHLTTKTISRSLGKGGSAPTSSSTSSST AEAQQQQQQQQQQQHLEQLLMDLQELLSRMENYRNLKLPRML TFKFYLPKQATELKDLQCLEDELGPLRHVLDLTQSKSFQLEDAENF ISNIRVTVVKLKGSDNTEFCQFDDESATVVDFLRRWIAFCQSIISTS <u>PQHHHHHH</u> **
16	D265A Fc /QQ6210 (nucleic acid sequence) (C-terminal 6x his tag is underlined)	ATGAGGGTCCCCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGCT CCCAGGTGCACGATGTGAGCCCAGAGTGCCCATAACACAGAAC CCCTGTCCTCCACTCAAAGAGTGTCCCCCATGCGCAGCTCCAGA CCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCA AGGATGTACTCATGATCTCCCTGAGCCCCATGGTCACATGTGTG GTGGTGGCCGTGAGCGAGGATGACCCAGACGTCCAGATCAGCT GGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAAC CCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCC CTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCA AATGCAAGGTCAACAACAGAGCCCTCCCATCCCCCATCGAGAA AACCATCTCAAAACCCAGAGGGGCCAGTAAGAGCTCCACAGGTA TATGTCTTGCCTCCACCAGCAGAAGAGATGACTAAGAAAGAGT TCAGTCTGACCTGCATGATCACAGGCTTCTTACCTGCCGAAATT GCTGTGGACTGGACCAGCAATGGGCGTACAGAGCAAACTACA AGAACACCGCAACAGTCCTGGACTCTGATGGTTCTTACTTCATG TACAGCAAGCTCAGAGTACAAAAGAGCACTTGGGAAAGAGGA AGTCTTTTCGCCTGCTCAGTGGTCCACGAGGGTCTGCACAATCA CCTTACGACTAAGACCATCTCCCGGTCTCTGGGTAAAGGAGGG GGCTCCGCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGA AGCACAACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA CCTGGAGCAGCTGTTGATGGACCTACAGGAACTCCTGAGTAGG ATGGAGGATCACAGGAACCTGAGACTCCCCAGGATGCTCACCT TCAAATTTTACTTGCCCCGAGCAGGCCACAGAATTGGAAGATCTT CAGTGCCTAGAAGATGAACTTGAACCACTGCGGCAAGTTCTGG ATTTGACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAA TTTCATCAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGC TCTGACAACACATTTGAGTGCCAATTTCGACGATGAGCCAGCAA CTGTGGTGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGC ATCATCTCAACAAGCCCTCAACACCATCACCACCATCACTGATA A
17	D265A Fc /QQ6210 (amino acid sequence) (C-terminal 6x his tag is underlined)	MRVPAQLLGLLLLWLPGARCEPRVPITQNPCPPLKECPPCAAPDLL GGPSVFIFPPKIKDVLMSLSPMVTCVVVAVSEDDPDVQISWVNN VEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNN RALPSPIEKTISKPRGPVRAPQVYVLPPPAEEMTKKEFSLTCMITGF LPAEIAVDWTSNGRTEQNYKNTATVLDSDGSYFMYSKLRVQKST WERGSLFACSVVHEGLHNHLTTKTISRSLGKGGSAPTSSSTSSST AEAQQQQQQQQQQQHLEQLLMDLQELLSRMEDHRNLRLPRML TFKFYLPQATELEDLQCLEDELEPLRQVLDLTQSKSFQLEDAENFI SNIRVTVVKLKGSDNTEFCQFDDEPATVVDFLRRWIAFCQSIISTSP <u>QHHHHHH</u>
18	D265A Fc / E76A (nucleic acid)	ATGAGGGTCCCCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGCT CCCAGGTGCACGATGTGAGCCCAGAGTGCCCATAACACAGAAC CCCTGTCCTCCACTCAAAGAGTGTCCCCCATGCGCAGCTCCAGA CCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCA

	sequence) (C-terminal 6x his tag is underlined)	AGGATGTACTCATGATCTCCCTGAGCCCCATGGTCACATGTGTG GTGGTGGCCGTGAGCGAGGATGACCCAGACGTCCAGATCAGCT GGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAAC CCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCC CTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCA AATGCAAGGTCAACAACAGAGCCCTCCCATCCCCCATCGAGAA AACCATCTCAAAACCCAGAGGGGCCAGTAAGAGCTCCACAGGTA TATGTCTTGCCTCCACCAGCAGAAGAGATGACTAAGAAAGAGT TCAGTCTGACCTGCATGATCACAGGCTTCTTACCTGCCGAAATT GCTGTGGACTGGACCAGCAATGGGCGTACAGAGCAAACTACA AGAACACCGCAACAGTCCTGGACTCTGATGGTTCTTACTTCATG TACAGCAAGCTCAGAGTACAAAAGAGCACTTGGGAAAGAGGA AGTCTTTTCGCCTGCTCAGTGGTCCACGAGGGTCTGCACAATCA CCTTACGACTAAGACCATCTCCCGGTCTCTGGGTAAAGGAGGG GGCTCCGCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGA AGCACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA CCTGGAGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGCAGG ATGGAGAATTACAGGAACCTGAAACTCCCCAGGATGCTCACCT TCAAATTTTACTTGCCCAAGCAGGCCACAGAATTGAAAGATCTT CAGTGCCTAGAAGATGCTCTTGGACCTCTGCGGCATGTTCTGGA TTTGACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAAT TTCATCAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCT CTGACAACACATTTGAGTGCCAATTCGATGATGAGTCAGCAAC TGTGGTGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGC ATCATCTCAACAAGCCCTCAACACCATCACCACCATCACTGATA A
19	D265A Fc / E76A (amino acid sequence) (C-terminal 6x his tag is underlined)	MRVPAQLLGLLLLWLPGARCEPRVPITQNPCPPLKECPPCAAPDLL GGPSVFIFPPKIKDVLMSLSPMVTCVVAVVSEDDPDVQISWVNN VEVHTAQQTTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNN RALPSPIEKTISKPRGPVRAPQVYVLPPPAEEMTKKEFSLTCMITGF LPAEIAVDWTSNGRTEQNYKNTATVLDSDGSYFMYSKLRVQKST WERGSLFACSVVHEGLHNHLTTKTISRSLGKGGSAPTSSSTSSST AEAQQQQQQQQQQQHHLEQLLMDLQELLSRMENYRNLKLPRML TFKFYLPKQATELKDLQCLEDALGPLRHVLDLTQSKSFQLEDAEN FISNIRVTVVKLKGSDNTEFCQFDDESATVVDFLRRWIAFCQSIIST SPQHHHHHH
20	D265A Fc / E76G (nucleic acid sequence) (C-terminal 6x his tag is underlined)	ATGAGGGTCCCCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGCT CCCAGGTGCACGATGTGAGCCCAGAGTGCCCATAACACAGAAC CCCTGTCTCTCCACTCAAAGAGTGTCCCCCATGCGCAGCTCCAGA CCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAGATCA AGGATGTACTCATGATCTCCCTGAGCCCCATGGTCACATGTGTG GTGGTGGCCGTGAGCGAGGATGACCCAGACGTCCAGATCAGCT GGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAAC CCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAGTGCC CTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAGTTCA AATGCAAGGTCAACAACAGAGCCCTCCCATCCCCCATCGAGAA AACCATCTCAAAACCCAGAGGGGCCAGTAAGAGCTCCACAGGTA TATGTCTTGCCTCCACCAGCAGAAGAGATGACTAAGAAAGAGT TCAGTCTGACCTGCATGATCACAGGCTTCTTACCTGCCGAAATT GCTGTGGACTGGACCAGCAATGGGCGTACAGAGCAAACTACA

		AGAACACCGCAACAGTCCTGGACTCTGATGGTTCTTACTTCATG TACAGCAAGCTCAGAGTACAAAAGAGCACTTGGGAAAGAGGA AGTCTTTTCGCCTGCTCAGTGGTCCACGAGGGTCTGCACAATCA CCTTACGACTAAGACCATCTCCCGGTCTCTGGGTAAAGGAGGG GGCTCCGCACCCACTTCAAGCTCCACTTCAAGCTCTACAGCGGA AGCACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA CCTGGAGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGCAGG ATGGAGAATTACAGGAACCTGAAACTCCCCAGGATGCTCACCT TCAAATTTTACTTGCCCAAGCAGGCCACAGAATTGAAAGATCTT CAGTGCCTAGAAGATGGTCTTGGACCTCTGCGGCATGTTCTGGA TTTGACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAAT TTCATCAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCT CTGACAACACATTTGAGTGCCAATTCGATGATGAGTCAGCAAC TGTGGTGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGC ATCATCTCAACAAGCCCTCAACACCATCACCACCATCACTGATA A
21	D265A Fc / E76G (amino acid sequence) (C-terminal 6x his tag is underlined)	MRVPAQLLGLLLLWLPGARCEPRVPITQNPCPPLKECPPCAAPDLL GGPSVFIFPPKIKDVLMSLSPMVTCVVAVVSEDDPDVQISWVNN VEVHTAQQTTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNN RALPSPIEKTISKPRGPVRAPQVYVLPPPAEEMTKKEFSLTCMITGF LPAEIAVDWTSNGRTEQNYKNTATVLDSDGSYFMYSKLRVQKST WERGSLFACSVVHEGLHNHLTTKTISRSLGKGGSAPTSSSTSSST AEAQQQQQQQQQQQHLEQLLMDLQELLSRMENYRNLKLPRML TFKFYLPKQATELKDLQCLEDGLGPLRHVLDLTQSKSFQLEDAEN FISNIRVTVVKLKGS DNTEFCQFDDESATVVDFLRRWIAFCQSIIST SPQH HHHHHH
22	mIL-2 QQ 6.2-4 (nucleic acid sequence)	GCACCCACCTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGG AGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGCAGGATGGA GGATTCCAGGAACCTGAGACTCCCCAGGATGCTCACCTTCAA TTTTACTTGCCCAAGCAGGCCACAGAATTGGAAGATCTTCAGTG CCTAGAAGATGAACTTGAACCTCTGCGGCAAGTTCTGGATTG ACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTCA TCAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCTCTGA CAACACATTTGAGTGCCAATTCGATGATGAGCCAGCAACTGTG GTGGGCTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCAT CTCAACGAGCCCTCAA
23	mIL-2 QQ 6.2-4 (amino acid sequence)	APTSSSTSSSTA EAQQQQQQQQQQQHLEQLLMDLQELLSRMEDS RNLRLPRMLTFKFYLPKQATELEDLQCLEDELEPLRQVLDLTQSKS FQLEDAENFISNIRVTVVKLKGS DNTEFCQFDDEPATVVGFLRRWI AFCQSIISTSPQ
24	mIL-2 QQ 6.2-8 (nucleic acid sequence)	GCACCCACCTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGG AGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGTAGGATGGAGGATCACAGGAAC CTGAGACTCCCCAGGATGCTCACCTTCAAATTTTACTTGCCCAA GCAGGCCACAGAATTGGAAGATCTTCAGTGCCTAGAAGATGAA CTTGAACCTCTGCGGCAAGTTCTGGATTGACTCAAAGCAAAA GCTTTCAATTGGAAGATGCTGAGAATTTTCATCAGCAATATCAG AGTAACTGTTGTAAAACTAAAGGGCTCTGACAACACATTTGAG TGCCAATTCGATGATGAGCCAGCAACTGTGGTGGACTTTCTGA

		GGAGATGGATAGCCTTCTGTCAAAGCATCATCTCAACAAGCCC TCGA
25	mIL-2 QQ 6.2-8 (amino acid sequence)	APTSSSTSSSTAEEAQQQQQQQQHLEQLLMDLQELLSRMEDHRNLR LPRMLTFKFYLPKQATELEDLQCLEDELEPLRQVLDLTQSKSFQLE DAENFISNIRVTVVKLKGSNDNTFECQFDDEPATVVDFLRRWIAFCQ SIISTSPR
26	mIL-2 QQ 6.2-10 (nucleic acid sequence)	GCACCCACCTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGG AGCAGCTGTTGATGGACCTACAGGAACTCCTGAGTAGGATGGA GGATCACAGGAACCTGAGACTCCCCAGGATGCTCACCTTCAAA TTTTACTTGCCCGAGCAGGCCACAGAATTGGAAGATCTTCAGTG CCTAGAAGATGAACTTGAACCACTGCGGCAAGTTCTGGATTTG ACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTCA TCAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCTCTGA CAACACATTTGAGTGCCAATTCGACGATGAGCCAGCAACTGTG GTGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATCAT CTCAACAAGCCCTCAG
27	mIL-2 QQ 6.2-10 (amino acid sequence)	APTSSSTSSSTAEEAQQQQQQQQQQHLEQLLMDLQELLSRMED HRNLRRLPRMLTFKFYLPEQATELEDLQCLEDELEPLRQVLDLTQSK SFQLEDAENFISNIRVTVVKLKGSNDNTFECQFDDEPATVVDFLRRW IAFCQSIISTSPQ
28	mIL-2 QQ 6.2-11 (nucleic acid sequence)	GCACCCACCTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGGAGCAGCTGTT GATGGACCTACAGGAGCTCCTGAGCAGGATGGAGGATTCCAGG AACCTGAGACTCCCCAGAATGCTCACCTTCAAATTTTACTTGCC CGAGCAGGCCACAGAATTGAAAGATCTCCAGTGCCTAGAAGAT GAACTTGAACCTCTGCGGCAAGTTCTGGATTTGACTCAAAGCA AAAGCTTTCAATTGGAAGATGCTGAGAATTTTCATCAGCAATAT CAGAGTAACTGTTGTAAAACTAAAGGGCTCTGACAACACATTT GAGTGCCAATTCGACGATGAGCCAGCAACTGTGGTGGACTTTC TGAGGAGATGGATAGCCTTCTGTCAAAGCATCATCTCAACAAG CCCTCAG
29	mIL-2 QQ 6.2-11 (amino acid sequence)	APTSSSTSSSTAEEAQQQQQQQQQQHLEQLLMDLQELLSRMEDSRNL RLPRMLTFKFYLPEQATELKDLDLQCLEDELEPLRQVLDLTQSKSFQL EDAENFISNIRVTVVKLKGSNDNTFECQFDDEPATVVDFLRRWIAFC QSIISTSPQ
30	mIL-2 QQ 6.2-13 (nucleic acid sequence)	GCACCCACCTCAAGCTCCACTTCAAGCTCTACAGCGGAAGCAC AACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTGG AGCAGCTGTTGATGGACCTACAGGAGCTCCTGAGTAGGATGGA GGATCACAGGAACCTGAGACTCCCCAGGATGCTCACCTTCAAA TTTTACTTGCCCGAGCAGGCCACAGAATTGAAAGATCTCCAGT GCCTAGAAGATGAACTTGAACCTCTGCGGCAGGTTCTGGATTT GACTCAAAGCAAAAGCTTTCAATTGGAAGATGCTGAGAATTTTC ATCAGCAATATCAGAGTAACTGTTGTAAAACTAAAGGGCTCTG ACAACACATTTGAGTGCCAATTCGATGATGAGCCAGCAACTGT GGTGGACTTTCTGAGGAGATGGATAGCCTTCTGTCAAAGCATC ATCTCAACAAGCCCTCAG
31	mIL-2 QQ 6.2-13 (amino acid)	APTSSSTSSSTAEEAQQQQQQQQQQHLEQLLMDLQELLSRMED HRNLRRLPRMLTFKFYLPEQATELKDLDLQCLEDELEPLRQVLDLTQS KSFQLEDAENFISNIRVTVVKLKGSNDNTFECQFDDEPATVVDFLRR

	sequence)	WIAFCQSIISTSPQ
32	Full length human IL-2 (nucleic acid sequence)	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGC ACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAA ACACAGCTACAACCTGGAGCATTTACTGCTGGATTTACAGATGA TTTTGAATGGAATTAATAATTACAAGAATCCCAAACCTCACCAG GATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAA CTGAAACATCTTCAGTGTCTAGAAGAAGAAGTCAAACCTCTGG AGGAAGTGCTAAATTTAGCTCAAAGCAAAAACCTTCACTTAAG ACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAA CTAAAGGGATCTGAAACAACATTCATGTGTAATATGCTGATGA GACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTTTT GTCAAAGCATCATCTCAACACTGACTTGA
33	Full length human IL-2 (amino acid sequence)	MYRMQLLSICIALSLALVTNSAPTSSSTKKTQLQLEHLLLDLQMILN GINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLN LAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFL NRWITFCQSIISTLT
34	Human IL-2 without signal peptide (nucleic acid sequence)	GCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACCTGG AGCATTTACTGCTGGATTTACAGATGATTTTGAATGGAATTAAT AATTACAAGAATCCCAAACCTCACCAGGATGCTCACATTTAAGT TTTACATGCCCAAGAAGGCCACAGAACTGAAACATCTTCAGTG TCTAGAAGAAGAAGTCAAACCTCTGGAGGAAGTGCTAAATTTA GCTCAAAGCAAAAACCTTCACTTAAGACCCAGGGACTTAATCA GCAATATCAACGTAATAGTTCTGGAAGTAAAGGGATCTGAAAC AACATTCATGTGTAATATGCTGATGAGACAGCAACCATTGTAG AATTTCTGAACAGATGGATTACCTTTTGTCAAAGCATCATCTCA ACACTGACTTGA
35	Human IL-2 without signal peptide (amino acid sequence)	APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFY MPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINV IVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT
36	Human serum albumin (amino acid sequence)	MDMRVPAQLLGLLLLWLPGARCAHAHKSEVAHRFKDLGEENFK ALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDS LHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDN PNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPEL LFFAKRYKAAFTTECCQAADKAACLLPKLDEL RDEGKASSAKQRL KCASLQKFGERAFAKAWAVARLSQRFPAEFAEVSKLVTDLT KVH TECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLEKSH CIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY EYARRHPDYSVVLRLAKTYETTLEKCCAAADPHECYAKVFDEF KPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTL VEVSRNLGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLHEKT PVSDRVTKCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADI CTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEK CCKADDKETCFAEEGKKLVAAASQAALGLGGGSAPTSSSTKKTQL QLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFM CEYADETATIVEFLNRWITFCQSIISTLTGGGS
37	Mature	DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNE

	HSA (amino acid sequence)	VTEFAKTCVADESAENCDSLHTLFGDKLCTVATLRETYGEMAD CCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETF LKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLL PKLDELRDEGKASSAKQRLKCASLQKFGERAFAKAWAVARLSQRF PKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQ DSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKD VCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLRLAKTYETTLE KCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKF QNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMP CAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALE VDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPK ATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQA ALGLGGGSAPTSSSTKKTKLQLEHLLLDLQMILNGINNYKNPKLTR MLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPR DLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIIST LTGGGS
38	Human serum albumin (nucleic acid sequence)	ATGGATATGCGGGTGCCTGCTCAGCTGCTGGGACTGCTGCTGCT GTGGCTGCCTGGGGCTAGATGCGCCGATGCTCACAAAAGCGAA GTCGCACACAGGTTCAAAGATCTGGGGGAGGAAAACTTTAAGG CTCTGGTGCTGATTGCATTCGCCCAGTACCTGCAGCAGTGCCCC TTTGAGGACCACGTGAAACTGGTCAACGAAGTGACTGAGTTCTG CCAAGACCTGCGTGCGCCGACGAATCTGCTGAGAATTGTGATAA AAGTCTGCATACTCTGTTTGGGGATAAGCTGTGTACAGTGGCCA CTCTGCGAGAAACCTATGGAGAGATGGCAGACTGCTGTGCCAA ACAGGAACCCGAGCGGAACGAATGCTTCCTGCAGCATAAGGAC GATAACCCCAATCTGCCTCGCCTGGTGCGACCTGAGGTGGACG TCATGTGTACAGCCTTCCACGATAATGAGGAAACTTTTCTGAAG AAATACCTGTACGAAATCGCTCGGAGACATCCTTACTTTTATGC ACCAGAGCTGCTGTTCTTTGCCAAACGCTACAAGGCCGCTTTCA CCGAGTGCTGTCAAGGCAGCCGATAAAGCTGCATGCCTGCTGCC TAAGCTGGACGAACTGAGGGATGAGGGCAAGGCCAGCTCCGCT AAACAGCGCCTGAAGTGTGCTAGCCTGCAGAAATTCGGGGAGC GAGCCTTCAAGGCTTGGGCAGTGGCACGGCTGAGTCAGAGATT CCCAAAGGCAGAATTTGCCGAGGTCTCAAAACTGGTGACCGAC CTGACAAAGGTGCACACCGAATGCTGTTCATGGCGACCTGCTGG AGTGCGCCGACGATCGAGCTGATCTGGCAAAGTATATTTGTGA GAACCAGGACTCCATCTCTAGTAAGCTGAAAGAATGCTGTGAG AAACCACTGCTGGAAAAGTCTCACTGCATTGCCGAAGTGGAGA ACGACGAGATGCCAGCTGATCTGCCCTCACTGGCCGCTGACTTC GTCGAAAGCAAAGATGTGTGTAAGAATTACGCTGAGGCAAAGG ATGTGTTCTGGAATGTTTCTGTACGAGTATGCCAGGCGCCAC CCAGACTACTCCGTGGTCCTGCTGCTGAGGCTGGCTAAAACAT ATGAAACCACACTGGAGAAGTGCTGTGCAGCCGCTGATCCCCA TGAATGCTATGCCAAAGTCTTCGACGAGTTTAAGCCCCTGGTGG AGGAACCTCAGAACCTGATCAAACAGAATTGTGAACTGTTTGA GCAGCTGGGCGAGTACAAGTTCCAGAACGCCCTGCTGGTGCGC TATACCAAGAAAGTCCCACAGGTGTCCACACCCACTCTGGTGG AGGTGAGCCGGAATCTGGGCAAAGTGGGGAGTAAATGCTGTAA GCACCCTGAAGCCAAGAGGATGCCATGCGCTGAGGATTACCTG AGTGTGGTCCTGAATCAGCTGTGTGTCCTGCATGAAAAAACAC

		CTGTCAGCGACCGGGTGACAAAGTGCTGTACTGAGTCACTGGT GAACCGACGGCCCTGCTTTAGCGCCCTGGAAGTCGATGAGACT TATGTGCCTAAAGAGTTCAACGCTGAGACCTTCACATTTACGC AGACATTTGTACCCTGAGCGAAAAGGAGAGACAGATCAAGAA ACAGACAGCCCTGGTCGAACTGGTGAAGCATAAACCCAAGGCC ACAAAAGAGCAGCTGAAGGCTGTCATGGACGATTTTCGCAGCCT TTGTGGAAAAATGCTGTAAGGCAGACGATAAGGAGACTTGCTT TGCCGAGGAAGGAAAGAACTGGTGGCTGCATCCCAGGCAGCT CTGGGACTGGGAGGAGGATCTGCCCCTACCTCAAGCTCCACTA AGAAAACCCAGCTGCAGCTGGAGCACCTGCTGCTGGACCTGCA GATGATTCTGAACGGGATCAACAATTACAAAAATCCAAAGCTG ACCCGGATGCTGACATTCAAGTTTTATATGCCCAAGAAAGCCA CAGAGCTGAAACACCTGCAGTGCCTGGAGGAAGAGCTGAAGCC TCTGGAAGAGGTGCTGAACCTGGCCCAGAGCAAGAATTTCCAT CTGAGACCAAGGGATCTGATCTCCAACATTAATGTGATCGTCCT GGAAGTGAAGGGATCTGAGACTACCTTTATGTGCGAATACGCT GACGAGACTGCAACCATTGTGGAGTTCCTGAACAGATGGATCA CCTTCTGCCAGTCCATCATTTCTACTCTGACAGGCGGGGGGAGC
39	EETI-II from Knottin Database	GC PRILMR CKQSDCLAGVCVCPNGFCG
40	AgRP from Knottin Database "- "indicates where mini protein can be formed	GCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCR- KLGTAMNPCSRT
41	Omega agatoxin from Knottin Database "- "indicates where mini protein can be formed	EDN--CIAEDYGKCTWGGTKCCRGRPCRC SMIGTN CECTPRLIMEGLSFA
42	EETI-II Library	GCXXXRGDXXXXXXCKQSDCLAGVCVCPNGFCG
43	EETI-II K15S Mutation Library	GCXXXRGDXXXXXCSQSDCLAGVCVCPNGFCG
44	2.5F- (K15S) mIgG2aFc Nucleic Acid Sequence	GGTTGTCCAAGACCAAGAGGTGATAATCCACCATTGACTTGTTT TCAAGATTCTGATTGTTTGGCTGGTTGTGTTTGTGGTCCAAATG GTTTTTGTGGTGGTTCGACTAGAGCCCAGAGTGCCCATAACACA GAACCCCTGTCCTCCACTCAAAGAGTGTCCCCCATGCGCAGCTC CAGACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAG ATCAAGGATGTACTCATGATCTCCCTGAGCCCCATGGTCACATG

		TGTGGTGGTGGATGTGAGCGAGGATGACCCAGACGTCCAGATC AGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACAC AAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAG TGCCCTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAG TTCAAATGCAAGGTCAACAACAGAGCCCTCCCATCCCCCATCG AGAAAACCATCTCAAAACCCAGAGGGCCAGTAAGAGCTCCACA GGTATATGTCTTGCCTCCACCAGCAGAAGAGATGACTAAGAAA GAGTTCAGTCTGACCTGCATGATCACAGGCTTCTTACCTGCCGA AATTGCTGTGGACTGGACCAGCAATGGGCGTACAGAGCAAAAC TACAAGAACACCGCAACAGTCCTGGACTCTGATGGTTCTTACTT CATGTACAGCAAGCTCAGAGTACAAAAGAGCACTTGGGAAAG AGGAAGTCTTTTCGCCTGCTCAGTGGTCCACGAGGGTCTGCACA ATCACCTTACGACTAAGACCATCTCCCGGTCTCTGGGTAAA
45	2.5F- (K15S) mIgG2aFc Amino Acid Sequence	GCPRPRGDNPPLTCSQSDCLAGCVCGPNGFCGEPRVPITQNPCPP LKECPPCAAPDLLGGPSVFIFPPKIKDVLMSLSPMVTCVVVDVSED DPDVQISWVFNNEVHTAQTQTHREDYNSTLRVVSALPIQHQDW MSGKEFKCKVNNRALPSPIEKTISKPRGPVRAPQVYVLPPPAEEMT KKEFSLTCMITGFLPAEIAVDWTSNGRTEQNYKNTATVLDSGGSY FMYSKLRVQKSTWERGSLFACSVVHEGLHNHLTTKTISRSLGK
46	2.5D- (K15S) mIgG2aFc Nucleic Acid Sequence	GGTTGTCCACAAGGCAGAGGTGATTGGGCTCCAACTTCTTGTTT TCAAGATTCTGATTGTTTGGCTGGTTGTGTTTGTGGTCCAAATG GTTTTTGTGGTGGTCGACTAGAGCCCAGAGTGCCCATAACACA GAACCCCTGTCCTCCACTCAAAGAGTGTCCCCCATGCGCAGCTC CAGACCTCTTGGGTGGACCATCCGTCTTCATCTTCCCTCCAAAG ATCAAGGATGTACTCATGATCTCCCTGAGCCCCATGGTCACATG TGTGGTGGTGGATGTGAGCGAGGATGACCCAGACGTCCAGATC AGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACAC AAACCCATAGAGAGGATTACAACAGTACTCTCCGGGTGGTCAG TGCCCTCCCCATCCAGCACCAGGACTGGATGAGTGGCAAGGAG TTCAAATGCAAGGTCAACAACAGAGCCCTCCCATCCCCCATCG AGAAAACCATCTCAAAACCCAGAGGGCCAGTAAGAGCTCCACA GGTATATGTCTTGCCTCCACCAGCAGAAGAGATGACTAAGAAA GAGTTCAGTCTGACCTGCATGATCACAGGCTTCTTACCTGCCGA AATTGCTGTGGACTGGACCAGCAATGGGCGTACAGAGCAAAAC TACAAGAACACCGCAACAGTCCTGGACTCTGATGGTTCTTACTT CATGTACAGCAAGCTCAGAGTACAAAAGAGCACTTGGGAAAG AGGAAGTCTTTTCGCCTGCTCAGTGGTCCACGAGGGTCTGCACA ATCACCTTACGACTAAGACCATCTCCCGGTCTCTGGGTAAA
47	2.5D- (K15S) mIgG2aFc Amino Acid Sequence	GCPQGRGDWAPTSCSQSDCLAGCVCGPNGFCGEPRVPITQNPCP PLKECPPCAAPDLLGGPSVFIFPPKIKDVLMSLSPMVTCVVVDVSE DDPDVQISWVFNNEVHTAQTQTHREDYNSTLRVVSALPIQHQD WMSGKEFKCKVNNRALPSPIEKTISKPRGPVRAPQVYVLPPPAEE MTKKEFSLTCMITGFLPAEIAVDWTSNGRTEQNYKNTATVLDSDG SYFMYSKLRVQKSTWERGSLFACSVVHEGLHNHLTTKTISRSLGK
48	2.5F- (K15S) hIgG1Fc Amino Acid Sequence	GCPRPRGDNPPLTCSQSDCLAGCVCGPNGFCGEPKSCDKTHTCP PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYS KLTVDKSRWQQGNVFSQSVMHENHHTQKSLSLSPGK

49	2.5F- (K15S) hIgG1Fc Fc Upper Hinge Deletion (ΔEPKSC) Amino Acid Sequence	GCPRPRGDNPLTCSQSDCLAGCVCGPNGFCGDKTHTCPPCPAP ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKC KVS NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL LVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
50	2.5D- (K15S) hIgG1Fc Amino Acid Sequence	GCPQGRGDWAPTSCSQSDCLAGCVCGPNGFCGEPKSCDKTHTC PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
51	2.5D- (K15S) hIgG1Fc Fc Upper Hinge Deletion (ΔEPKSC) Amino Acid Sequence	GCPQGRGDWAPTSCSQSDCLAGCVCGPNGFCGDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLT CLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
52	hPD-1 amino acid sequence	MQIPQAPWPVVWAVLQLGWRPGWFLDSPDPWNPPFTFFPALLVVT EGDNATFTCSFSNTSESVLNWYRMSPSNQTDKLAAPEDRSQPG QDCRFRVTQLPNGRDFHMSVVRARRNDSGT YLCGAISLAPKAQIK ESLRAELRV TERRAEVPTAHPSPSRPAGQFQTLVVG VVGGLLGS L VLLVWVLAVICSRAARGTIGARRTGQPLKEDPSAVPVFSVDY GEL DFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPR SAQPLRPEDGHCSWPL
53	hPD-L-1 amino acid sequence	MRIFAVFIFMTYWHLNNAFTVTVPKDL YVVEYGSNM TIECKFPVE KQLDLAALIVY WEMEDKNIIQFVHGEEDLKVQHSSYRQRARLLK DQLSLGNAALQITDVKLQDAGVYRCMISYGGADYKRITVKVNAP YNKINQRILV VDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGK TTTTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAELVI PELPLAHPPNERTHLVILGAILLC LGVALTFIFR LRKGRMMDVKKCGIQDTNSK KQSDTHLEET
54	hCTLA-4 amino acid sequence	MACLGFQRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPA VVLASS RGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYM MGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMY PPPY Y LGIGNGTQIY VIDPEPCPDS DFLLWILAAVSSGLFFYSFL LTAVSLSKML KKRSP LTTGVYVKMPPEPE CEKQFQPYFI PIN
55	hLAG3 amino acid sequence	MWEAQFLGLLFLQPLWVAPVKPLQPGAEPVVVWAQEGAPAQLP CSPTIPLQDLSLLRRAGVTWQHQPDSGPPAAAPGHPLAPGPHPAAP SSWGPRPRRYTVLSVGPGLRSGRLPLQPRVQLDERGRQRGDFSL

		WLRPAR RADAGEYRAAVHLRDRALSCRLRLRLGQASMTASPPGSLR ASDWVILNCSFSRPDRPASVHWFRNRGQGRVPVRESPHHHLAESF LFLPQVSPMDSGPWGCILTYRDGFNVSIMYNLTVLGLEPPTPLTVY AGAGSRVGLPCRLPAGVGTRSFLTAKWTPPGGGPDLLVTGDNGD FTLRL EDVSQAQAGT YTCHIHLEQ QLNATVTLAI ITVTPKSFGS PGSLGKLLCEVTPVSGQERFVWSSLDTPSQRSFSGPWLEAQEAQLL SQPWQCQLYQGERLLGAAVYFTEL SSPGAQRSGR APGALPAGHL LLFLILGVLS LLLVLTGAFG FHLWRRQWRPRRFSALEQGI HPPQAQSKIE ELEQEPEPEP EPEPEPEPEP EPEQL
56	hTIM3 amino acid sequence	MFSHLPFDCVLLLLLLLLLRSSEVEYRAEVGQNAYLPCFYTPAAPG NLVPVCWGKGACPVFECGNVVLRTDERDVNYWTSRYWLNNGDFR KGDVSLTIENVTLADSGIYCCRIQIPGIMNDEKFNLKLVIKPAKVTP APTR QRDFTAAFR MLTTRGHGPA ETQTLGSLPD INLTQISTLA NELRDSRLANDLRDSGATIRGIYIGAGICAGLALALIFGALIFKWYS HSKEKIQNLSLISLANLPPSGLANAVAEGIRSEENIYTIENVYEVEE PNEYCYVSSRQQPSQPLGCRFAMP
57	hB7-H3 amino acid sequence	MLRRRGSPGMGVHVGAAALGALWFCLTGALEVQVPEDPVVALVG TDATLCC SFSPEPGFSLQLNLIWQLT DTKQLVHSFA EGQDQGSAYA NRTALFPDLLAQGNASRLRQVRVADEGSFCFVSIRDFGSAAVSL QVAA PYSKPSMTLE PNKDLRPGDT VTITCSSYQG YPEAEVFWQD GQGVPLTGNVTTSQMANEQGLFDVHSILRVVLGANGTYSCLVRN PVLQQD AHSSVTITPQRSPTGAVEVQVPEDPVVALVGTDATLRCSF SPEPGFSLAQLNLIWQLTDTKQLVHSFTEGRDQGSAYANRTALFP DLLAQ GNASRLRQVR RVADEGSFTC FVSIRDFGSA AVSLQVAAPY SKPSMTLEPNKDLRPGDVTITCSSYRGYPEAEVFWQDGQGVPLT GNVTT SQMANEQGLFDVHSVLRVVLGANGTYSCLVRNPVLQQDAH GSVTITGQPMTFPEALWVTVGLSVCLIALLLVALAFVCWRKIKQSC EEN AGAEDQDGEGEKSKTALQPLKHSDSKEDDGQEIA
58	hB7-H4 amino acid sequence	MASLGQILFWSIISIIILAGAIALIGFGISAFSMPEVNVVDYNASSETL RCEAPRWFPQPTVVWASQVDQGANFSEVSNTSFELNSENVTMKV VSVLYN VTINNTYSCM IENDIAKATGDIKVTSEIKRRSHLQLLNS KASLCVSSFFAISWALLPLSPYLMLK
59	Anti-VEGF clone B20- 4.1.1 Heavy Chain nucleic acid sequence	ATGGGCTGGTCCCTGATCCTGCTGTTCCCTGGTGGCTGTGGCCACTG GCGTGCACTCTGAAGTGCAGCTGGTGGAATCTGGCGGCGGA CTGGTGCAGCCTGGCGGATCTCTGAGACTGAGCTGTGCCG CCAGCGGCTTCAGCATCAGCGGCAGCTGGATCTTCTGGGTG CGCCAGGCCCCCTGGAAAGGGCCTGGAATGGGTGGGAGCCA TCTGGCCTTTTGGCGGCTACACCCACTACGCCGACAGCGTG AAGGGCCGGTTTACCATCAGCGCCGACACCAGCAAGAACA CCGCCTACCTCCAGATGAACAGCCTGCGGGCCGAGGACAC CGCCGTGTACTATTGTGCCAGATGGGGCCACAGCACCTCCC CCTGGGCCATGGATTATTGGGGCCAGGGAACCCTCGTGAC CGTGTCCTCTGCCAAAACAACAGCCCCATCGGTCTATCCACTG GCCCCTGTGTGTGGAGGTACAACCTGGCTCCTCGGTGACTCTAGG

		ATGCCTGGTCAAGGGTTATTTCCCTGAGCCAGTGACCTTGACCT GGA ACTCTGGCTCACTGTCCAGTGGTGTGCACACCTTCCCAGCT CTCCTCCAATCTGGCCTCTACACCCTCAGCAGCTCAGTGACTGT AACCTCGAACACCTGGCCCAGCCAGACCATCACCTGCAATGTG GCCCACCCGGCAAGCAGCACCAAAGTGGACAAGAAAATTGAG CCCAGAGTGGCCATAACACAGAACCCCTGTCCTCCACTCAAAG AGTGTCCCCCATGCGCAGCTCCAGACCTCTTGGGTGGACCATCC GTCTTCATCTTCCCTCCAAAGATCAAGGATGTACTCATGATCTC CCTGAGCCCCATGGTCACATGTGTGGTGGTGGATGTGAGCGAG GATGACCCAGACGTCCAGATCAGCTGGTTTGTGAACAACGTGG AAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAA CAGTACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAGG ACTGGATGAGTGGCAAGGAGTTCAAATGCAAGGTCAACAACAG AGCCCTCCCATCCCCCATCGAGAAAACCATCTCAAAACCCAGA GGGCCAGTAAGAGCTCCACAGGTATATGTCTTGCCTCCACCAG CAGAAGAGATGACTAAGAAAGAGTTCAGTCTGACCTGCATGAT CACAGGCTTCTTACCTGCCGAAATTGCTGTGGACTGGACCAGC AATGGGCGTACAGAGCAAACTACAAGAACACCGCAACAGTC CTGGACTCTGATGGTTCTTACTTCATGTACAGCAAGCTCAGAGT ACAAAAGAGCACTTGGGAAAGAGGAAGTCTTTTCGCCTGCTCA GTGGTCCACGAGGGTCTGCACAATCACCTTACGACTAAGACCA TCTCCCGGTCTCTGGGTAAA
60	Anti-VEGF clone B20- 4.1.1 Heavy Chain amino acid sequence	MGWSLILLFLVAVATGVHSEVQLVESGGGLVQPGGSLRLSCAASG FSISGSWIFWVRQAPGKGLEWVGAIWPFGGYTHYADSVKGRF TISADTSKNTAYLQMNSLRAEDTAVYYCARWGHSTSPWAMDY WGQGTLVTVSSAKTTAPSVYPLAPVCGGTTGSSVTLGCLVKGYF PEPVTLTWNSGSLSSGVHTFPALLQSGLYTLSSSVTVTSNTWPSQTI TCNVAHPASSTKVDKKIEPRVPITQNPCPPLKECPPCAAPDLLGGPS VFIFPPKIKDVLMI~~SL~~SPMVTCVVVDVSEDDPDVQISW~~FV~~NNVEVH TAQTQTHREDYNSTLRVVSALPIQH~~QD~~WMSGKEFKCKVNNRALP SPIEKTISKPRGPVRAPQVYVLPPPAEEMTKKEFSLT~~CM~~ITGFLPAEI AVDWT~~S~~NGRTEQNYKNTATVLDSDGSYFMYSKLRVQKSTW~~ERG~~ SLFACSVVHEGLHNHLTKTISRSLGK
61	Anti-VEGF clone B20- 4.1.1 Light Chain nucleic acid sequence	ATGGACATGAGAGTGCCCCGCCAGCTGCTGGGACTTCTGCTGCTGT GGCTGCCAGGCGCCAGATGCGACATCCAGATGACCCAGAGCC CCAGCAGCCTGTCTGCCAGCGTGGGCGACAGAGTGACCAT CACCTGTAGAGCCTCTCAGGGCGTGCGGACAAGCCTGGCC TGGTATCAGCAGAAGCCTGGCAAGGCCCCCAAGCTGCTGAT CTACGATGCCAGCTCTCTGGCCAGCGGCGTGCCCAGCAGAT TTTCTGGCAGCGGCTCCGGCACCGACTTCACCCTGACAATC AGCTCCCTCCAGCCCCGAGGACTTCGCCACCTACTACTGCCA GCAGAGCTACAAGAGCCCCCTGACCTTTGGCCAGGGCACC AAGGTGGAAATCAAGCGGGCTGATGCTGCACCAACTGTATCC ATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGCCTC AGTCGTGTGCTTCTTGAACAAC~~TT~~CTACCCCAAAGACATCAATG TCAAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCT GAACAGTTGGACTGATCAGGACAGCAAAGACAGCACCTACAGC ATGAGCAGCACCCCTCACGTTGACCAAGGACGAGTATGAACGAC ATAACAGCTATACCTGTGAGGGCCACTCACAAGACATCAACTTC

		ACCCATTGTCAAGAGCTTCAACAGGAATGAGTGT
62	Anti-VEGF clone B20-4.1.1 Light Chain amino acid sequence	MDMRVPAQLLGLLLLWLPGARCDIQMTQSPSSLSASVGDRVITITC RASQGVRTSLAWYQQKPGKAPKLLIYDASSLASGVPSRFSGSG SGTDFTLTISLQPEDFATYYCQQSYKSPLTFGQGTKVEIKRAD AAPTVISIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSRQ NGVLNSWTDQDSKDSTYSMSSTLTTLTKDEYERHNSYTCEATHKTS TSPIVKSFNRNEC
63	Anti-CTLA-4 clone 9D9 Heavy Chain nucleic acid sequence	ATGGGCTGGTCCCTGATCCTGCTGTTCCCTGGTGGCTGTGGCCACC GGCGTGCACCTCTGAAGCCAAGCTCCAGGAATCCGGCCCTGTG CTCGTGAAGCCTGGCGCCTCTGTGAAGATGAGCTGCAAGG CCAGCGGCTACACCTTTACCGACTACTACATGAACTGGGTC AAGCAGAGCCACGGCAAGTCTCTGGAATGGATCGGCGTGA TCAACCCCTACAACGGCGACACCAGCTACAACCAGAAAGTTC AAGGGCAAGGCCACCCTGACCGTGGACAAGAGCAGCAGCA CCGCCTACATGGAAGTGAACAGCCTGACCAGCGAGGACAG CGCCGTGTACTATTGCGCCCGGTACTACGGCAGTTGGTTTCG CCTATTGGGGCCAGGGCACCCCTGATCACCGTGTCCACAGCC AAAACAACAGCCCCATCGGTCTATCCACTGGCCCCTGTGTGTG GAGGTACAAGTGGCTCCTCGGTGACTCTAGGATGCCTGGTCAA GGGTTATTTCCCTGAGCCAGTGACCTTGACCTGGAAGTCTGGCT CACTGTCCAGTGGTGTGCACACCTTCCCAGCTCTCCTCCAATCT GGCCTCTACACCCTCAGCAGCTCAGTGACTGTAACCTCGAACA CCTGGCCCAGCCAGACCATCACCTGCAATGTGGCCCACCCGGC AAGCAGCACCAAAGTGGACAAGAAAATTGAGCCCAGAGTGCC CATAACACAGAACCCCTGTCCTCCACTCAAAGAGTGTCCCCCAT GCGCAGCTCCAGACCTCTTGGGTGGACCATCCGTCTTCATCTTC CCTCCAAAGATCAAGGATGTACTCATGATCTCCCTGAGCCCCAT GGTCACATGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGAC GTCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAG CTCAGACACAAACCCATAGAGAGGATTACAACAGTACTCTCCG GGTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGT GGCAAGGAGTTCAAATGCAAGGTCAACAACAGAGCCCTCCCAT CCCCCATCGAGAAAACCATCTCAAAACCCAGAGGGCCAGTAAG AGCTCCACAGGTATATGTCTTGCCTCCACCAGCAGAAGAGATG ACTAAGAAAGAGTTCAGTCTGACCTGCATGATCACAGGCTTCTT ACCTGCCGAAATTGCTGTGGACTGGACCAGCAATGGGCGTACA GAGCAAAACTACAAGAACACCGCAACAGTCCTGGACTCTGATG GTTCTTACTTCATGTACAGCAAGCTCAGAGTACAAAAGAGCAC TTGGGAAAGAGGAAGTCTTTTCGCCTGCTCAGTGGTCCACGAG GGTCTGCACAATCACCTTACGACTAAGACCATCTCCCGGTCTCT GGGTAAA
64	Anti-CTLA-4 clone 9D9 Heavy Chain amino acid sequence	MGWSLILLFLVAVATGVHSEAKLQESGPVLVKPGASVKMSCKAS GYTFTDYIMNWVKQSHGKSLEWIGVINPYNGDTSYNQKFKG KATLTVDKSSSTAYMELNSLTSEDSAVYYCARYYGSWFAYWG QGTLITVSTAKTTAPSVYPLAPVCGGTTGSSVTLGCLVKGYFPEP VTLTWNSGSLSSGVHTFPALLQSGLYTLSSSVTVTSNTWPSQTITC NVAHPASSTKVDKKIEPRVPITQNPCPPLKECPPCAAPDLLGGPSVF IFPPKIKDVLMISSLPMVTCVVDVSEDDPDVQISWVNNVEVHTA QTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNRALPSPI EKTISKPRGPRAPQVYVLPPPAEEMTKKEFSLTCMITGFLPAEIAV

		DWTSNGRTEQNYKNTATVLDSDSYFMYSKLRVQKSTWERSLF ACSVVHEGLHNHLTTKTISRSLGK
65	Anti-CTLA-4 clone 9D9 Light Chain nucleic acid sequence	ATGGACATGAGAGTGCCCCGCCAGCTGCTGGGACTTCTGCTGCTGT GGCTGCCAGGCGCCAGATGCGACATCGTGATGACCCAGACCA CCCTGAGCCTGCCTGTGTCCCTGGGAGATCAGGCCAGCATC AGCTGTCGGAGCAGCCAGAGCATCGTGACAGCAACGGCA ACACCTACCTGGAATGGTATCTCCAGAAGCCCCGGCCAGAGC CCCAAGCTGCTGATCTACAAGGTGTCCAACCGGTTTCAGCGG CGTGCCCGACAGATTTTCTGGCAGCGGCTCCGGCACCGACT TCACCCTGAAGATCTCCCGGGTGGAAGCCGAGGACCTGGG CGTGTACTACTGTTTTCAAGGCAGCCACGTGCCCTACACCT TCGGCGGAGGCACCAAGCTGGAAATCAAGCGGGCTGATGCT GCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAAC ATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACCTTCTACC CCAAAGACATCAATGTCAAGTGGAAGATTGATGGCAGTGAACG ACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAAA GACAGCACCTACAGCATGAGCAGCACCCCTCACGTTGACCAAGG ACGAGTATGAACGACATAACAGCTATACCTGTGAGGCCACTCA CAAGACATCAACTTCACCCATTGTCAAGAGCTTCAACAGGAAT GAGTGT
66	Anti-CTLA-4 clone 9D9 Light Chain amino acid sequence	MDMRVPAQLLGLLLLWLPGARCDIVMTQTTLSPVSLGDQASISC RSSQSIVHSNGNTYLEWYLQKPGQSPKLLIYKVSNRFSGVPDRF SGSGSGTDFTLKISRVEAEDLGVYYCFQGSHVPTYFGGGTKLE IKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKID GSERQNGVLNSWTDQDSKDSTYSMSSTLTTLKDEYERHNSYTCE ATHKTSTSPIVKSFNRNEC

CLAIMS

We claim:

1. A method for treating cancer in a subject comprising administering to the subject an effective amount of interleukin (IL)-2 and an integrin-binding-Fc fusion protein, wherein the integrin-binding-Fc fusion protein comprises (i) an integrin-binding polypeptide comprising an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain.
2. The method of claim 1, wherein IL-2 is an extended pharmacokinetic (PK) IL-2.
3. The method of claim 1, wherein the extended-PK IL-2 comprises a fusion protein.
4. The method of claim 3 wherein the fusion protein comprises an IL-2 moiety and a moiety selected from the group consisting of an immunoglobulin fragment, human serum albumin, and Fn3.
5. The method of claim 4, wherein the fusion protein comprises an IL-2 moiety operably linked to an immunoglobulin Fc domain.
6. The method of claim 4, wherein the fusion protein comprises an IL-2 moiety operably linked to human serum albumin.
7. The method of claim 1, wherein the extended-PK IL-2 comprises an IL-2 moiety conjugated to a non-protein polymer.
8. The method of claim 7, wherein the non-protein polymer is a polyethylene glycol.
9. The method of claim 1, wherein the integrin-binding polypeptide binds to a tumor-associated integrin selected from the group consisting of $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$, or combination thereof.
10. The method of claim 1, wherein the integrin-binding polypeptide binds to $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$.

11. The method of claim 1, wherein the knottin polypeptide scaffold comprises at least three cysteine disulfide linkages or crosslinked cysteine residues, and wherein the integrin-binding loop is adjacent to cysteine residues of the knottin polypeptide scaffold.
12. The method of claim 11, wherein the integrin-binding loop comprises an RGD peptide sequence.
13. The method of claim 11, wherein the knottin polypeptide scaffold is derived from a knottin protein selected from the group consisting of EETI-II, AgRP, and agatoxin.
14. The method of claim 13, wherein the knottin protein is EETI-II.
15. The method of claim 1, wherein the integrin-binding loop comprises an RGD peptide sequence and the knottin polypeptide scaffold is derived from EETI-II.
16. The method of claim 1, wherein the knottin polypeptide scaffold is derived from EETI-II and the integrin-binding loop comprises the sequence $X_1X_2X_3RGDX_7X_8X_9X_{10}X_{11}$, wherein each X represents any amino acid, wherein the loop is inserted between 2 cysteine residues in the EETI-II sequence and replaces the native EETI-II sequence.
17. The method of claim 16, wherein the integrin-binding loop is inserted after the first cysteine in the native EETI-II sequence.
18. The method of claim 1, wherein the integrin-binding polypeptide comprises the amino acid sequence set forth SEQ ID NO: 42 or 43, wherein X_1 is selected from the group consisting of A, V, L, P, F, Y, S, H, D, and N; X_2 is selected from the group consisting of G, V, L, P, R, E, and Q; X_3 is selected from the group consisting of G, A, and P; X_7 is selected from the group consisting of W and N; X_8 is selected from the group consisting of A, P, and S; X_9 is selected from the group consisting of P and R; X_{10} is selected from the group consisting of A, V, L, P, S, T, and E; and X_{11} is selected from the group consisting of G, A, W, S, T, K, and E.
19. The method of claim 1, wherein the integrin-binding polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 59-125.

20. The method of claim 1, wherein the integrin-binding polypeptide comprises the amino acid sequence of SEQ ID NO: 86 or 88.
21. The method of claim 1, wherein the Fc domain is a human IgG1 Fc domain.
22. The method of claim 1, wherein the integrin-binding polypeptide is operably linked with or without a linker to the Fc domain.
23. The method of claim 1, wherein the integrin-binding polypeptide is operably linked to the N-terminus of the Fc domain.
24. The method of claim 1, wherein the integrin-binding polypeptide is operably linked to the C-terminus of the Fc domain.
25. The method of claim 1, wherein the integrin-binding-Fc fusion protein comprises the amino acid sequence of SEQ ID NO: 48, 49, 50, or 51.
26. The method of any one of claims 1-25, wherein the extended-PK IL-2 and the integrin-binding -Fc fusion protein are administered simultaneously or sequentially.
27. The method of any one of claims 1-25, further comprising administering an immune checkpoint blocker.
28. The method of claim 27, wherein the immune checkpoint blocker is an antibody or antibody fragment targeting a protein selected from the group consisting of PD-1, PD-L1, CTLA4, TIM3, LAG3, and a member of the B7 family.
29. The method of claim 28, wherein the immune checkpoint blocker is an antibody or antibody fragment targeting PD-1.
30. The method of claim 28, wherein the immune checkpoint blocker is an antibody or antibody fragment targeting CTLA4.
31. The method of any one of claims 1-25, further comprising administering an antagonist of VEGF.

32. The method of claim 31, wherein the antagonist of VEGF is an antibody or antibody fragment thereof that binds VEGF, an antibody or antibody fragment thereof that binds VEGF receptor, a small molecule inhibitor of the VEGF receptor tyrosine kinase, a dominant negative VEGF, or a VEGF receptor.

33. The method of any one of the claims 1-32 wherein the cancer is selected from the group consisting of melanoma, leukemia, lung cancer, breast cancer, prostate cancer, ovarian cancer, colon cancer, renal cell carcinoma, pancreatic cancer, cervical cancer, and brain cancer.

34. A method for inhibiting growth and/or proliferation of tumor cells in a subject comprising administering to the subject an effective amount of an extended-pharmacokinetic (PK) interleukin (IL)-2, and an integrin-binding-Fc fusion protein, wherein the integrin-binding-Fc fusion protein comprises (i) an integrin-binding polypeptide comprising an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain.

35. A method for inhibiting growth and/or proliferation of tumor cells in a subject comprising administering to the subject an effective amount of an integrin-binding-Fc fusion protein, wherein the integrin-binding-Fc fusion protein comprises (i) an integrin-binding polypeptide comprising an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain, and an immune checkpoint blocker.

36. The method of claim 35, wherein the immune checkpoint blocker is an antibody or antibody fragment targeting a protein selected from the group consisting of PD-1, PD-L1, CTLA4, TIM3, LAG3, and a member of the B7 family.

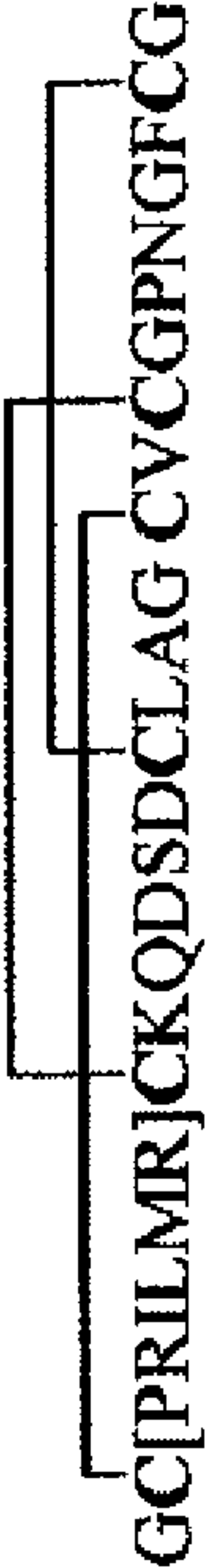
37. The method of claim 36, wherein the immune checkpoint blocker is an antibody or antibody fragment targeting PD-1.

38. The method of claim 36, wherein the immune checkpoint blocker is an antibody or antibody fragment targeting CTLA4.

39. A method for inhibiting growth and/or proliferation of tumor cells in a subject comprising administering to the subject an effective amount of an integrin-binding-Fc fusion protein, wherein the integrin-binding-Fc fusion protein comprises (i) an integrin-binding polypeptide comprising an integrin-binding loop and a knottin polypeptide scaffold; and (ii) an immunoglobulin Fc domain, wherein the integrin-binding polypeptide is operably linked to the Fc domain, and an antagonist of VEGF.
40. The method of claim 39, wherein the antagonist of VEGF is an antibody or antibody fragment thereof that binds VEGF, an antibody or antibody fragment thereof that binds VEGF receptor, a small molecule inhibitor of the VEGF receptor tyrosine kinase, a dominant negative VEGF, or a VEGF receptor.

Figure 1

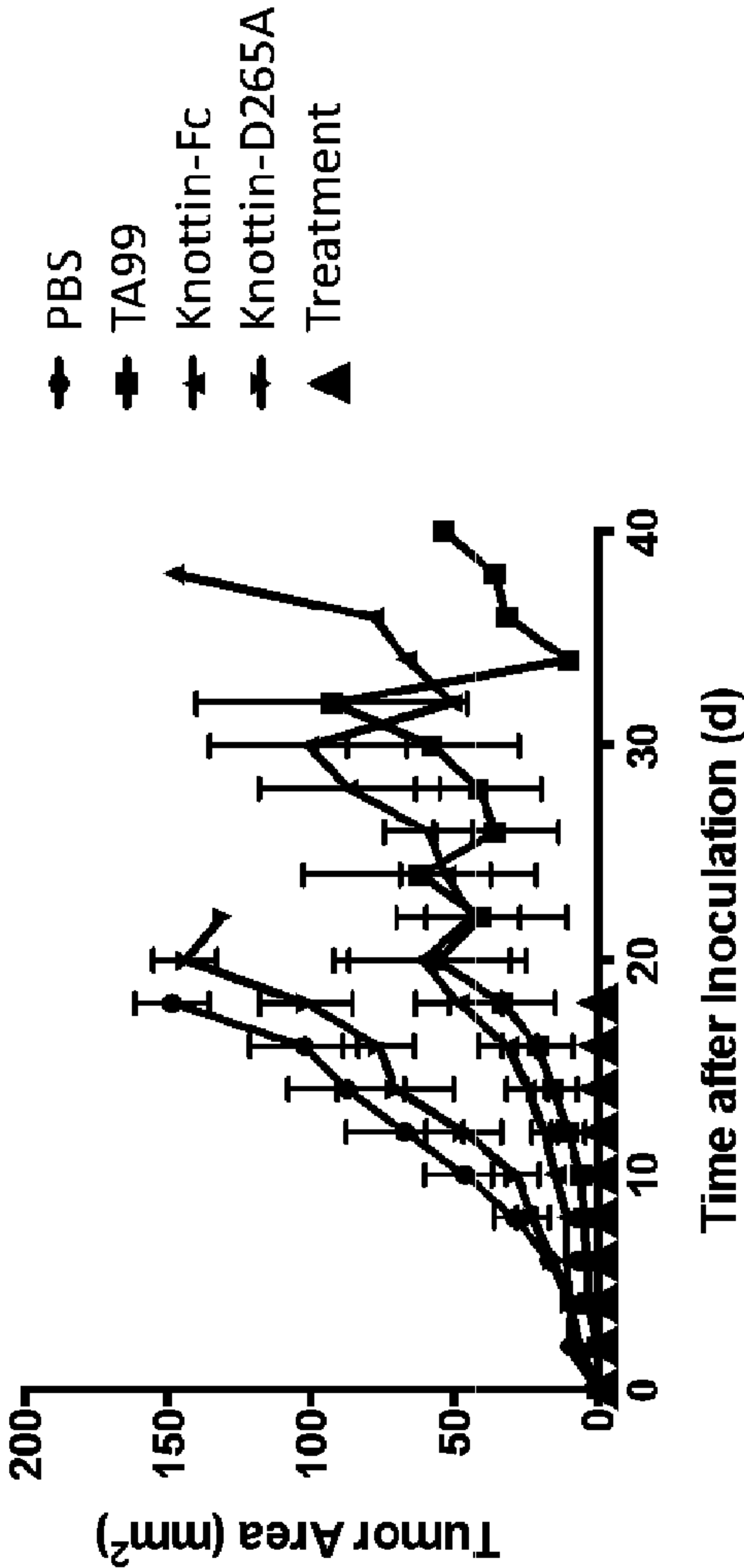
EETI-II



AgRp



Figure 2



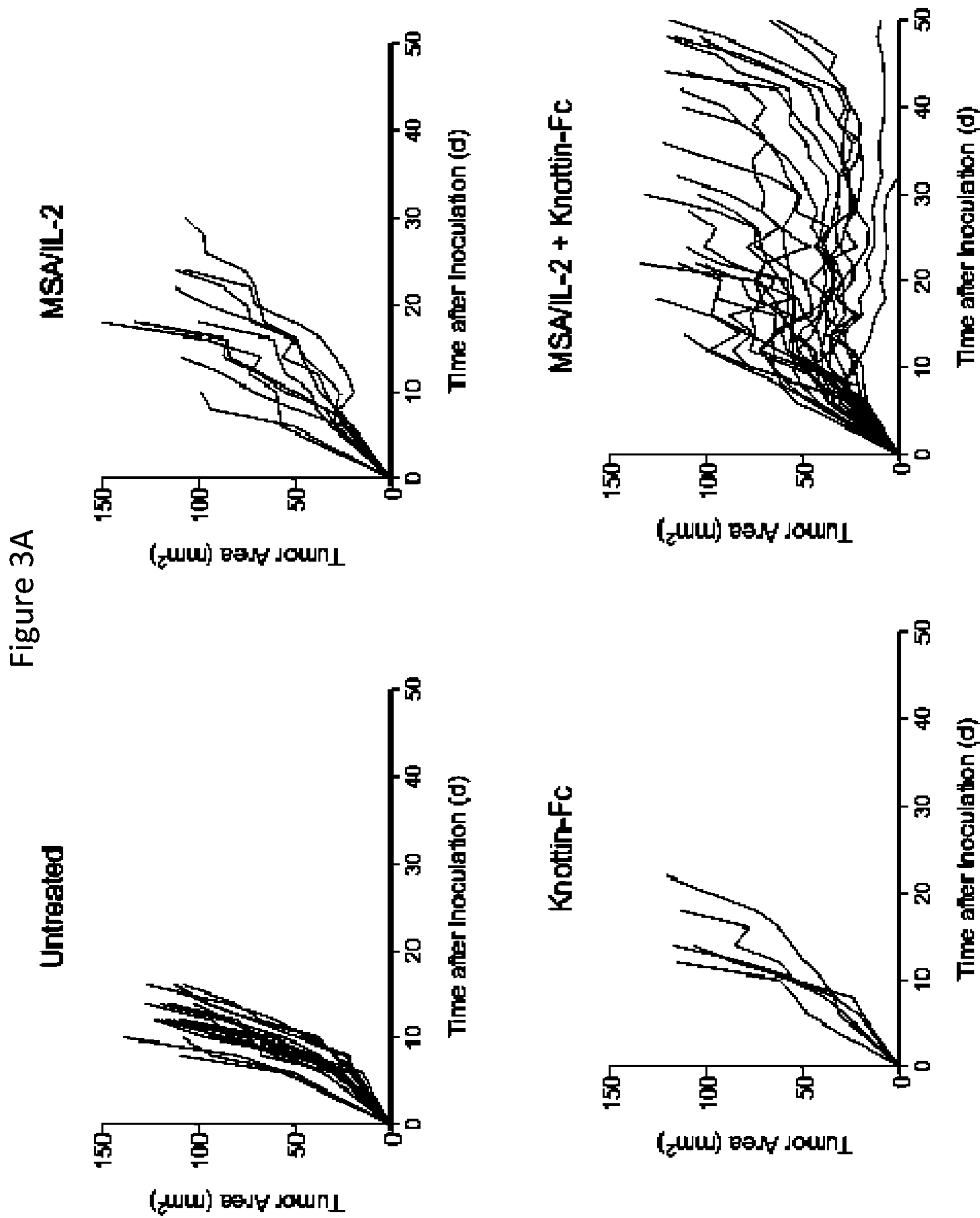


Figure 3B

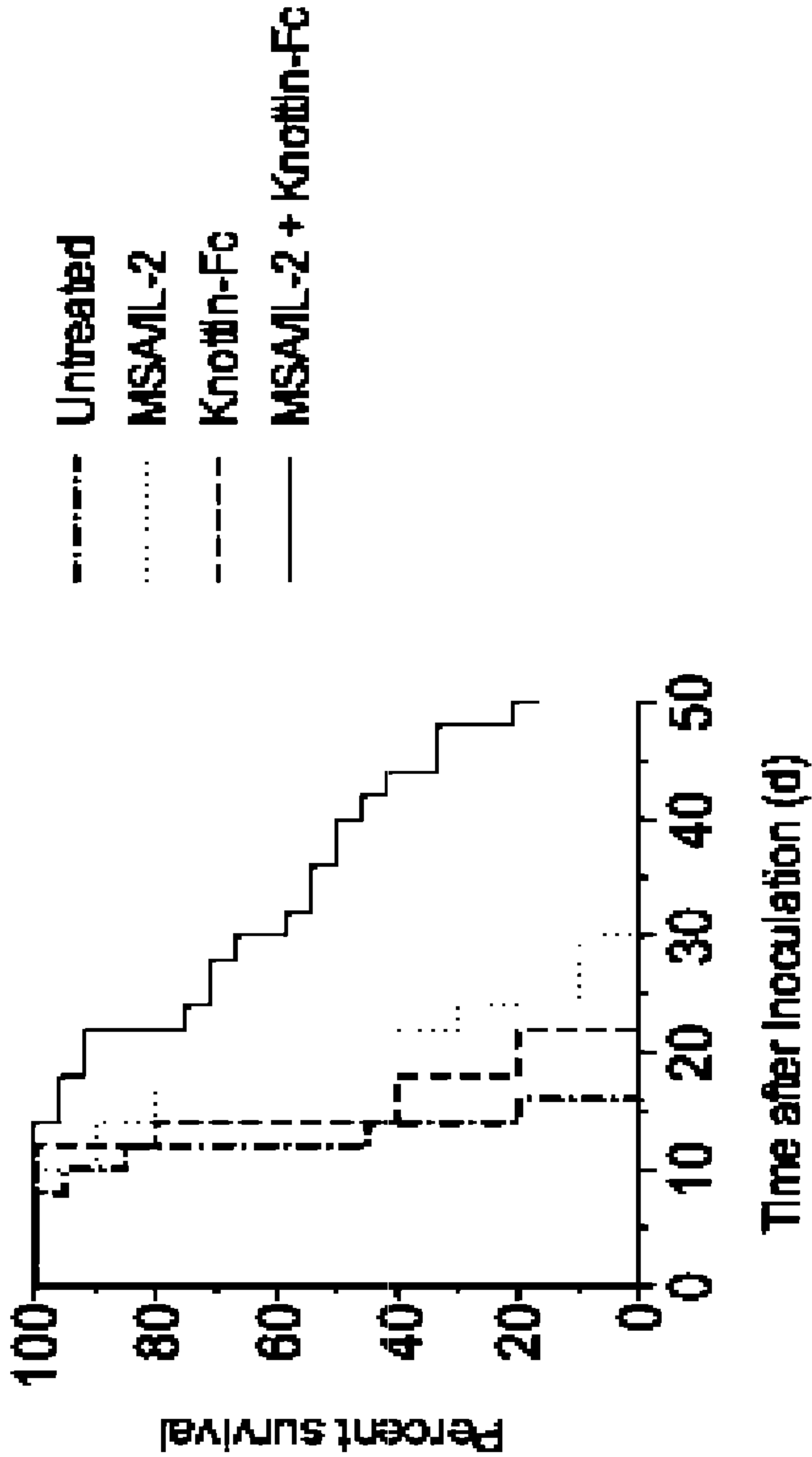
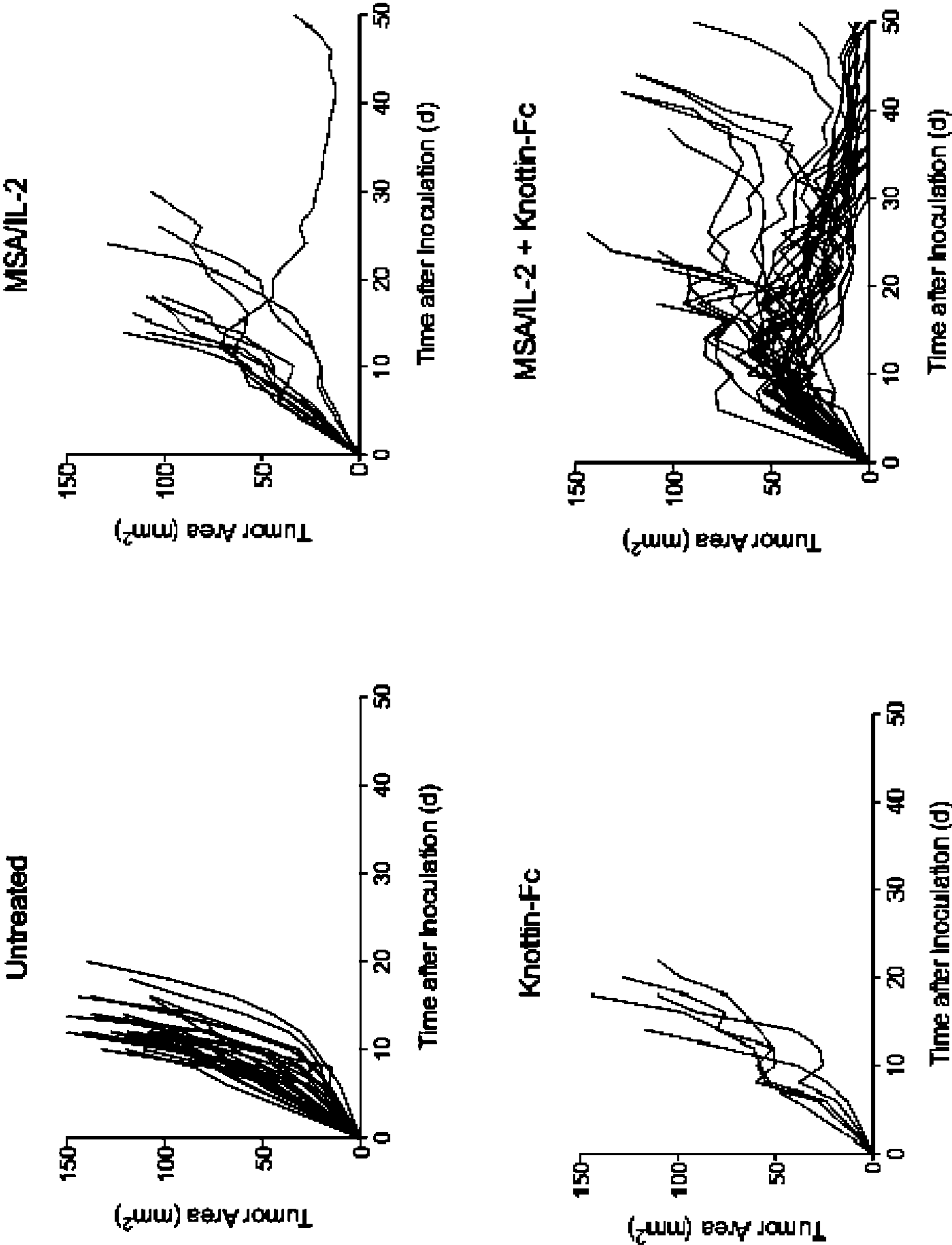


Figure 4A



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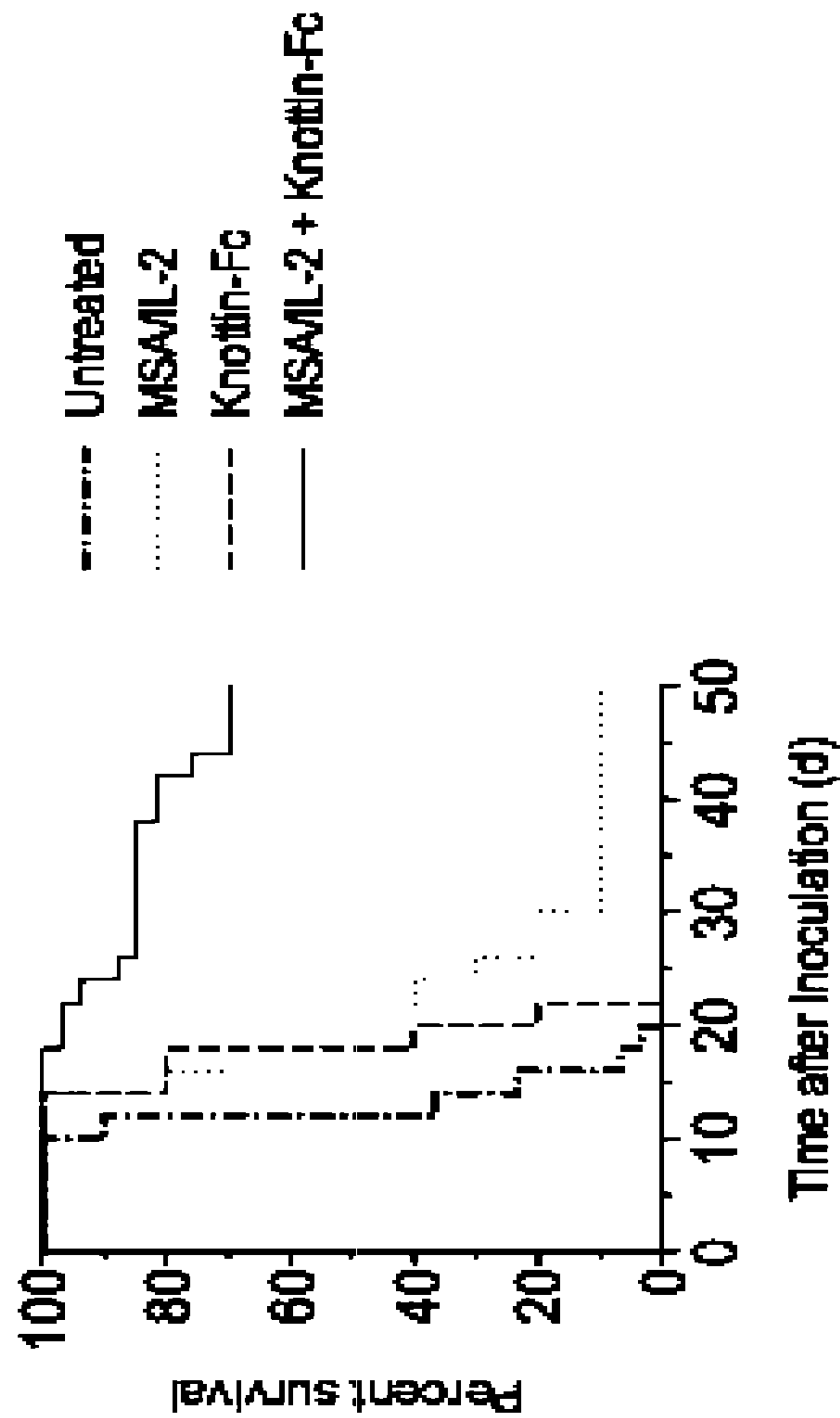


Figure 4B

Figure 5A

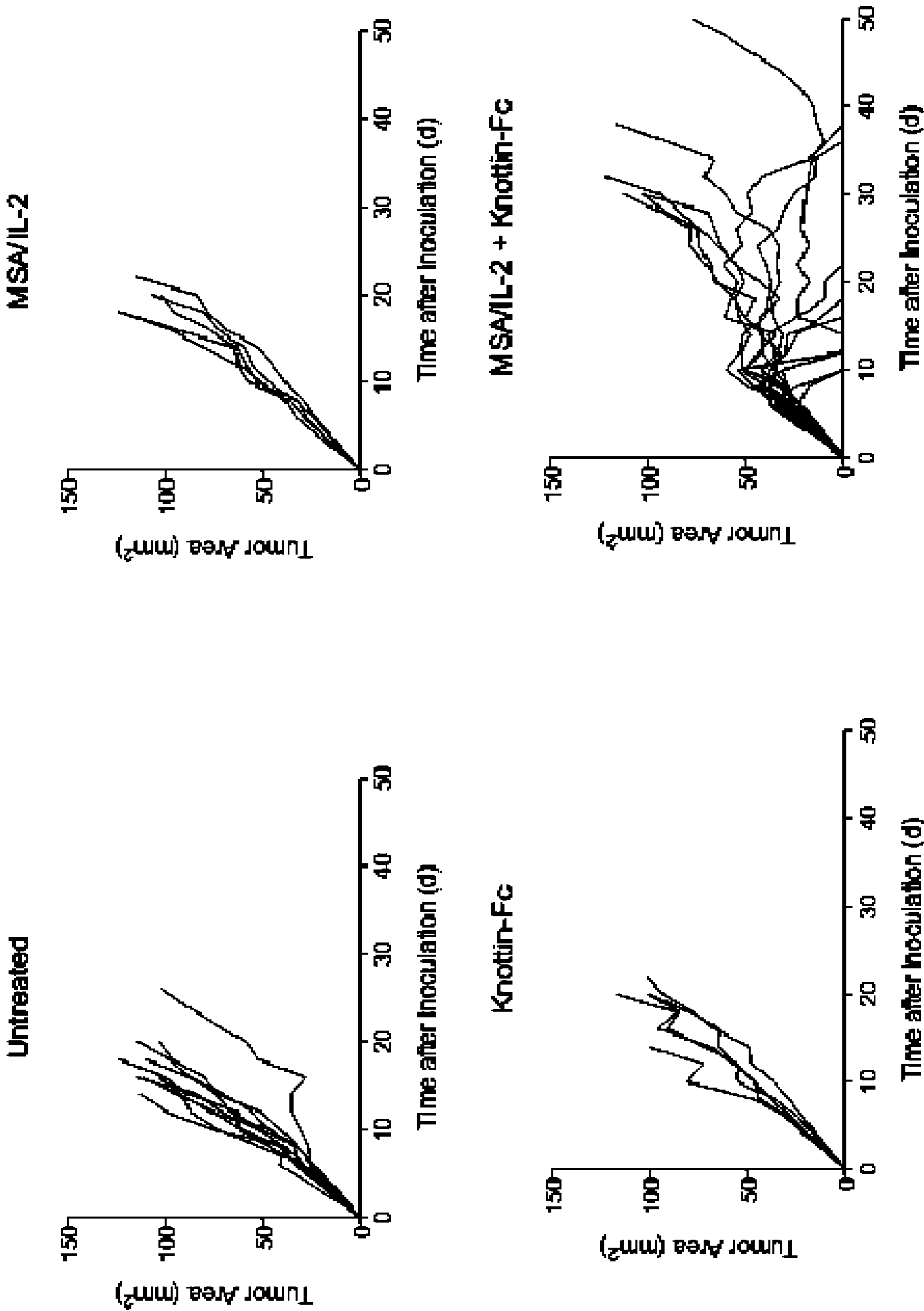


Figure 5B

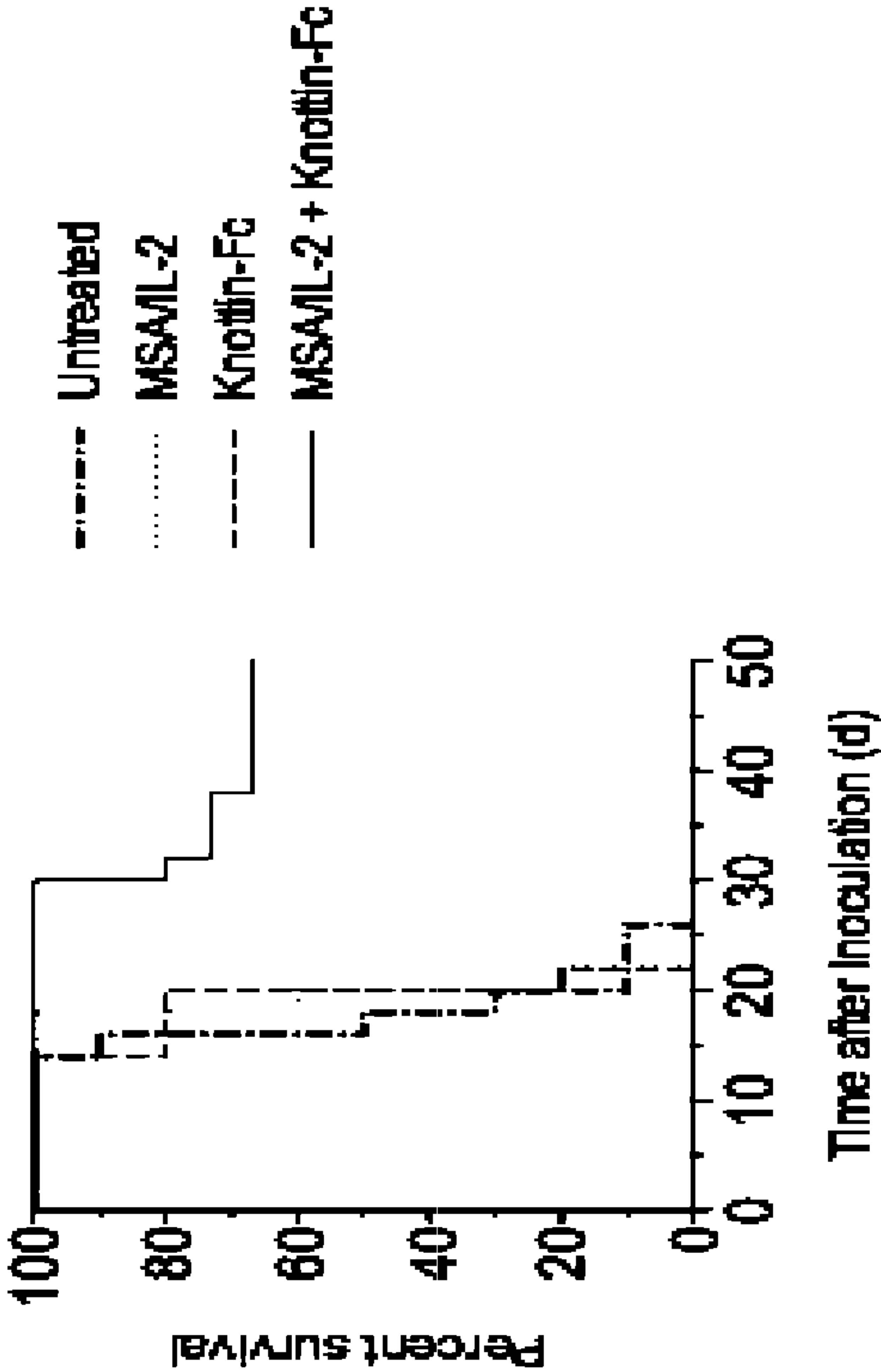


Figure 6A

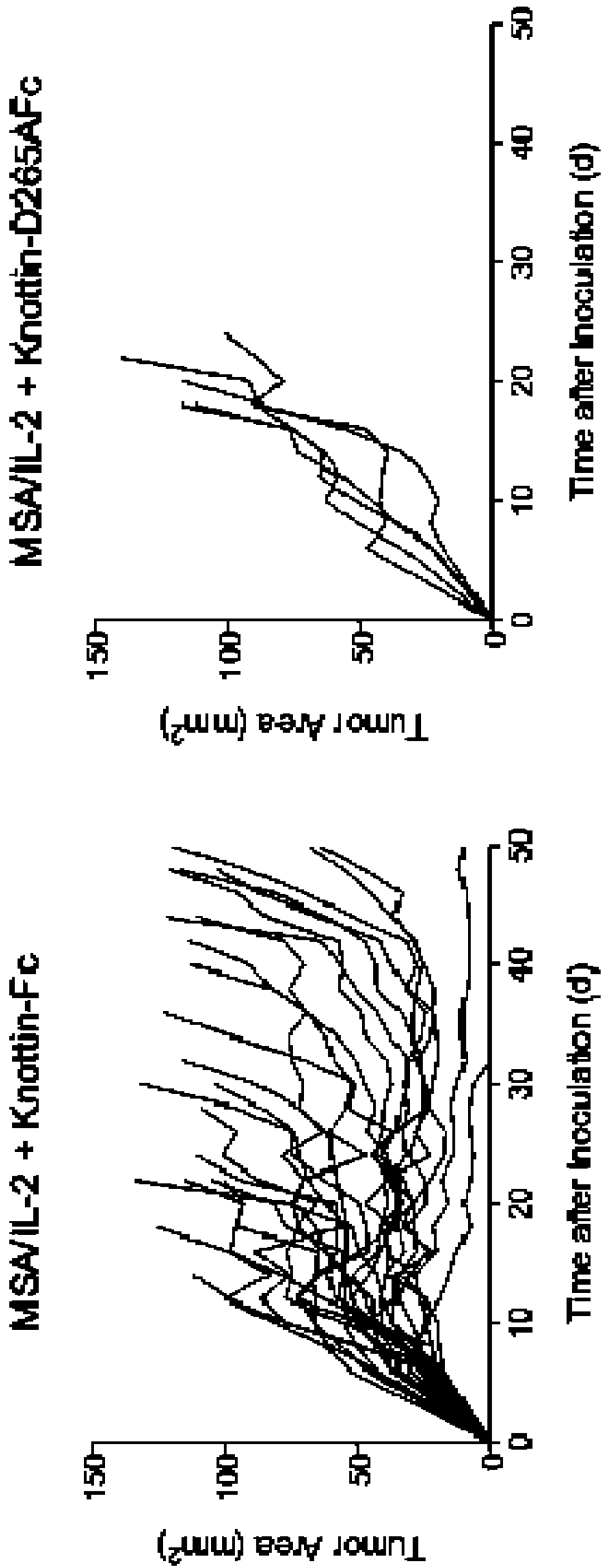


Figure 6B

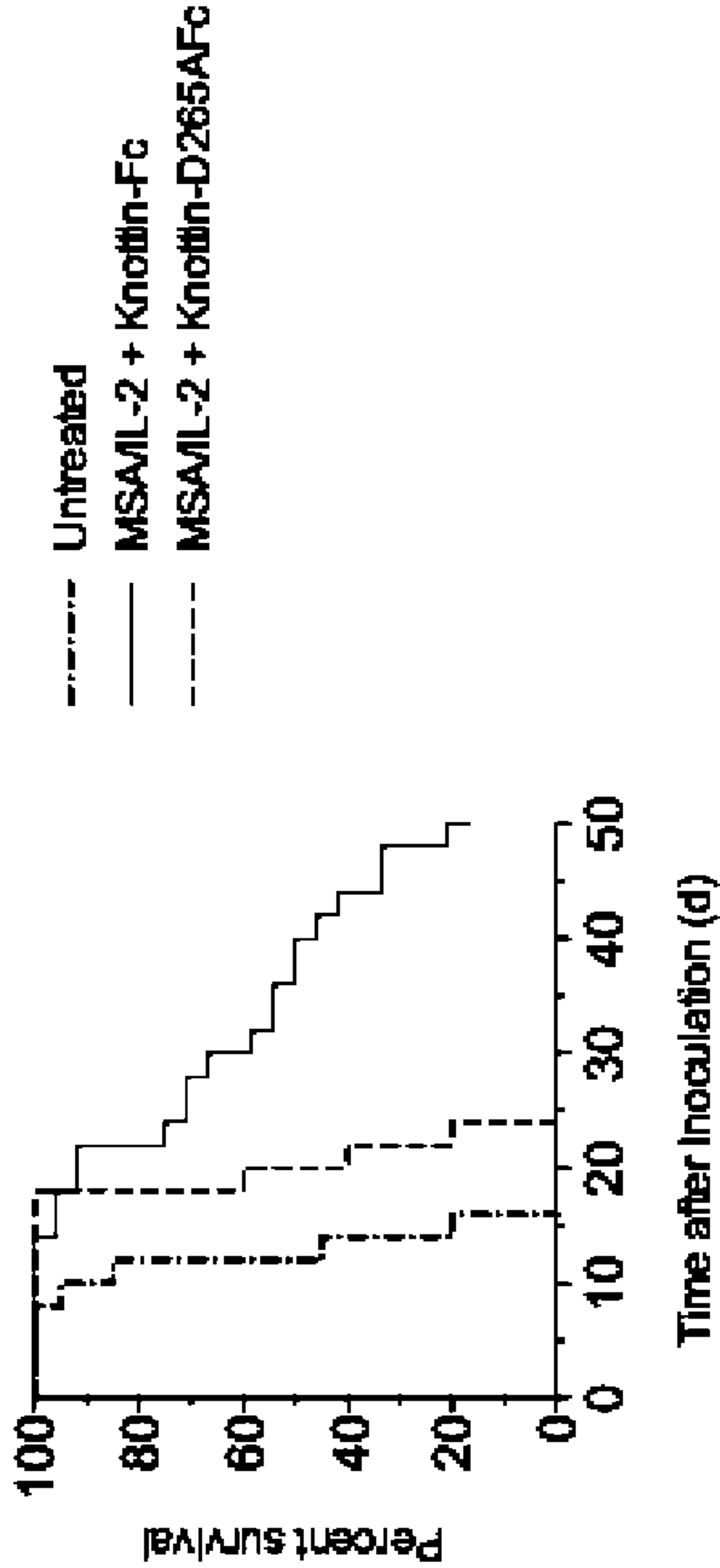


Figure 7A

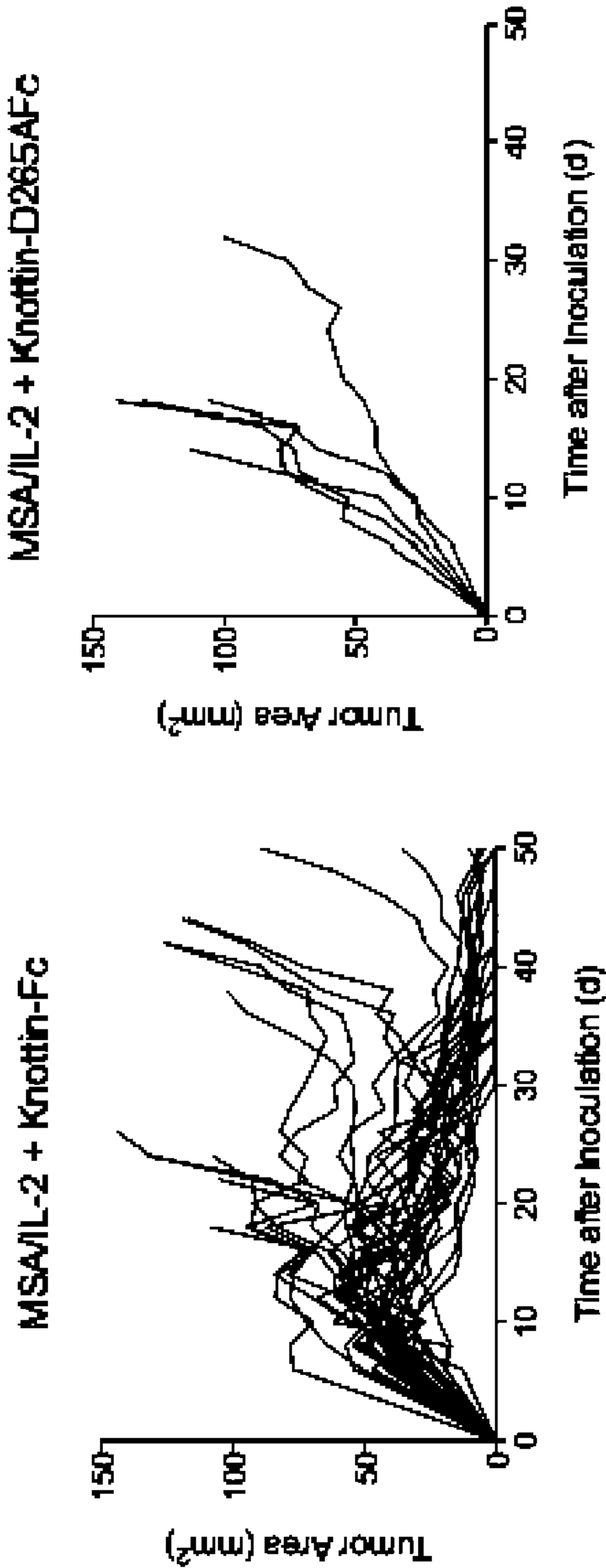


Figure 7B

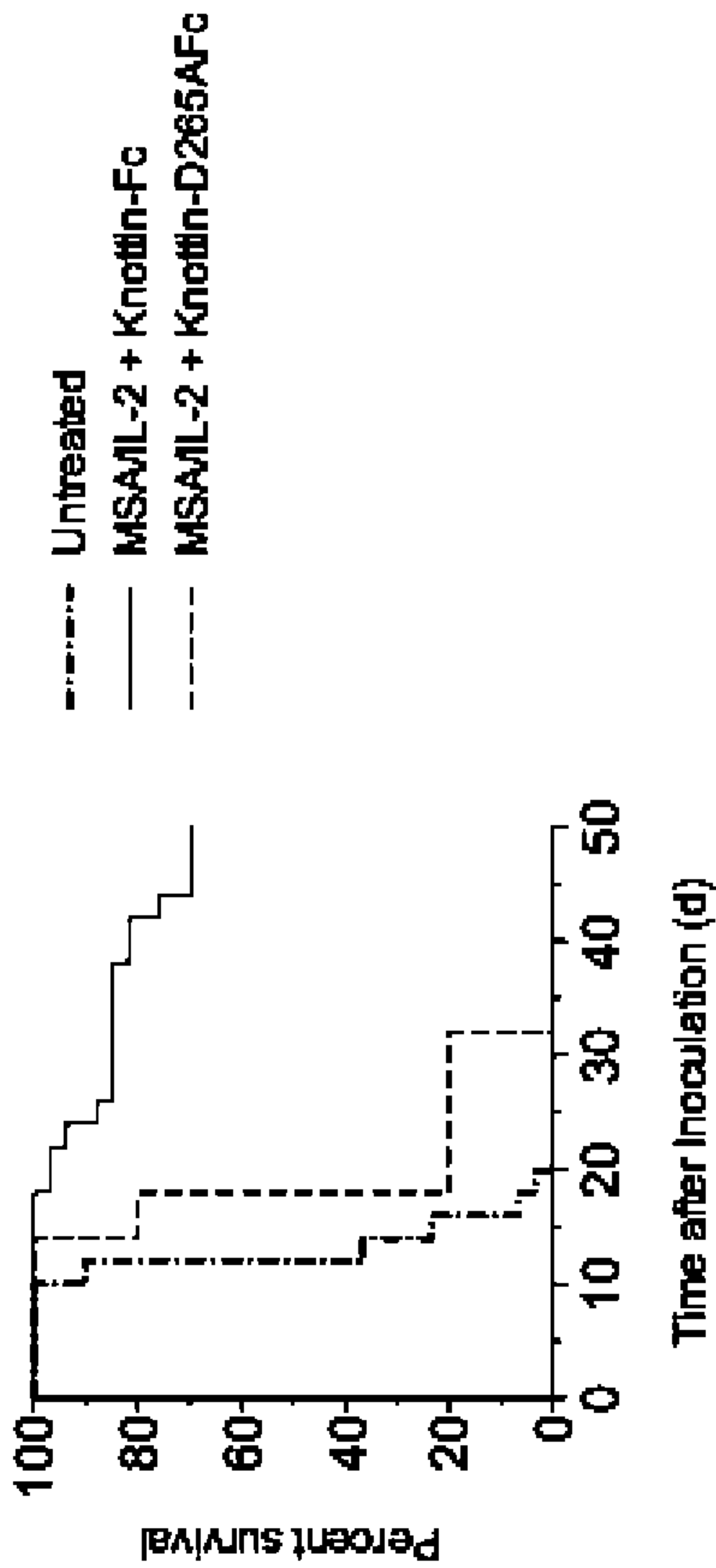
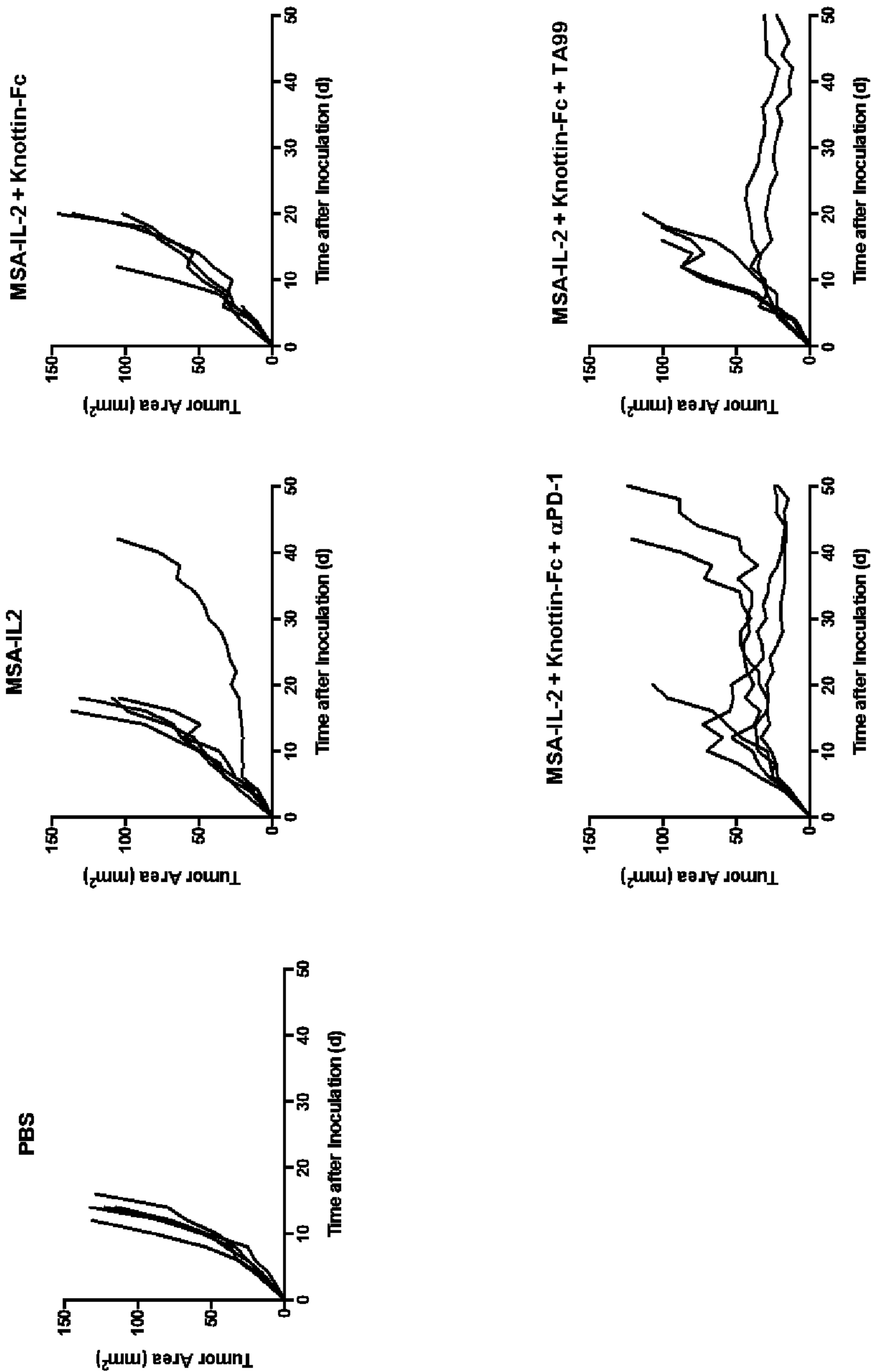


Figure 8A



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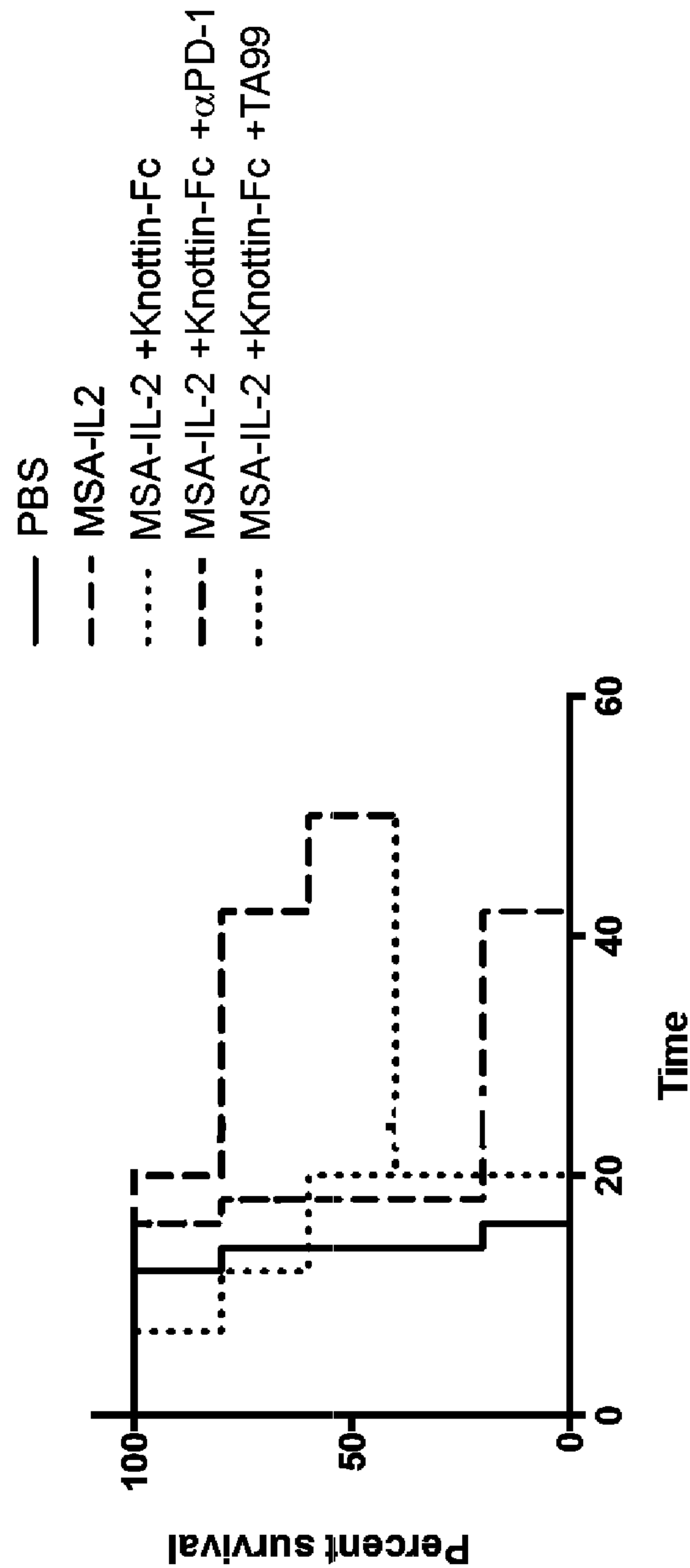


Figure 8B

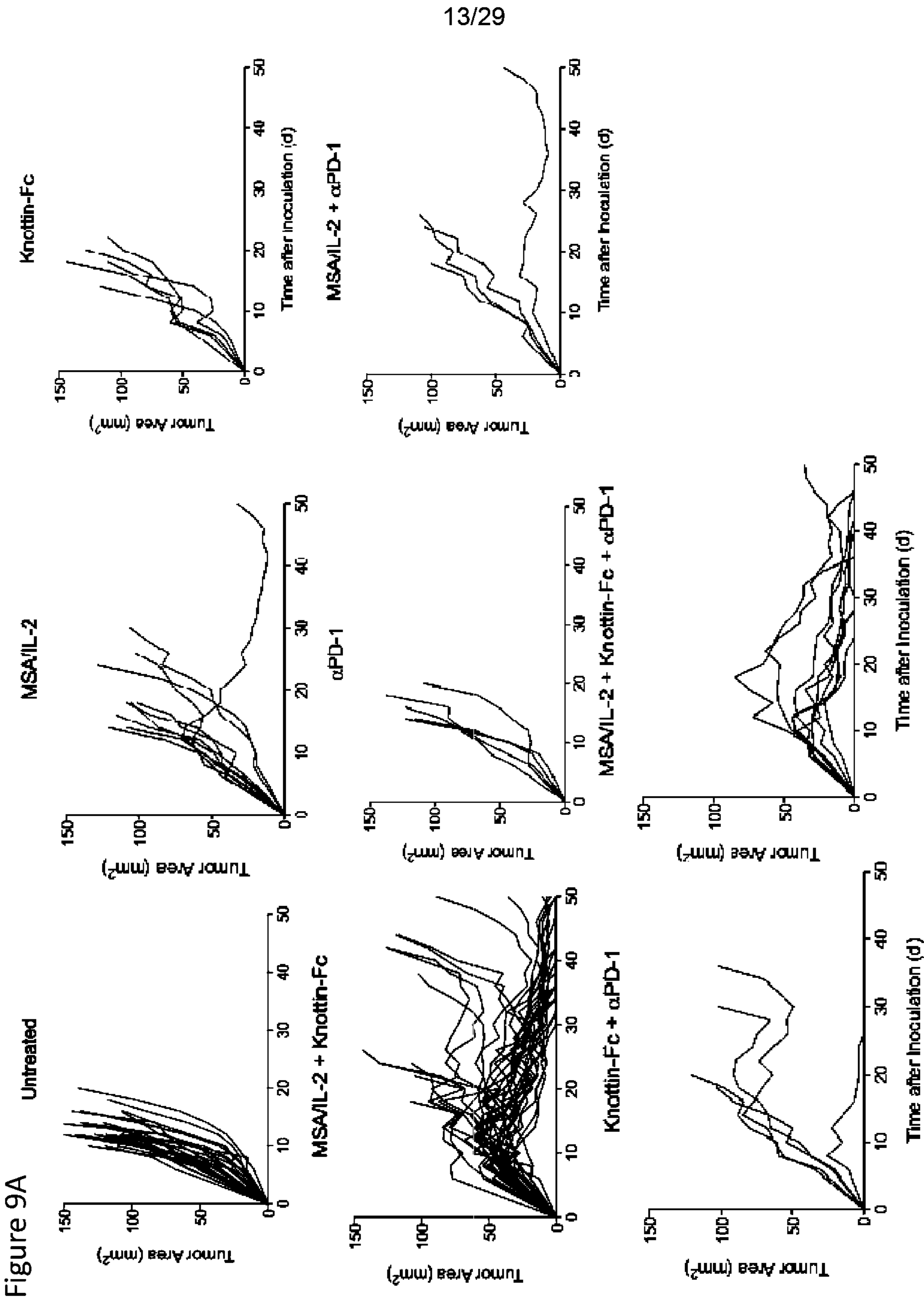


Figure 9B

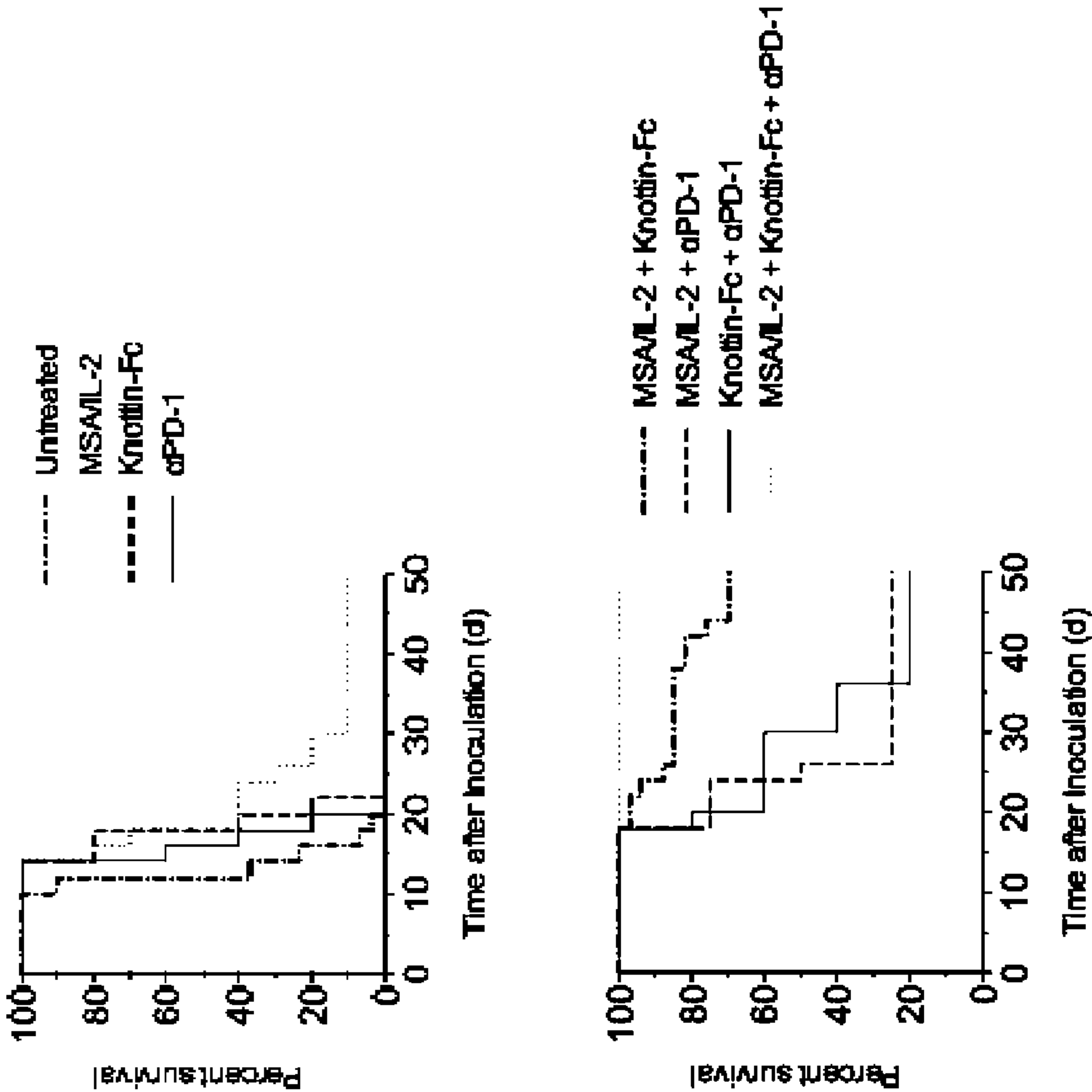


Figure 10

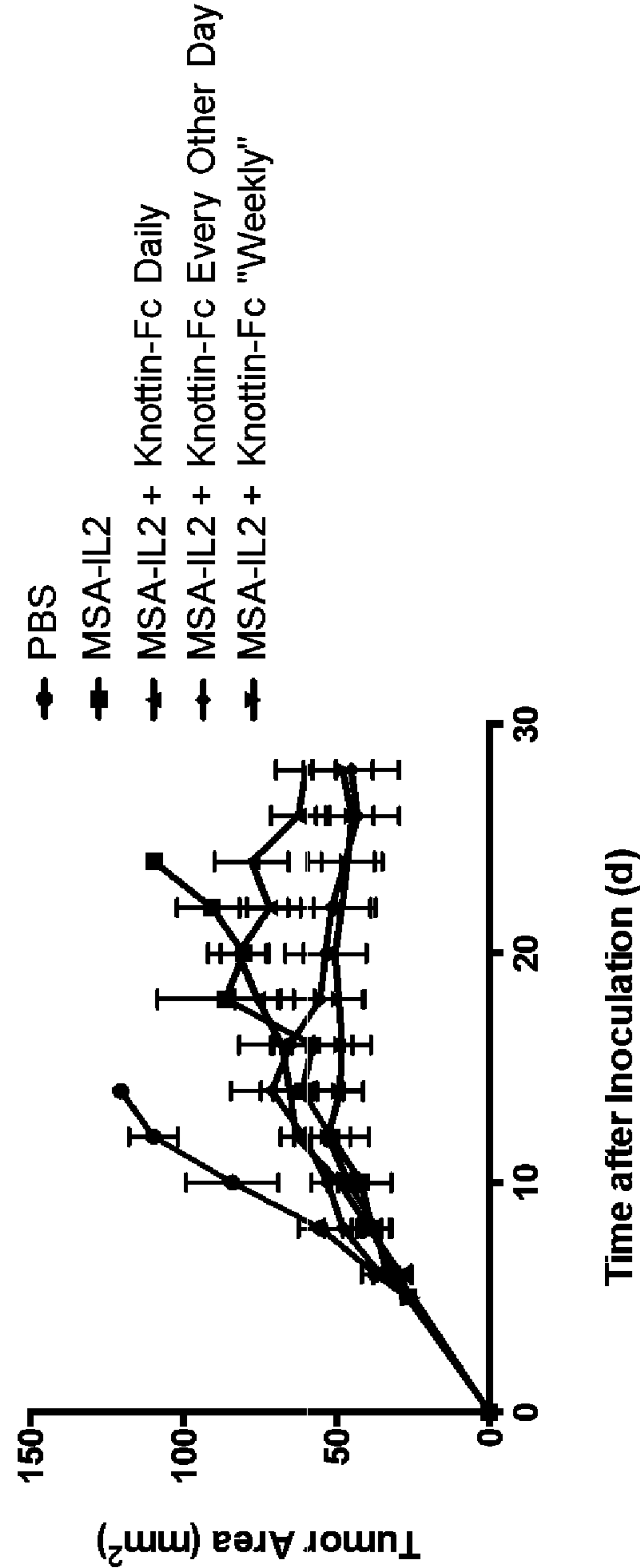


Figure 11

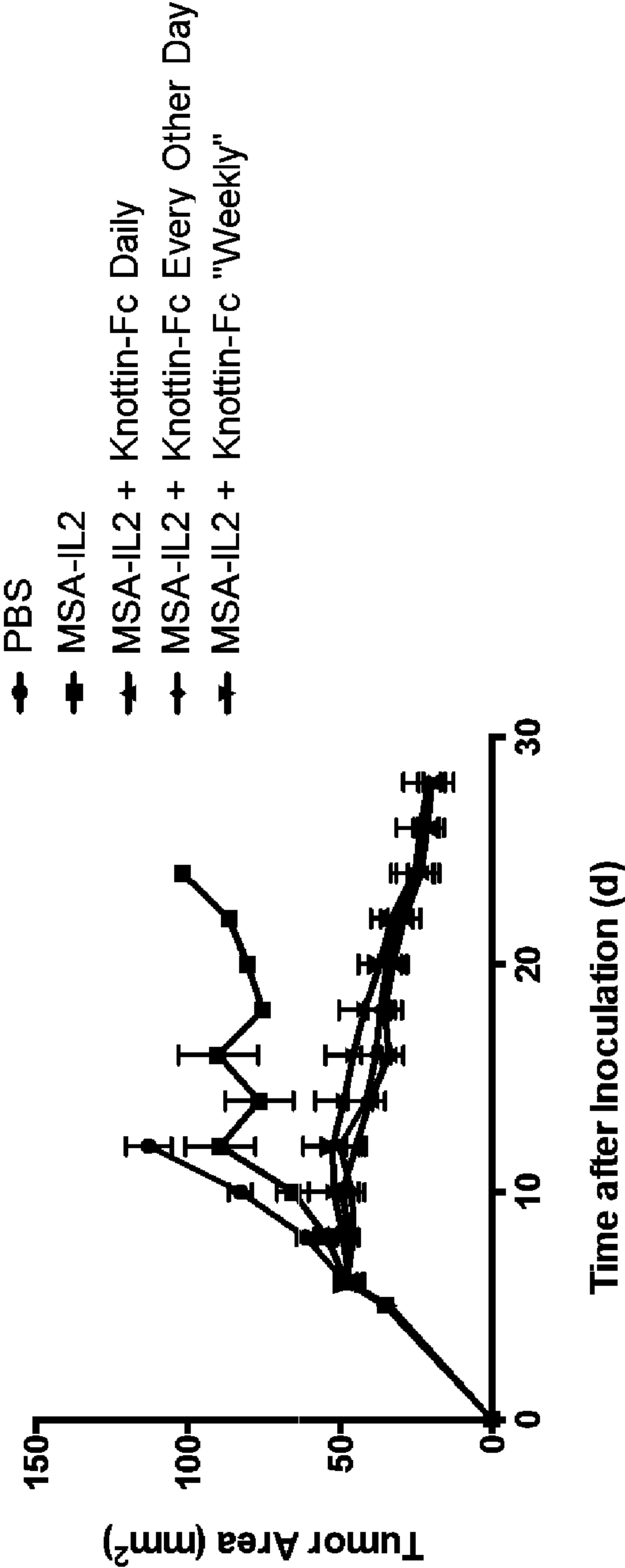
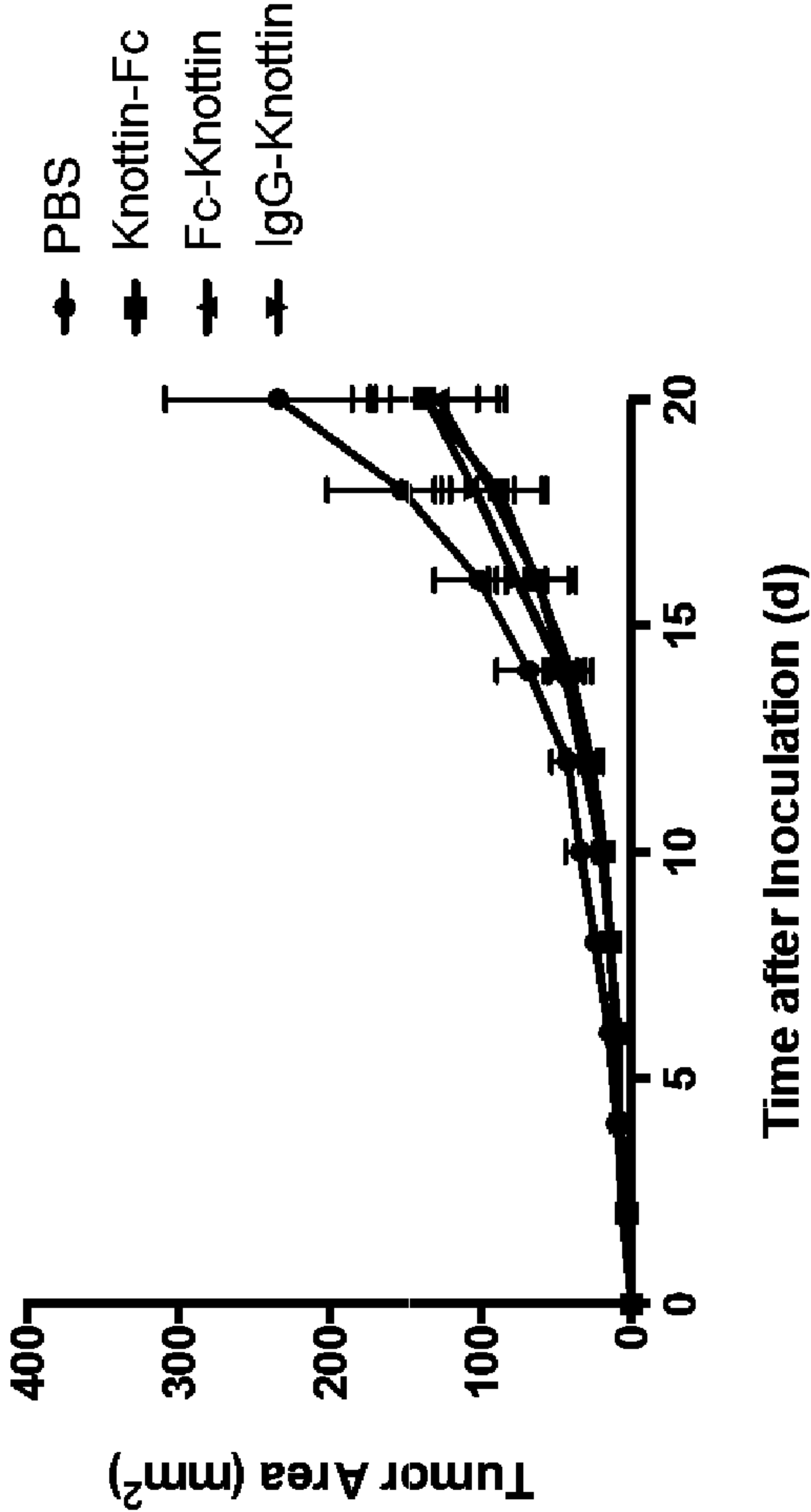


Figure 12



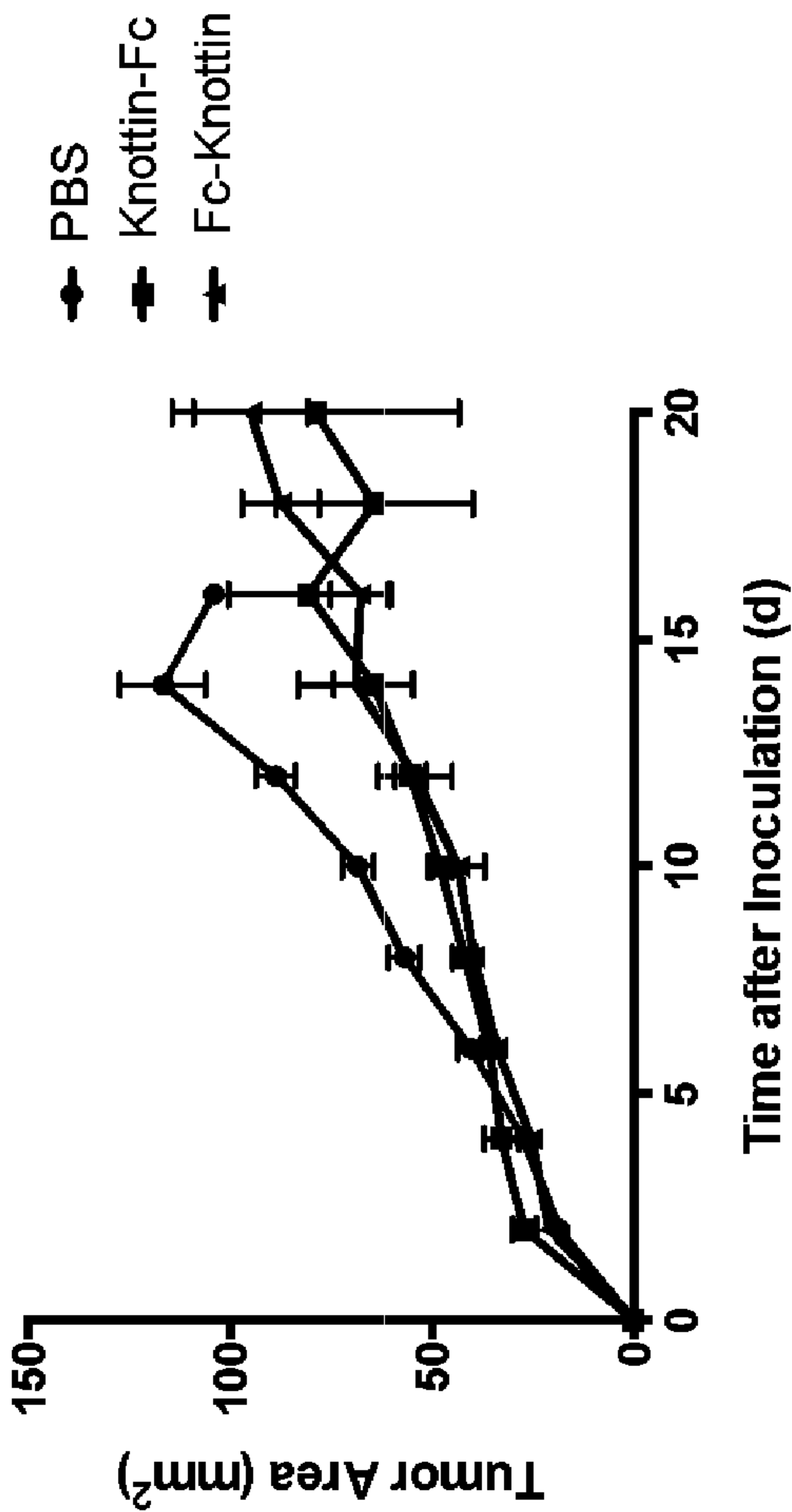


Figure 13

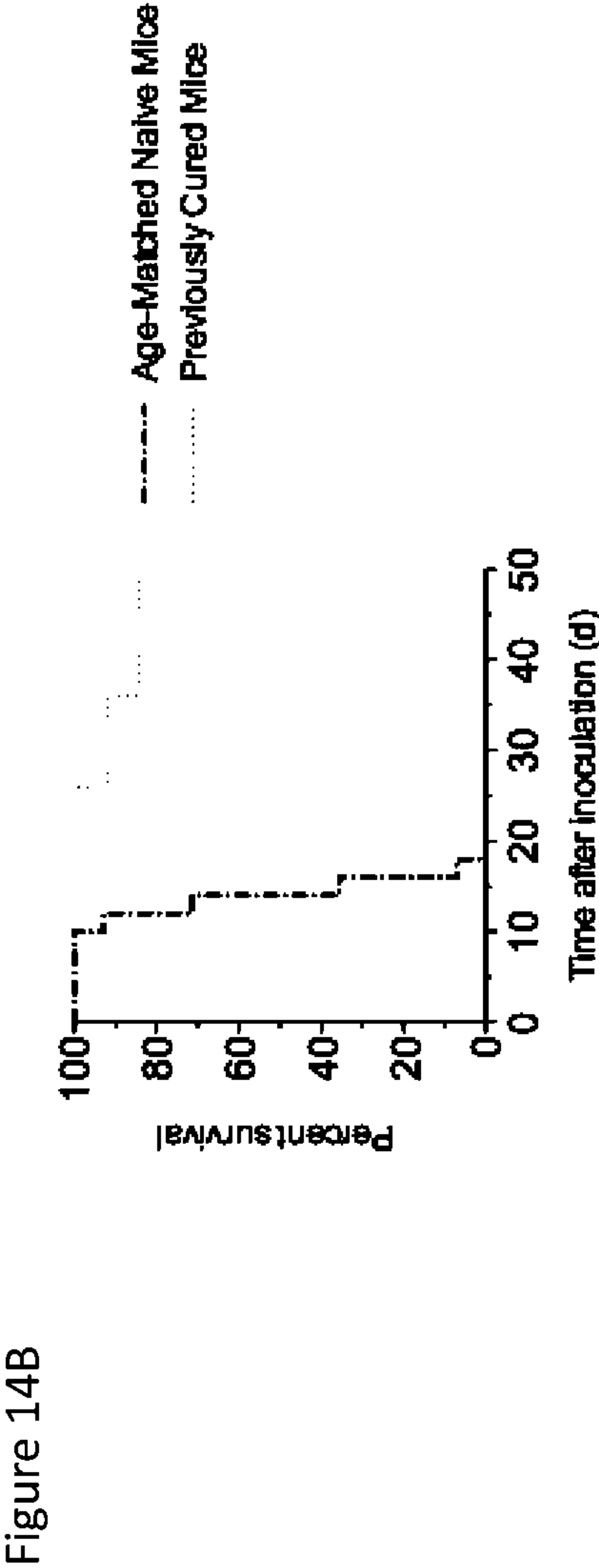
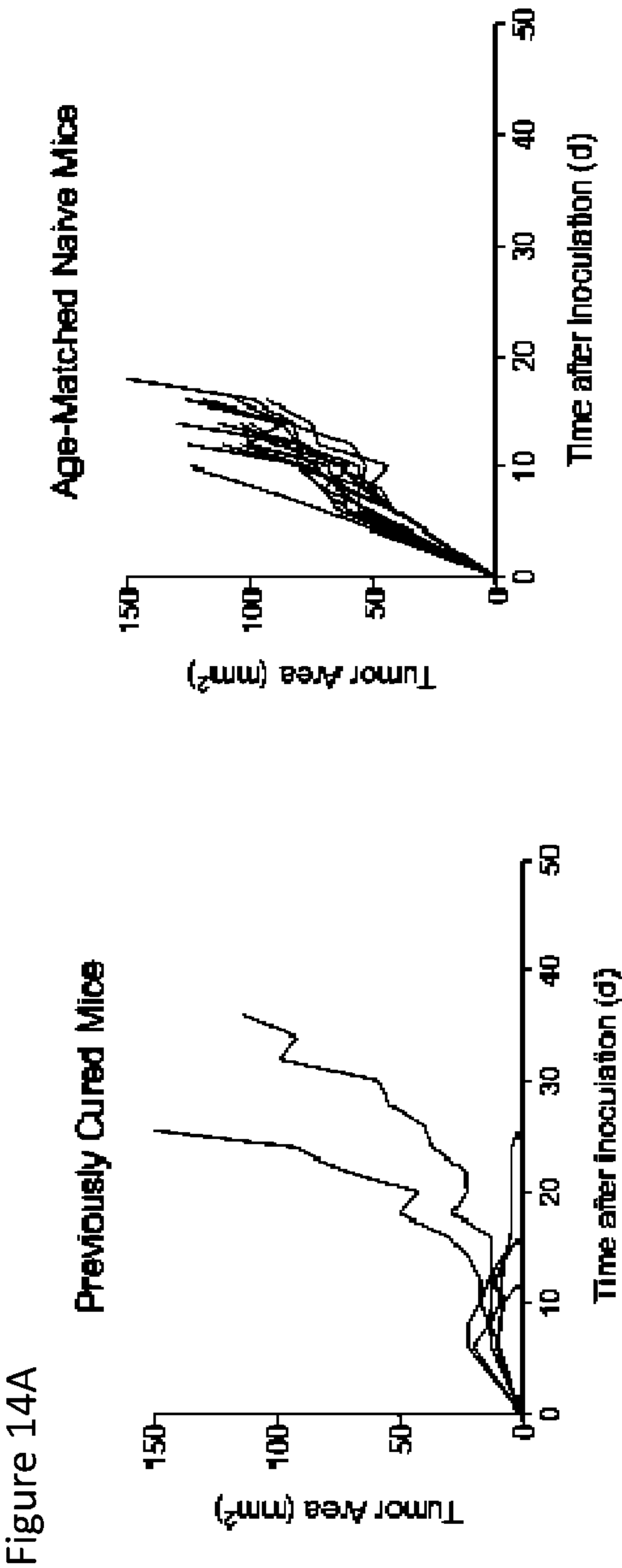


Figure 15A

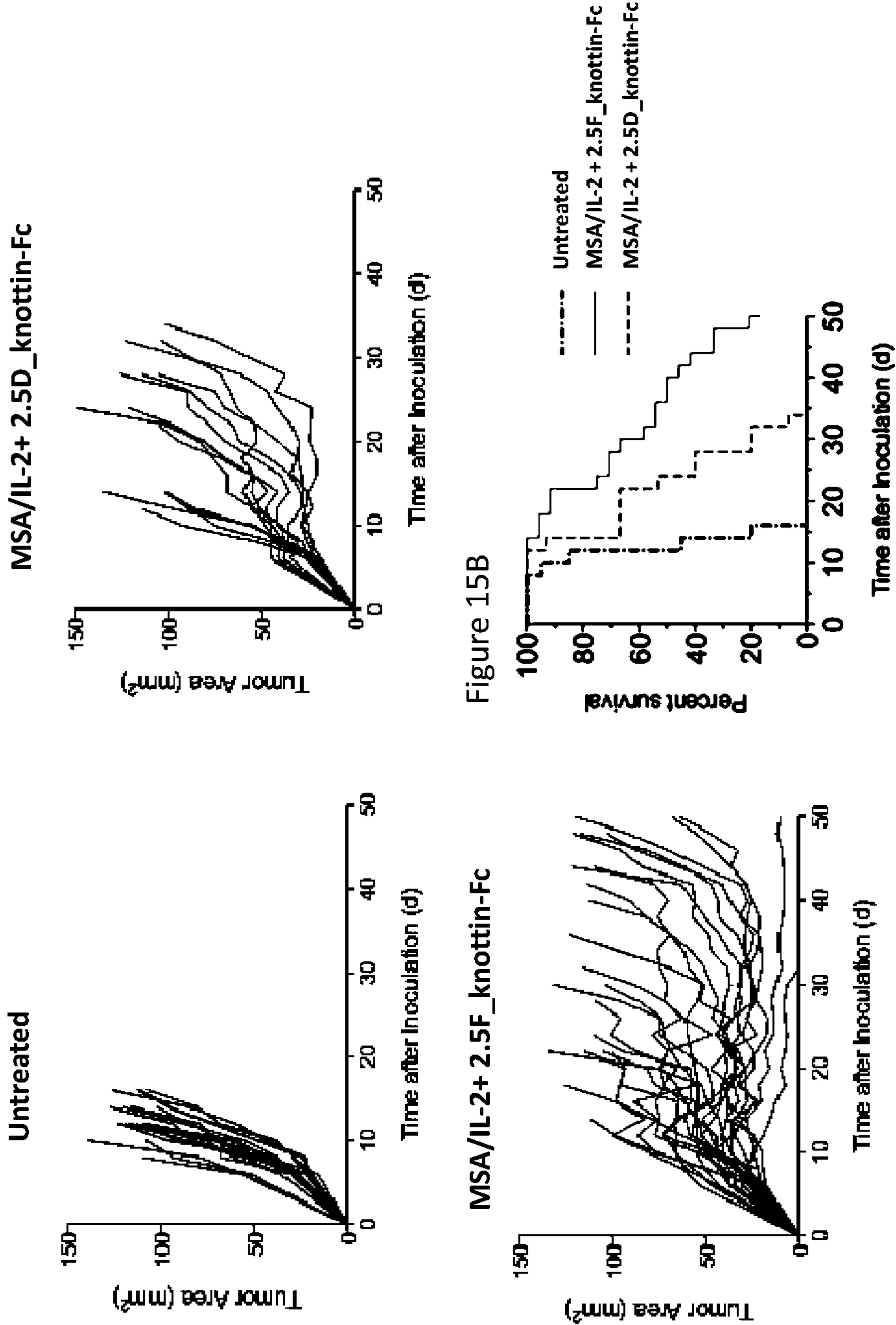


Figure 16A

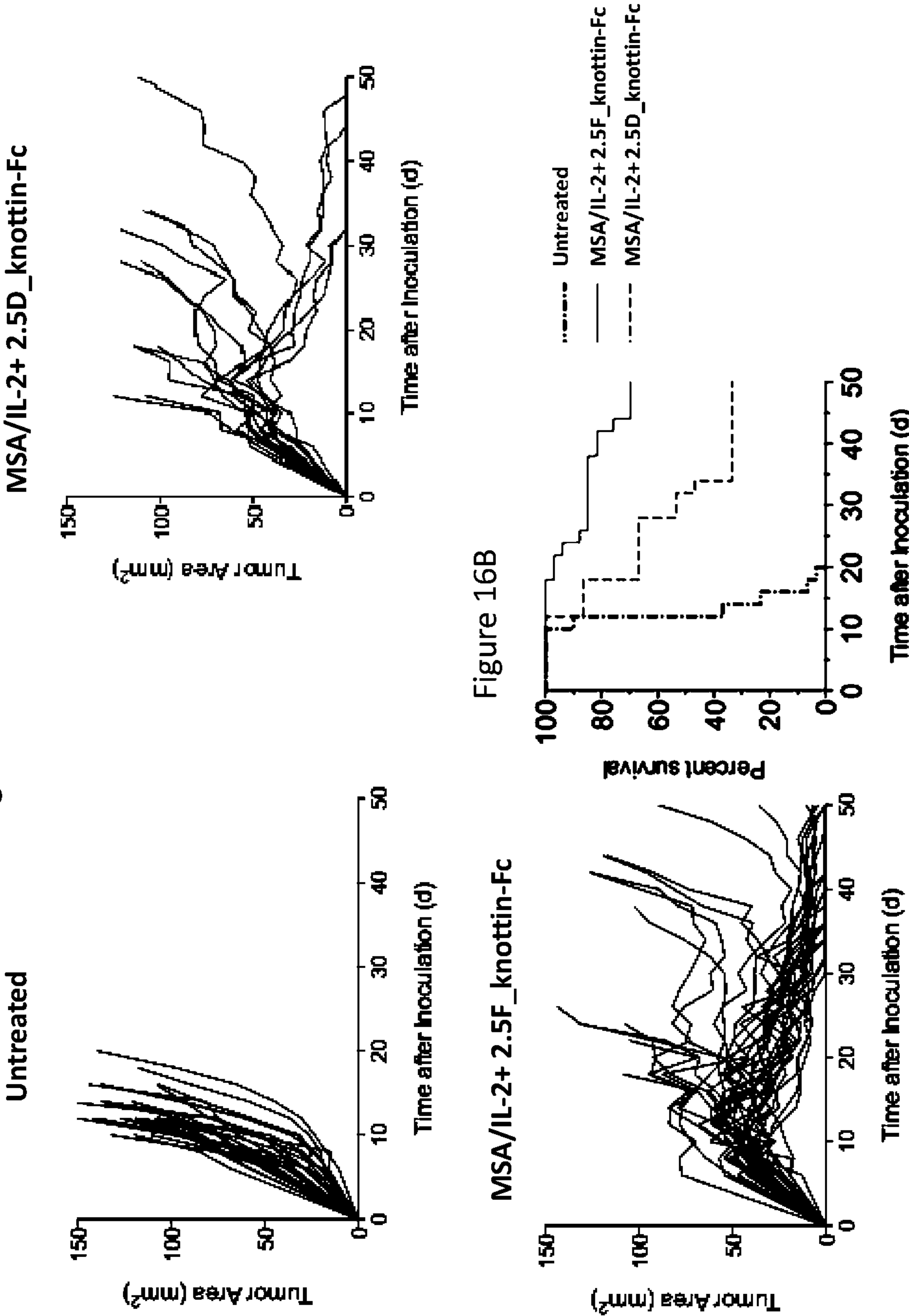


Figure 17A

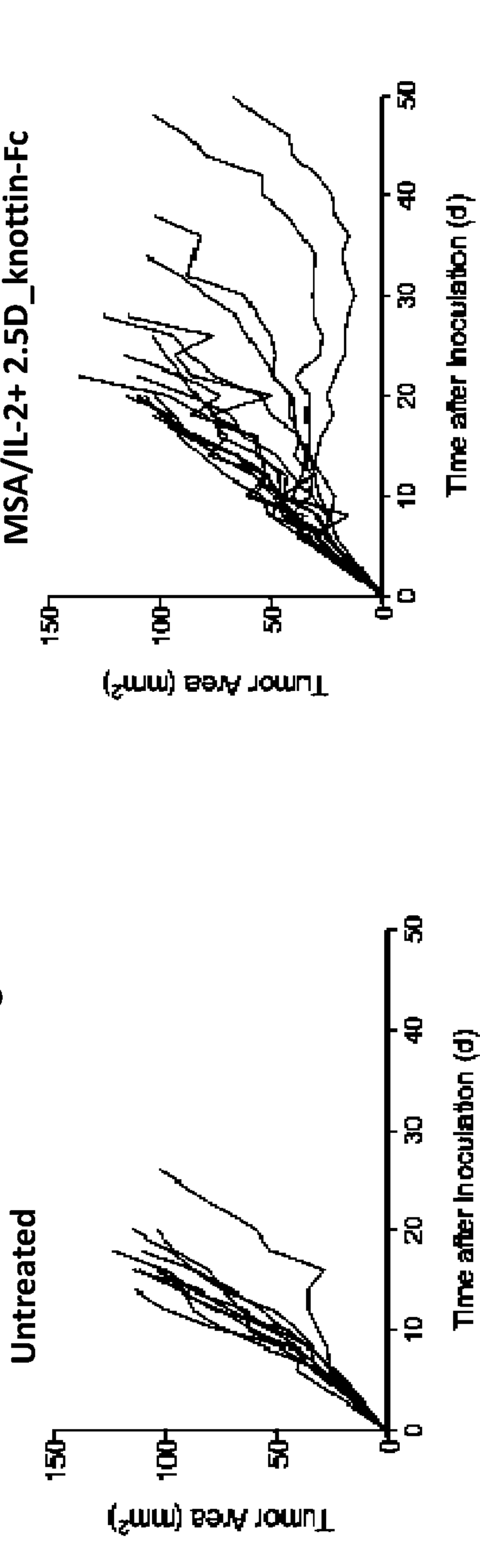
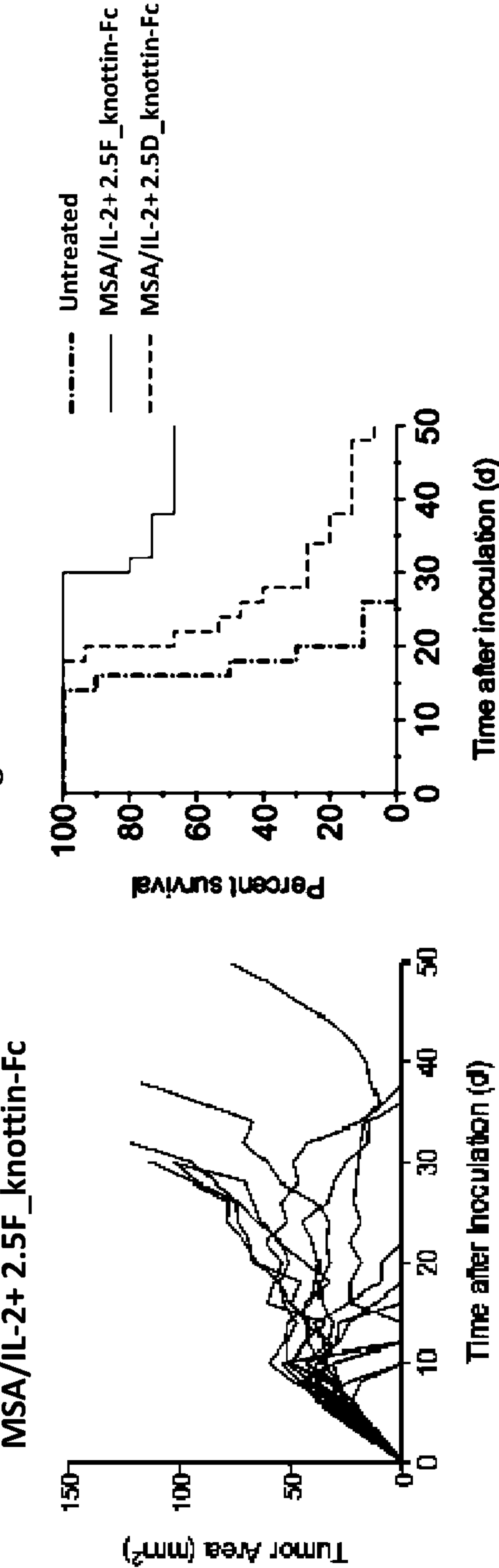
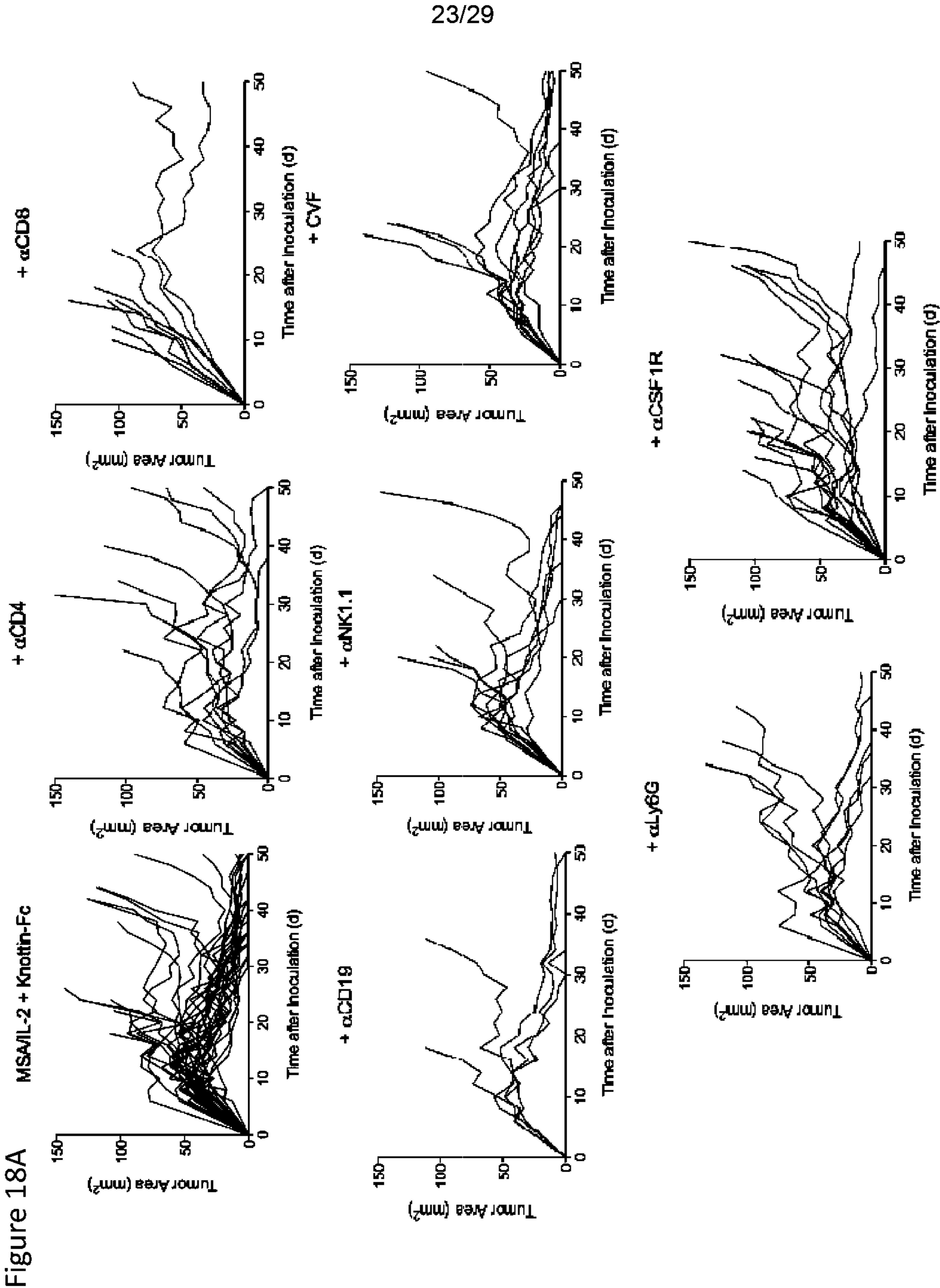
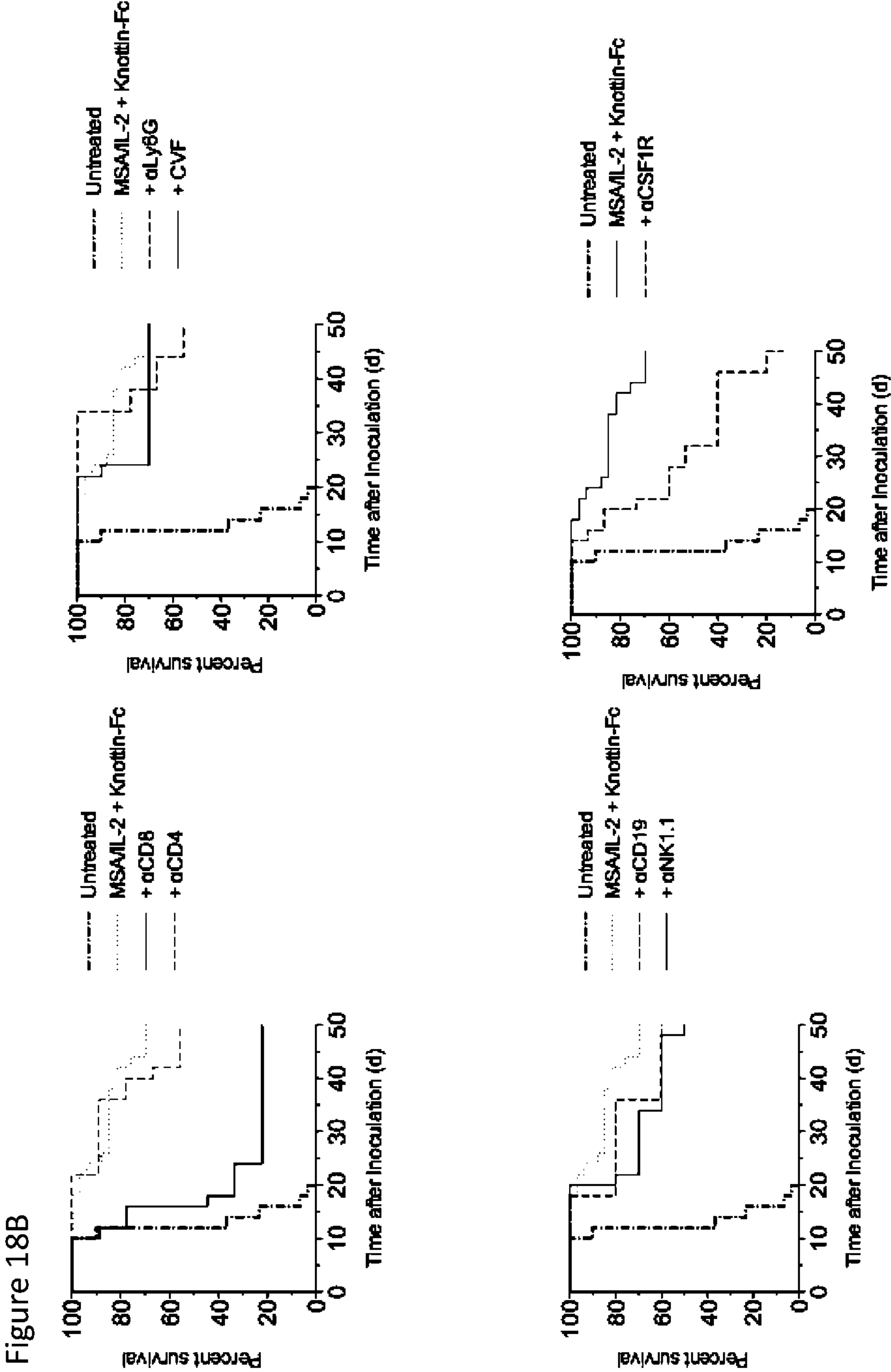


Figure 17B







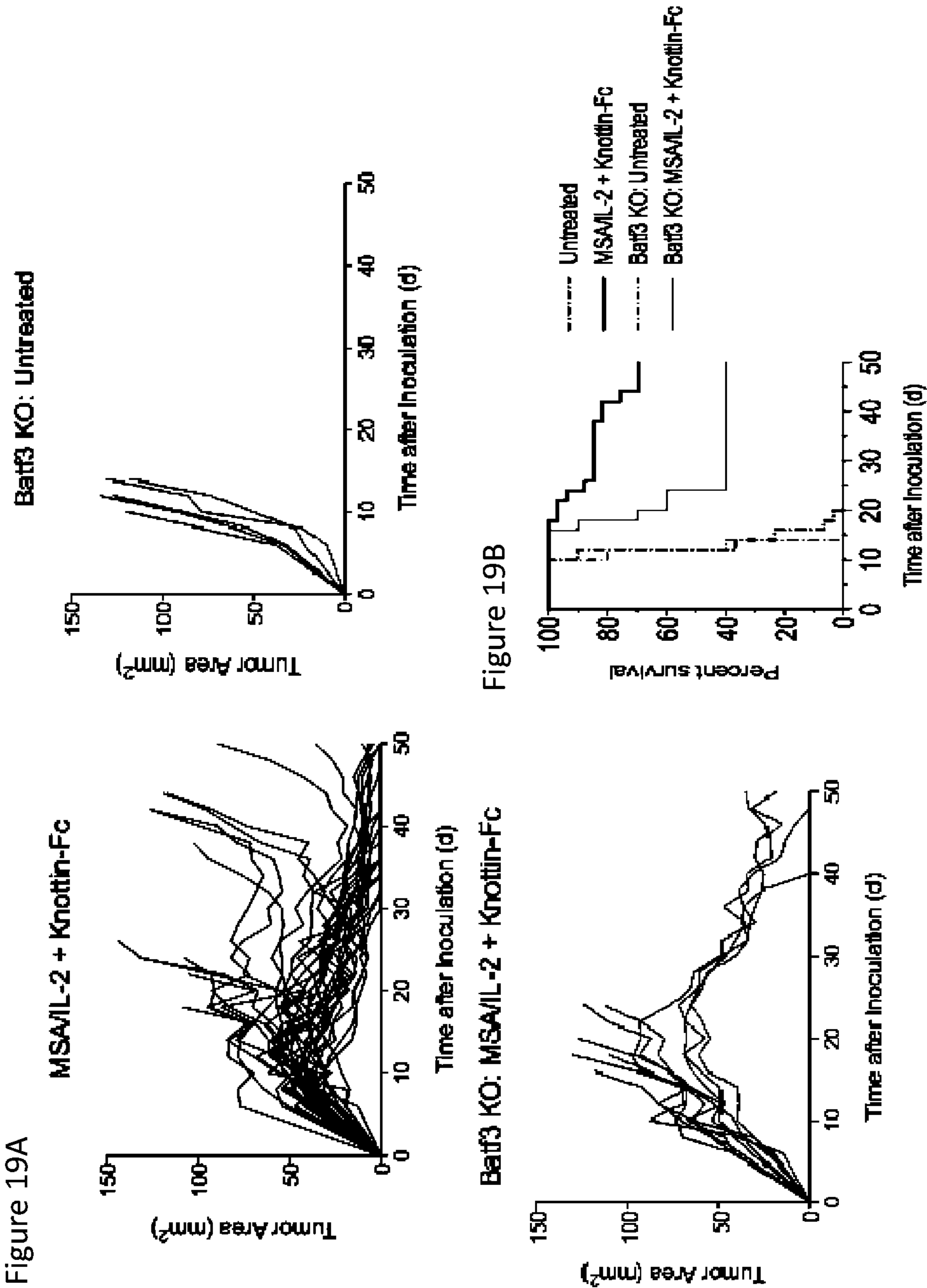
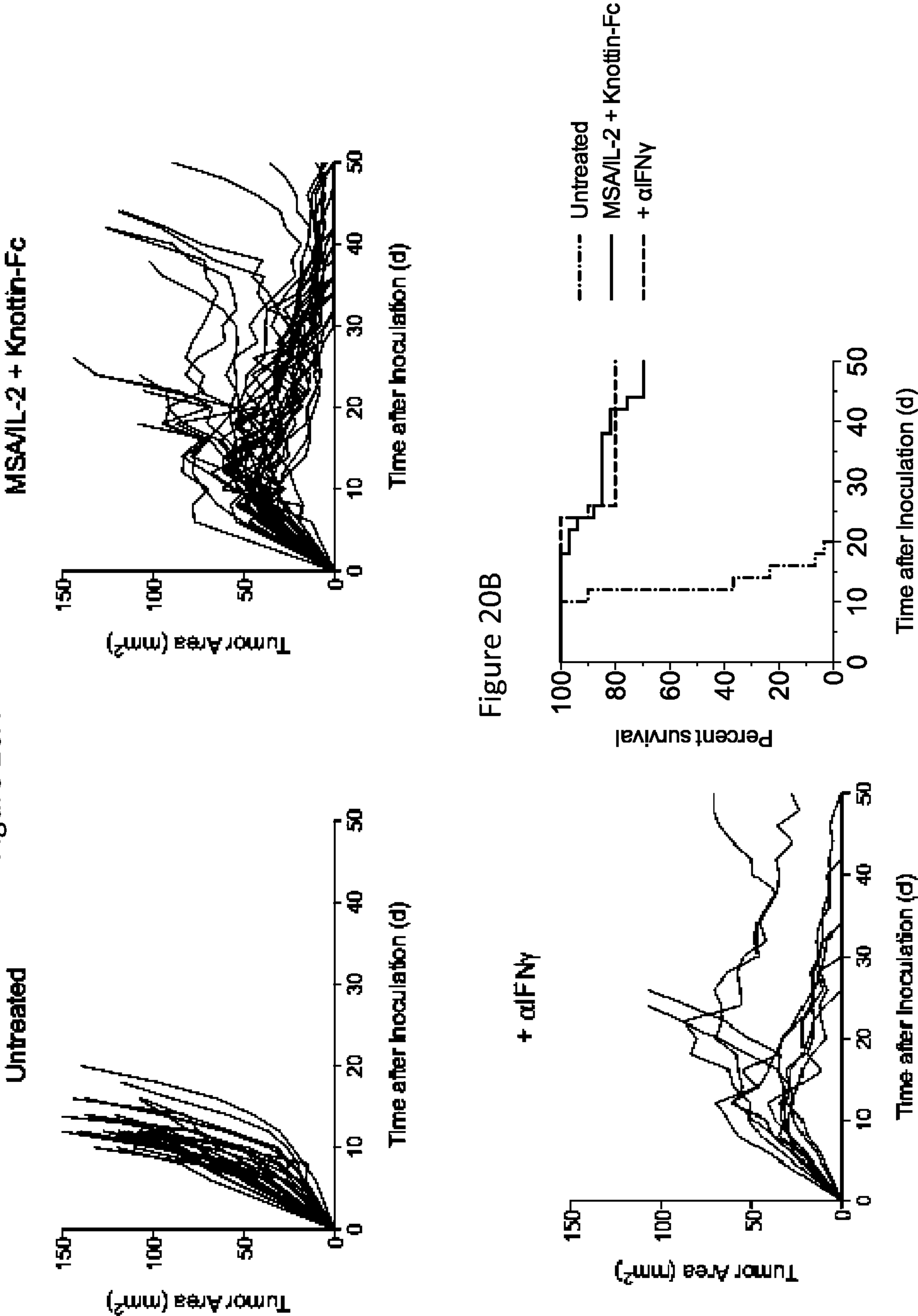


Figure 20A



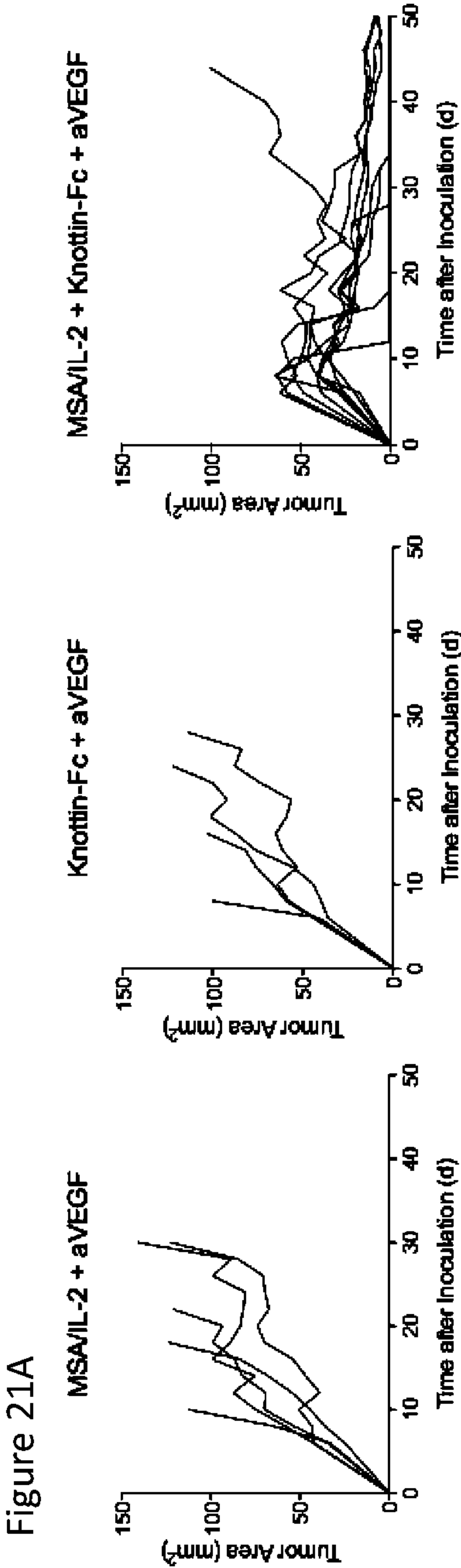


Figure 21B

