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(54) Title: IL-6Rα/IL-8R BISPECIFIC BINDING AGENTS FOR INHIBITING CANCER CELL MIGRATION

(57) Abstract: The present disclosure relates to bispecific binding agents with a novel format that bind IL-6Rα and IL-8R. The present disclosure also relates to methods of using such bispecific binding agents in the treatment of cancer.

WO 2020/243489 A1

IL-6Rα/IL-8R BISPECIFIC BINDING AGENTS FOR INHIBITING CANCER CELL MIGRATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application 62/855,625 filed on May 31, 2019. The contents of that application is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present disclosure relates to bispecific binding agents with a novel format that bind IL-6Rα and IL-8R. The present disclosure also relates to methods of using such bispecific binding agents in the treatment of cancer.

BACKGROUND

Metastasis is the spread of cancer from a primary site to a distal site through the circulatory or lymphatic systems. Conventional development of anti-cancer therapeutics assumes that drugs that target tumor growth will also target metastasis, or that interrupting metastasis is not necessary in the face of overwhelming growth inhibition.

As a result, metastasis has largely not been targeted specifically and separately from tumor growth in cancer drug development.

Monoclonal antibodies can be used in immunotherapy and disease treatment and are a cornerstone of the pharmaceutical market. Monoclonal antibodies can possess high affinity, pinpoint specificity, stability, extended in vivo-half life, and multi-tiered mechanisms of action. However, monoclonal antibodies are not without limitations, such as, acquired resistance or side effects. Immunotherapy and disease treatment with more than one monoclonal antibody also requires dosing ratio optimization.

Thus, there is a need for improved antibody methods for immunotherapy and disease treatment.

SUMMARY

Provided herein is a novel bispecific binding agent that combines a knobs-in- holes dimerization strategy and a single-chain Fab expression approach to generate a bispecific binding agent with specificity for: IL-6Rα and IL-8R. Bispecific binding agents provided herein can be used in the treatment of cancer (alone or in combination with other therapeutics), particularly in the inhibition of metastasis. Bispecific binding agents provided herein can also be used in any of a variety of in vitro systems and assays.

Bispecific binding agents simultaneously engage two different targets with increased affinity, avidity, potency, and selectivity over monoclonal antibodies. Provided herein are novel bispecific binding agents that bind IL-6Rα and IL-8R, which bispecific binding agents combine a knobs-in-holes approach with a single-chain Fab having a flexible linker, resulting in a novel bispecific agent format (FIG. 1D). In some embodiments, bispecific binding agents provided herein include a knobs-in-holes format in which amino acid substitutions are introduced into the third heavy chain constant domains of the antibody heavy chains. Such a knobs-in-holes approach can enforce proper heterodimerization over homodimerization of the antibody heavy chains. In some embodiments, bispecific binding agents provided herein include a single-chain Fab format that results the C-terminus of the light chain constant domain (CL) to the N- terminus of the variable heavy (VH) chain with a flexible linker. The single-chain Fab construction enforces proper variable heavy and variable light chain pairing. Combination of a knobs-in-holes approach with a single-chain Fab format results in a novel bispecific format that exhibits improved characteristics over conventional antibody-derivative formats.

In some embodiments, provided herein are bispecific binding agents that include: first polypeptide comprising a first antibody heavy chain or portion thereof, a linker, and a first antibody light chain or portion thereof, wherein the first linker connects the first antibody heavy chain or portion thereof and the first antibody light chain or portion thereof, and wherein the first antibody heavy chain or portion thereof and the first antibody light chain or portion thereof form a first binding site specific for IL-6Rα; a second polypeptide comprising a second polypeptide antibody heavy chain or portion thereof, a second linker, and a second polypeptide antibody light chain or portion thereof, wherein the second linker connects the second antibody heavy chain or portion thereof and the second antibody light chain or portion thereof, and wherein the second antibody heavy chain or portion thereof and the second antibody light chain or portion thereof

form a second binding site specific for IL-8R, wherein the first antibody heavy chain or portion thereof comprises one or more amino acid substitutions, the second antibody heavy chain or portion thereof comprises one or more amino acid substitutions, or both, such that the first polypeptide antibody heavy chain or portion thereof and the second polypeptide antibody heavy chain or portion thereof preferentially associate with each other to form the bispecific binding agent.

In some embodiments of bispecific binding agents provided herein, the first antibody heavy chain or portion thereof comprises a CH1 domain or portion thereof, a CH2 domain or portion thereof, a CH3 domain or portion thereof, and a VH domain or portion thereof. In some embodiments, the second antibody heavy chain or portion thereof, comprises a CH1 domain or portion thereof, a CH2 domain or portion thereof, a CH3 domain or portion thereof, and a VH domain or portion thereof. In some embodiments, the first polypeptide and the second polypeptide preferentially associate with each other as compared to a corresponding first polypeptide comprising an antibody heavy chain that lacks the one or more amino acid substitutions, a corresponding second polypeptide comprising a second antibody heavy chain that lacks the one or more amino acid substitutions, or both. In some embodiments, the first antibody light chain comprises a CL domain or portion thereof and a VL domain or portion thereof. In some embodiments, the second antibody light chain comprises a CL domain or portion thereof and a VL domain or portion thereof. In some embodiments, the first polypeptide linker connects a CL domain of the first antibody light chain to a VH domain of the first antibody heavy chain. In some embodiments, the second polypeptide linker connects a CL domain of the second antibody light chain to a VH domain of the second antibody heavy chain. In some embodiments, the first polypeptide linker comprises a polypeptide having at least 80% sequence identity to SEQ ID NO. 13. In some embodiments, the second polypeptide linker comprises a polypeptide having at least 80% sequence identity to SEQ ID NO. 13. In some embodiments, the one or more amino acid substitutions in the first antibody heavy chain or portion thereof comprises an amino acid substitution at a one or more of positions 645, 647, and 686 of SEQ ID NO. 9. In some embodiments, the one or more amino acid substitutions in the second antibody heavy chain or portion thereof comprises an amino acid substitution at a one or more of positions 642 of SEQ ID NO. 11.

In some embodiments of bispecific binding agents provided herein, the first antibody heavy chain or portion thereof comprises a VH domain comprising: a heavy chain CDR1 domain comprising SEQ ID NO. 16, a heavy chain CDR2 domain comprising SEQ ID NO. 17, and a heavy chain CDR3 domain comprising SEQ ID NO. 18; and the first antibody light chain or portion thereof comprises a VL domain comprising: a light chain CDR1 domain comprising SEQ ID NO. 19, a light chain CDR2 domain comprising SEQ ID NO. 20, and a light chain CDR3 domain comprising SEQ ID NO. 21.

In some embodiments of bispecific binding agents provided herein, the second antibody heavy chain or portion thereof comprises a VH domain comprising: a heavy chain CDR1 domain comprising SEQ ID NO. 22, a heavy chain CDR2 domain comprising SEQ ID NO. 23, and a heavy chain CDR3 domain comprising SEQ ID NO. 24; and the second antibody light chain or portion thereof comprises a VL domain comprising: a light chain CDR1 domain comprising SEQ ID NO. 25, a light chain CDR2 domain comprising SEQ ID NO. 26, and a light chain CDR3 domain comprising SEQ ID NO. 27.

In some embodiments of bispecific binding agents provided herein, the first antibody heavy chain or portion thereof comprises a VH domain comprising: a heavy chain CDR1 domain comprising SEQ ID NO. 16, a heavy chain CDR2 domain comprising SEQ ID NO. 17, and a heavy chain CDR3 domain comprising SEQ ID NO. 18; the first antibody light chain or portion thereof comprises a VL domain comprising: a light chain CDR1 domain comprising SEQ ID NO. 19, a light chain CDR2 domain comprising SEQ ID NO. 20, and a light chain CDR3 domain comprising SEQ ID NO. 21; the second antibody heavy chain or portion thereof comprises a VH domain comprising: a heavy chain CDR1 domain comprising SEQ ID NO. 22, a heavy chain CDR2 domain comprising SEQ ID NO. 23, and a heavy chain CDR3 domain comprising SEQ ID NO. 24; and the second antibody light chain or portion thereof comprises a VL domain comprising: a light chain CDR1 domain comprising SEQ ID NO. 25, a light chain CDR2 domain comprising SEQ ID NO. 26, and a light chain CDR3 domain comprising SEQ ID NO. 27.

In some embodiments of bispecific binding agents provided herein, the first binding site comprises: the VH domain comprising residues 278-396 of SEQ ID NO. 9, and the VL domain comprising residues 24-130 of SEQ ID NO. 9. In some embodiments of bispecific binding agents

provided herein, the second binding site comprises: the VH domain comprising residues 280-393 of SEQ ID NO. 11, and the VL domain comprising residues 24-132 of SEQ ID NO. 11.

In some embodiments, provided herein are pharmaceutical compositions that include any of the bispecific binding agents provided herein.

In some embodiments, provided herein are methods of treating a disease in a subject in need thereof that include administering a therapeutically effective amount any of the bispecific binding agents provided herein or any of the pharmaceutical compositions that include any of the bispecific binding agents provided herein. In some embodiments, the disease is cancer. In some embodiments, the method inhibits metastatic cell migration of the cancer. In some embodiments, the cancer is a breast cancer. In some embodiments, the cancer is a triple negative breast cancer. In some embodiments, the cancer is a pancreatic cancer. In some embodiments, the cancer is a pancreatic ductal adenocarcinoma.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used.

Other features and advantages of the invention will be apparent from the following Detailed Description, and from the claims. It should be understood, however, that the Detailed Description and the specific examples, while indicating certain embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. All publications mentioned herein, including patents, patent application publications, and scientific papers, are incorporated by reference in their entirety.

DESCRIPTION OF DRAWINGS

Figure 1. Exemplary bispecific binding agent format combining a knobs-in-holes assembly strategy and a single-chain Fab expression approach. A) Schematic of a tandem scFv. B) Schematic of scFv-IgG fusion (BS2 format). C) Schematic of scFv-Fc fusion showing the knobs-in-holes approach. D) Schematic of bispecific binding agent provided herein.

Figure 2. Exemplary bispecific binding agent format combining a knobs-in-holes assembly strategy and a single-chain Fab expression approach with tocilizumab (anti-IL-6R) and 10H2 (anti-IL-8R) antibody variable heavy and light chains, "BS1."

Figure 3. Exemplary bispecific antibody format with tocilizumab (anti-IL-6R) scFvs linked to light chains of 10H2 (anti-IL-8R) IgG antibody, denoted "BS2."

Figure 4. Expression of recombinant bispecific antibodies, BS1 and BS2. Size exclusion chromatography traces from FPLC purification of bispecific binding agents secreted in a mammalian cell expression system. Non-reducing and reducing SDS-PAGE analyses demonstrated that the proteins were purified to homogeneity and migrate at the expected molecular weights.

Figure 5. Bispecific antibodies BS1 and BS2, which contain the variable heavy and light chains of the anti-IL-6Rα antibody tocilizumab, bind to the IL-6Rα extracellular domain (ECD). Equilibrium bio-layer interferometry (BLI) titrations are shown of immobilized human IL-6Rα ECD with tocilizumab (anti-IL-6R), 10H2 (anti-IL-8R), and bispecific antibodies BS1 and BS2.

Figure 6. Bispecific antibodies BS1 and BS2 competitively inhibit IL-6/IL-6Rα binding. Titration of recombinant IL-6Rα ECD on IL-6-expressing yeast, as measured by flow cytometry (A). Competitive inhibition of the IL-6/IL-6Rα interaction by tocilizumab (anti-IL-6R), 10H2 (anti-IL-8R), and bispecific antibodies BS1 and BS2 (B). The anti-IL-8RB antibody 10H2 does not compete with the cytokine/receptor binding, whereas the anti-IL-6Rα antibody tocilizumab and the engineered tocilizumab-containing Bispecific antibodies block binding in accordance with their affinities. Data represent mean± s.d.

Figure 7. Bispecific antibodies BS1 and BS2 specifically bind IL-6Rα- and IL-8R-expressing human embryonic kidney (HEK 293T) cell lines. Binding titrations of tocilizumab (anti-IL-6R), 10H2 (anti-IL-8R), and bispecific antibodies BS1 and BS2 on (A) IL-6Rα^{+}/IL-8R^{-}, (B) IL-6Rα^{-}/IL-8R^{+}, (C) IL-6Rα^{+}/IL-8R^{+}, and (D) IL-6Rα^{-}/IL-8R^{-} HEK 293T cells. Both bispecific antibodies bind functional IL-6Rα and IL-8R on cells, whereas their constituent monoclonal antibodies bind only to either IL-6Rα or IL-8R. Antibody binding to cells was detected via flow cytometry. Data represent mean± s.d.

Figure 8. Bispecific antibodies BS1 and BS2 competitively inhibit both IL-6/IL-6Rα and IL-8/IL-8R interactions. (A) Cell surface competition assays between soluble IL-6 cytokine and tocilizumab (anti-IL-6R), 10H2 (anti-IL-8R), and bispecific antibodies BS1 and BS2 on IL-

6Rα$^+$/IL-8R$^-$ HEK 293T cells. Tocilizumab, BS1, and BS2 compete with IL-6 engagement of IL-6Rα. (B) Cell surface competition assays between soluble IL-8 cytokine and either tocilizumab, 10H2, BS1, or BS2 on IL-6Rα$^-$/IL-8R$^+$ HEK 293T cells. 10H2, BS1, and BS2 compete with IL-8 engagement of IL-8R. Binding of IL-6 was measured via flow cytometry. Data represent mean ± s.d.

Figure 9. Bispecific antibodies competitively inhibit IL-6 signaling. IL-6-mediated phosphorylation of STAT3 on HepG2 lung cancer cells in the presence of tocilizumab (anti-IL-6R), 10H2 (anti-IL-8R), and bispecific antibodies BS1 and BS2. Tocilizumab, BS1, and BS2 inhibit IL-6-induced signaling, whereas 10H2 does not. Signaling was measured via flow cytometry. Data represent mean ± s.d.

Figure 10. Bispecific antibodies dramatically reduce migration of cancer cells. Randomly selected trajectories of triple negative breast cancer cells (MDA-MB-231) (A) and fibrosarcoma cells (HT-1080) (B) show that bispecific antibodies (BS1 and BS2) are extremely effective at reducing the migration of cancer cells, outperforming the combination of the monoclonal antibodies tocilizumab and 10H2 (anti-IL-6R+anti-IL-8R) and matching or exceeding the effect of tocilizumab plus reparixin (T+R). Each color represents a single cell trajectory, with sixteen trajectories overlaid from a common origin for each condition (scale bar = 10 μm).

Figure 11. Bispecific antibodies robustly inhibit migration of triple negative breast cancer and fibrosarcoma cells. Motility (as measured by mean squared displacement [MSD]) (A)+(D), total diffusivity (B)+(E), and persistence (C)+(F) for untreated MDA-MB-231 triple negative breast cancer cells and HT-1080 fibrosarcoma cells versus cells treated with tocilizumab plus reparixin (T+R), tocilizumab+10H2 (anti-IL-6R+anti-IL-8R antibodies), BS1, or BS2. Total diffusivity and persistence were calculated using the high throughput 3D cell migration model previously developed (Fraley, S. I.; Feng, Y.; Krishnamurthy, R.; Kim, D.-H.; Celedon, A.; Longmore, G. D.; Wirtz, D. A Distinctive Role for Focal Adhesion Proteins in Three-Dimensional Cell Motility. *Nat. Cell Biol.* 2010, *12* (6), 598–604. https://doi.org/10.1038/ncb2062; Giri A, Bajpai S, Trenton N, Jayatilaka H, Longmore GD, Wirtz D. The Arp2/3 complex mediates multigeneration dendritic protrusions for efficient 3-dimensional cancer cell migration. FASEB J Off Publ Fed Am Soc Exp Biol. 2013 Oct;27(10):4089– 4099. PMCID: PMC4046187). Bispecific antibodies (BS1 and BS2) elicit superior inhibition to combined treatment with anti-IL-6R+anti-IL-8R monoclonal antibodies

and the T+R antibody/small molecule combination. A minimum of three independent experiments were run for each treatment condition for each cell line. In all panels, data are represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001 (unpaired student's *t*-test).

Figure 12. Bispecific antibodies target cell migration without affecting cell growth. Relative cell proliferation of MDA-MB-231 triple negative breast cancer cells (A) and HT-1080 fibrosarcoma cells (B) embedded in the 3D model was determined based on metabolic activity of the cells 48 hours after treatment was administered. Untreated cells were compared to cells treated with tocilizumab plus reparixin (T+R), tocilizumab+10H2 (anti-IL-6R+anti-IL-8R antibodies), BS1, or BS2. None of the treatment conditions had a significant effect on growth, demonstrating that bispecific antibodies effectively inhibit migration while not impacting proliferation of the cells. Error bars represent s.d. and statistical comparisons were performed via unpaired student's *t*-test.

Figure 13. Bispecific antibodies induce potent inhibition of metastasis in orthotopic breast cancer xenograft studies in mice. To maximize the information gained from in vivo studies, three pilot experiments were completed to determine the optimum timeline and dose of the bispecific antibodies. (A) The first study was carried out with five mice, four of which were injected with $1x10^6$ MDA-MB-231 triple negative breast cancer cells into the mammary fat pad at day 0. Starting 3 weeks post-injection, the lungs from one mouse were extracted each week and tested for human genomic content. The cycle threshold shows that there is very little noise in the measurement, as the noise level of HK2 in the healthy control is very close to the maximum cycle number, giving essentially a 0 reading for human genomic content. The trend of the cycle threshold of HK2 decreasing over time was expected, and gave us confidence that ended the study at 35 days would yield measurable metastatic burden. (B) In the second study, five different doses of BS1 were given to each mouse, all mice were treated for the same duration and then the lungs were tested for metastatic burden. (C) The third study (which also exposed all animals to the same duration of treatment) confirmed that a dose of 1 mg/kg or lower of BS1 would effectively reduce lung metastases in this model.

Figure 14. Bispecific antibody treatment does not affect orthotopic breast cancer tumor growth. Mice bearing orthotopic MDA-MB-231 xenograft tumors were left untreated or treated with tocilizumab plus reparixin (T+R), tocilizumab+10H2 (anti-IL-6R+anti-IL-8R antibodies), BS1, or BS2 every 3 days for 3 weeks beginning on day 10 post-inoculation, and tumor volume

was tracked over time. Bispecific antibodies had no effect on the growth of the tumor, as expected from in vitro proliferation assays and past work with the combination of tocilizumab plus reparaxin (T+R).

Figure 15. Bispecific antibody treatment does not affect orthotopic breast cancer tumor weight. Mice bearing orthotopic MDA-MB-231 xenograft tumors were left untreated or treated with tocilizumab plus reparixin (T+R), tocilizumab+10H2 (anti-IL-6R+anti-IL-8R antibodies), BS1, or BS2 every 3 days for 3 weeks beginning on day 10 post-inoculation, and on day 35 the study was concluded and the tumors were extracted. Tumor weights confirm that there was no difference between any of the treatment groups in terms of tumor growth. Data is represented as mean ± s.e.m.

Figure 16. Bispecific antibodies significantly reduce metastatic burden in an orthotopic breast cancer tumor model. Mice bearing orthotopic MDA-MB-231 xenograft tumors were left untreated or treated with tocilizumab plus reparixin (T+R), tocilizumab+10H2 (anti-IL-6R+anti-IL-8R antibodies), BS1, or BS2. Human genomic content in mouse lungs was quantified using qPCR to determine the metastatic burden. Bispecific antibody (BS1 and BS2) treatment significantly outperformed the anti-IL-6R+anti-IL-8R monoclonal antibodies, as well as the anti-IL-6R+anti-IL-8R and T+R conditions. Data is represented as mean ± s.e.m. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (unpaired student's *t*-test).

Figure 17. Bispecific antibodies greatly diminish lung metastases in an orthotopic breast cancer tumor model, as visualized by tissue analysis. Histological analysis of the lung tissue from mice bearing orthotopic MDA-MB-231 xenograft tumors that were left untreated or treated with tocilizumab plus reparixin (T+R), tocilizumab+10H2 (anti-IL-6R+anti-IL-8R antibodies), BS1, or BS2. Lung tissue was fixed in formalin and stained with H&E. Visual evaluation of the H&E slides show that there are identifiable micro-metastases in the lung tissue of all conditions, and there are significantly more micrometastases in the control, anti-IL-6R+anti-IL-8R monoclonal antibody and T+R conditions compared with the bispecific antibody conditions.

DETAILED DESCRIPTION

Metastasis is the spread of cancer from a primary site to a distal site through the circulatory or lymphatic systems and is responsible for 90% of cancer related deaths (Weinberg RA. The biology of cancer. Second edition. New York: Garland Science, Taylor & Francis

Group; 2014). Conventional development of anti-cancer therapeutics assumes that drugs that target tumor growth will also target metastasis, or that interrupting metastasis is not necessary in the face of overwhelming growth inhibition.

As a result, the process of metastasis has typically not been targeted specifically and separately from tumor growth in cancer drug development, and many therapies currently used in the clinic can actually induce metastasis (Steeg PS. Targeting metastasis. Nat Rev Cancer. 2016 Apr;16(4):201–218. PMID: 27009393; Karagiannis GS, Pastoriza JM, Wang Y, Harney AS, Entenberg D, Pignatelli J, Sharma VP, Xue EA, Cheng E, D'Alfonso TM, Jones JG, Anampa J, Rohan TE, Sparano JA, Condeelis JS, Oktay MH. Neoadjuvant chemotherapy induces breast cancer metastasis through a TMEM-mediated mechanism. Sci Transl Med. 2017 05;9(397). PMCID: PMC5592784; Obenauf AC, Zou Y, Ji AL, Vanharanta S, Shu W, Shi H, Kong X, Bosenberg MC, Wiesner T, Rosen N, Lo RS, Massagué J. Therapy-induced tumour secretomes promote resistance and tumour progression. Nature. 2015 Apr 16;520(7547):368–372. PMCID: PMC4507807; Martin OA, Anderson RL, Narayan K, MacManus MP. Does the mobilization of circulating tumour cells during cancer therapy cause metastasis? Nat Rev Clin Oncol. 2017 Jan;14(1):32–44. PMID: 27550857).

Targeting cancer metastasis depends on the elucidation of metastatic mechanisms. A synergistic paracrine signaling pathway between the interleukin-6 cytokine (IL-6) and the interleukin-8 chemokine (IL-8) is unique to tumorigenic, metastatic cells (Jayatilaka H, Tyle P, Chen JJ, Kwak M, Ju J, Kim HJ, Lee JSH, Wu P-H, Gilkes DM, Fan R, Wirtz D. Synergistic IL-6 and IL-8 paracrine signalling pathway infers a strategy to inhibit tumour cell migration. Nat Commun. 2017 26;8:15584. PMCID: PMC5458548). The mechanism underlying this pathway couples tumor cell proliferation and migration, two key drivers of metastasis, via local tumor cell density (number of cells/unit volume). As tumor cells proliferate and local cell density increases, both IL-6 and IL-8 expression is enhanced, causing an increase in tumor cell migration (i.e., cell density-dependent migration).

Pharmacological inhibition of this synergistic pathway through targeted blockade of the IL-6 and IL-8 receptors (IL-6Rα and IL-8R), using a combination of the anti-IL-6 receptor antibody drug tocilizumab (currently used to treat rheumatoid arthritis) (Nakashima Y, Kondo M, Fukuda T, Harada H, Horiuchi T, Ishinishi T, Jojima H, Kuroda K, Miyahara H, Maekawa M, Nishizaka H, Nagamine R, Nakashima H, Otsuka T, Shono E, Suematsu E, Shimauchi T,

Tsuru T, Wada K, Yoshizawa S, Yoshizawa S, Iwamoto Y. Remission in patients with active rheumatoid arthritis by tocilizumab treatment in routine clinical practice: results from 3 years of prospectively registered data. Mod Rheumatol. 2014 Mar;24(2):258–264. PMID: 24593201) and the anti-IL-8 receptor small molecule drug reparixin (currently in phase II clinical trials against breast cancer), (Goldstein LJ, Perez RP, Yardley DA, Han LK, Reuben JM, McCanna S, Butler B, Ruffini PA, Chang JC. Abstract CT057: A single-arm, preoperative, pilot study to evaluate the safety and biological effects of orally administered reparixin in early breast cancer patients who are candidates for surgery. Cancer Res. 2016 Jul 15;76(14 Supplement):CT057–CT057; Schott AF, Goldstein LJ, Cristofanilli M, Ruffini PA, McCanna S, Reuben JM, Perez RP, Kato G, Wicha M. Phase Ib Pilot Study to Evaluate Reparixin in Combination with Weekly Paclitaxel in Patients with HER-2-Negative Metastatic Breast Cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2017 Sep 15;23(18):5358–5365. PMCID: PMC5600824) significantly decreases cell motility in 3D models of migration (Jayatilaka H, Tyle P, Chen JJ, Kwak M, Ju J, Kim HJ, Lee JSH, Wu P-H, Gilkes DM, Fan R, Wirtz D. Synergistic IL-6 and IL-8 paracrine signalling pathway infers a strategy to inhibit tumour cell migration. Nat Commun. 2017 26;8:15584. PMCID: PMC5458548). Furthermore, pre-clinical testing in mouse models revealed that combination treatment with tocilizumab and reparixin markedly suppresses metastasis to the lungs, liver, and lymph nodes. However, translation of a combination therapy regimen is complicated by the need for dosing ratio optimization and increased regulatory hurdles. Moreover, small molecule drugs such as reparixin face significant clinical challenges in terms of specificity.

Provided herein is a modular and compact bispecific binding agent that allows for the engagement of two different target proteins: IL-6Rα (e.g., human IL-6Rα extracellular domain (ECD)) and IL-8R (e.g., human IL-8RB ECD). The bispecific binding agent can engage IL-6Rα through a first binding site. The first binding site can include a first antigen-binding domain. The bispecific binding agent can engage IL-8R through a second binding site. The second binding site can include a second antigen- binding domain. In some embodiments, a bispecific binding agent provided herein can closely approximate the binding properties of a conventional monoclonal antibody.

In some embodiments, the bispecific binding agent combines a knobs-in-holes assembly strategy that facilitates heterodimerization over homodimerization. In some embodiments, the

knobs-in-holes assembly strategy includes complementary substitutions into the heavy chain constant domain of one or both of a first polypeptide or portion thereof of the bispecific binding agent that binds IL-6Rα (e.g., human IL-6Rα ECD) and a second polypeptide or portion thereof of the bispecific binding agent that binds IL-8R (e.g., human IL-8R ECD, e.g., IL-8RA or IL-8RB). In some embodiments, such amino acid substitutions result in one or more cavities or "holes" in the first polypeptide and one or more "knobs" or protuberances in the second polypeptide, such that the first polypeptide or portion thereof and second polypeptide or portion thereof preferentially associate with each other rather than a corresponding first polypeptide or portion thereof or second polypeptide or portion thereof, or both, without the amino acid substitutions (FIG. 1D). In some embodiments the amino acid substitutions are in the C_H3 domain of the first polypeptide or potion thereof. In some embodiments the amino acid substitutions are in the C_H3 domain of the second polypeptide or portion thereof. This assembly strategy has been shown to achieve >95% purity of the heterodimer (Merchant AM, Zhu Z, Yuan JQ, Goddard A, Adams CW, Presta LG, Carter P. An efficient route to human bispecific IgG. Nat Biotechnol. 1998 Jul;16(7):677). In some embodiments, bispecific binding agents provided herein include a single-chain Fab design, wherein the C-terminus of the C_L domain or portion thereof (e.g., of an anti-IL-6Rα antibody, or of an anti-IL-8R antibody) is connected to the N-terminus of the V_H domain or portion thereof (e.g., of an anti-IL-6Rα antibody, or of an anti-IL-8R antibody) using a long, flexible linker (Koerber JT, Hornsby MJ, Wells JA. An improved single-chain Fab platform for efficient display and recombinant expression. J Mol Biol. 2015 Jan 30;427(2):576–586. PMCID: PMC4297586). In some embodiments, the first polypeptide or portion thereof comprises a linker from the C-terminus of the first polypeptide C_L domain or portion thereof connected to the V_H domain of the first polypeptide or portion thereof. In some embodiments, the second polypeptide or portion thereof comprises a linker from the C- terminus of the second polypeptide CL domain or portion thereof connected to the VH domain of the second polypeptide or portion thereof.

Various non-limiting embodiments of bispecific binding agents are described herein, and can be used in any combination without limitation. Additional aspects of various components of bispecific binding agents are known in the art.

As used herein, the word "a" before a noun refers to one or more of the particular noun. As used herein, the term "affinity" refers to the strength of the sum total of non-covalent

interactions between an antigen-binding site and its binding partner (e.g., an antigen or epitope). Unless indicated otherwise, as used herein, "affinity" refers to intrinsic binding affinity, which reflects a 1:1 interaction between the participating members of an antigen-binding domain and an antigen or epitope. The affinity of a molecule X for its partner Y can be represented by the dissociation equilibrium constant (KD). Affinity can be measured by common methods known in the art, including those described herein. Affinity can be determined, for example, using surface plasmon resonance (SPR) technology (e.g., BIACORE®) or biolayer interferometry (e.g., FORTEBIO®). Additional methods for determining the affinity for an antigen-binding domain and its corresponding antigen or epitope are known in the art.

As used herein, the term "antibody" refers to an intact antibody, or an antigen binding fragment thereof. An antibody may comprise a complete antibody molecule (including polyclonal, monoclonal, chimeric, humanized, or human versions having full length heavy and/or light chains), or comprise an antigen binding fragment thereof. Antibody fragments include, without limitation, F(ab′)2, Fab, Fab′, Fv, Fc, and Fd fragments, single domain antibodies, monovalent antibodies, single-chain antibodies, maxibodies, minibodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR and bis-scFv (See e.g., Hollinger and Hudson, 2005, Nature Biotechnology, 23, 9, 1126-1136). Antibody polypeptides are also disclosed in U.S. Pat. No. 6,703,199, including fibronectin polypeptide monobodies. Other antibody polypeptides are disclosed in U.S. Patent Publication 2005/0238646, which are single-chain polypeptides. Monovalent antibody fragments are disclosed in US Patent Publication 20050227324.

Antigen binding fragments derived from an antibody can be obtained, for example, by proteolytic hydrolysis of the antibody, for example, pepsin or papain digestion of whole antibodies according to conventional methods. By way of example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment termed F(ab′)2. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab′ monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using papain produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. No. 4,331,647, Nisonoff et al., Arch. Biochem. Biophys. 89:230, 1960; Porter, Biochem. J. 73:119, 1959; Edelman et al., in Methods in Enzymology 1:422 (Academic Press 1967); and by Andrews, S.

M. and Titus, J. A. in Current Protocols in Immunology (Coligan J. E., et al., eds), John Wiley & Sons, New York (2003), pages 2.8.1-2.8.10 and 2.10A.1-2.10A.5. Other methods for cleaving antibodies, such as separating heavy chains to form monovalent light-heavy chain fragments (Fd), further cleaving of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

An antibody fragment may also be any synthetic or genetically engineered protein. For example, antibody fragments include, without limitation, isolated fragments that include the light chain variable region, "Fv" fragments that include the variable regions of the heavy and light chains, and recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker (scFv proteins).

Another form of an antibody fragment is a peptide comprising one or more complementarity determining regions (CDRs) of an antibody. CDRs (also termed "minimal recognition units", or "hypervariable region") can be obtained by constructing polynucleotides that encode the CDR of interest. Such polynucleotides are prepared, for example, by using the polymerase chain reaction to synthesize the variable region using mRNA of antibody-producing cells as a template (see, for example, Larrick et al., Methods: A Companion to Methods in Enzymology 2:106, 1991; Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in Monoclonal Antibodies: Production, Engineering and Clinical Application, Ritter et al. (eds.), page 166 (Cambridge University Press 1995); and Ward et al., "Genetic Manipulation and Expression of Antibodies," in Monoclonal Antibodies: Principles and Applications, Birch et al., (eds.), page 137 (Wiley-Liss, Inc. 1995)).

As used herein, the term "antigen" refers generally to a binding partner specifically recognized by an extracellular antigen-binding domain described herein. Exemplary antigens include different classes of molecules, such as, but not limited to, polypeptides and peptide fragments thereof, small molecules, lipids, carbohydrates, and nucleic acids. Non-limiting examples of antigen or antigens that can be specifically bound by any of the extracellular antigen-binding domains are described herein. Additional examples of antigen or antigens that can be specifically bound by any of the extracellular antigen-binding domains are known in the art.

As used herein, the term "antigen-binding domain" refers to one or more protein domain(s) (e.g., formed from amino acids from a single polypeptide or formed from amino acids

from two or more polypeptides (e.g., the same or different polypeptides)) that is capable of specifically binding to one or more different antigen(s) (e.g., an identifying antigen and/or a control antigen). In some embodiments, an antigen-binding domain can bind to an antigen or epitope with specificity and affinity similar to that of naturally-occurring antibodies. In some embodiments, the antigen-binding domain can be an antibody or a fragment thereof. In some embodiments, an antigen-binding domain can include an alternative scaffold. Non-limiting examples of antigen-binding domains are described herein. Additional examples of antigen-binding domains are known in the art. In some embodiments, an antigen-binding domain can bind to a single antigen (e.g., an identifying antigen or a control antigen). In some embodiments, an antigen-binding domain can bind to two or more antigens (e.g., an identifying antigen and a control antigen).

As used herein, the term "bispecific antibody" refers to an antibody derivative that has, in the same antibody molecule, variable regions that recognize two different epitopes. A bispecific antibody may be an antibody that recognizes two different antigens, or an antibody that recognizes two different epitopes on a same antigen.

In some embodiments of bispecific binding agents provided herein, the first binding site, the second binding site, or both, can be or can be derived from: an antibody, a VHH-scAb, a VHH-Fab, a Dual scFab, a F(ab')2, a diabody, a crossMab, a DAF (two- in-one), a DAF (four-in-one), a DutaMab, a DT-IgG, a knobs-in-holes common light chain, a knobs-in-holes assembly, a charge pair, a Fab-arm exchange, a SEEDbody, a LUZ-Y, a Fcab, a κλ-body, an orthogonal Fab, a DVD-IgG, a IgG(H)-scFv, a scFv- (H)IgG, IgG(L)-scFv, scFv-(L)IgG, IgG(L,H)-Fv, IgG(H)-V, V(H)-IgG, IgG(L)-V, V(L)- IgG, KIH IgG-scFab, 2 scFv-IgG, IgG-2 scFv, scFv4-Ig, Zybody, DVI-IgG, Diabody- CH3, a triple body, a miniantibody, a minibody, a TriBi minibody, scFv-C_H3 KIH, Fab- scFv, a F(ab')2-scFv2, a scFv-KIH, a Fab-scFv-Fc, a tetravalent HCAb, a scDiabody-Fc, a Diabody-Fc, a tandem scFv-Fc, an Intrabody, a dock and lock, a ImmTAC, an IgG-IgG conjugate, a Cov-X-Body, and a scFv1-PEG-scFv2. The previous examples are meant to be illustrative rather than limiting.

As used herein, the term "connect" means to fuse, join, couple, attach, combine, interconnect or any other similar word generally describing the physical adjoining of one or more polypeptide domain to each other via a linker.

As used herein, the term "epitope" refers to a portion of an antigen that is specifically bound by an antigen-binding domain through a set of physical interactions between: (i) all monomers (e.g. individual amino acid residues, sugar side chains, and post-translationally modified amino acid residues) on the portion of the antigen-binding domain that specifically binds the antigen, and (ii) all monomers (e.g. individual amino acid residues, sugar side chains, post-translationally modified amino acid residues) on the portion of the antigen that is specifically bound by the antigen-binding domain. Epitopes can include, without limitation, surface-accessible amino acid residues, sugar side chains, phosphorylated amino acid residues, methylated amino acid residues, and/or acetylated amino acid residues and may have specific three-dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former, but not the latter, may be lost in the presence of denaturing solvents. In some embodiments, an epitope is defined by a linear amino acid sequence of at least about 3 to 6 amino acids, or about 10 to 15 amino acids. In some embodiments, an epitope refers to a portion of a full-length protein or a portion thereof that is defined by a three-dimensional structure (e.g., protein folding). In some embodiments, an epitope is defined by a discontinuous amino acid sequence that is brought together via protein folding. In some embodiments, an epitope is defined by a discontinuous amino acid sequence that is brought together by quaternary structure (e.g., a cleft formed by the interaction of two different polypeptide chains). The amino acid sequences between the residues that define the epitope may not be critical to three- dimensional structure of the epitope. A conformational epitope may be determined and screened using assays that compare binding of antigen-binding protein construct to a denatured version of the antigen, such that a linear epitope is generated. An epitope may include amino acid residues that are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding. Methods for identifying an epitope to which an antigen-binding domain specifically binds are known in the art, e.g., structure-based analysis (e.g. X-ray crystallography, NMR, and/or electron microscopy) (e.g. on the antigen and/or the antigen-antigen-binding domain complex) and/or mutagenesis-based analysis (e.g. alanine scanning mutagenesis, glycine scanning mutagenesis, and homology scanning mutagenesis) wherein mutants are measured in a binding assay with a binding partner, many of which are known in the art.

The term "paratope" refers to a portion of an antigen-binding domain that specifically binds to an antigen through a set of physical interactions between: (i) all monomers (e.g. individual amino acid residues, sugar side chains, post-translationally modified amino acid residues) on the portion of the antigen-binding domain that specifically binds the antigen, and (ii) all monomers (e.g. individual amino acid residues, sugar side chains, post-translationally modified amino acid residues) on the portion of the antigen that is specifically bound by the antigen-binding domain. Paratopes can include, without limitation, surface-accessible amino acid residues and may have specific three- dimensional structural characteristics, as well as specific charge characteristics. In some embodiments, a paratope refers to a portion of a full-length antigen-binding domain or a portion thereof that is defined by a three-dimensional structure (e.g., protein folding). In some embodiments, a paratope is defined by a discontinuous amino acid sequence that is brought together via protein folding. In some embodiments, a paratope is defined by a discontinuous amino acid sequence that is brought together by quaternary structure (e.g., a cleft formed by the interaction of two different polypeptide chains). In some embodiments, the amino acid sequences between the residues that define the paratope are not critical to three-dimensional structure of the paratope. A paratope may, e.g., comprise amino acid residues that are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding. Methods for identifying a paratope to which an antigen-binding domain specifically binds are known in the art, e.g., structure-based analysis (e.g., X-ray crystallography, NMR, and/or electron microscopy) (e.g. on the antigen-binding domain, and/or the antigen-binding domain-antigen complex), and/or mutagenesis-based analysis (e.g., alanine scanning mutagenesis, glycine scanning mutagenesis, and homology scanning mutagenesis) wherein mutants are measured in a binding assay with a binding partner, many of which are known in the art. As used herein, the term "IL-8R" refers to an isotype of an interleukin 8 receptor, e.g., IL-8RA or IL-8RB.

As used herein, the term "knobs-in-holes" generally refers to an antibody assembly strategy. In a non-limiting way, complementary sets of mutations can be introduced into the C_H3 domain that can enforce heterodimerization over homodimerization. For example, an exemplary set of substitutions commonly used, include, in a non-limiting way, a "knob" created via a T366W substitution in the C_H3 domain, and "holes" created via substitutions T366S, L368A, and Y407V in the corresponding C_H3 domain. (*See*, for example, Ridgway et al., *Protein Eng.*

(1996); Merchant et al., An efficient route to human bispecific IgG, *Nat Biotechnol.* 16:677-81 (1998); Koerber JT, Hornsby MJ, Wells JA. An improved single-chain Fab platform for efficient display and recombinant expression. J Mol Biol. 2015 Jan 30;427(2):576–586. PMCID: PMC4297586; Carter P. Bispecific human IgG by design. J Immunol Methods 2001; 248:7-15; PMID:11223065; Atwell S, Ridgway JB, Wells JA, Carter P. Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library. J Mol Biol 1997; 270:26-35; PMID:9231898; Ridgway JB, Presta LG, Carter P. "Knobs-into-holes" engineering of antibody CH3 domains for heavy chain heterodimerization. Protein Eng 1996; 9:617-21; PMID:8844834). Generally, in a knobs- in-holes assembly approach, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers (e.g., heterodimers that are recovered from recombinant cell culture). In some embodiments, the interface comprises at least a part of the C_H3 domain of an antibody constant domain. In some embodiments, one or more amino acid substitutions in the first antibody (e.g., tyrosine or tryptophan) generate knobs or "protuberances." Compensatory "cavities" (holes) are generated on the interface of the second antibody by one or more amino acid substitutions (e.g., alanine, serine, or valine). Such a strategy provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. The use of knobs-in-holes as a method of producing bispecific antibodies and/or one-armed antibodies and/or immunoadhesins is well known in the art. See US Pat. No. 5,731,168 granted 24 March 1998 assigned to Genentech, PCT Pub. No. W02009089004 published 16 July 20, 2009 and assigned to Amgen, and US Pat. Pub. No. 20090182127 published 16 July 2009 and assigned to Novo Nordisk A/S. See also Marvin and Zhu, Acta Pharmacologica Sincia (2005) 26(6):649-658 and Kontermann (2005) Acta Pharacol. Sin., 26:1-9. Other heterodimerization formats are known and can include heterodimerization variants such as pI variants, charge pairs (a subset of steric variants e.g., knobs-in-holes), isosteric variants, and SEED body ("strand-exchange 8 CA 02902739 2015-08-26 WO 2014/145806 PCT/US2014/030634 engineered domain"; see Klein et al., mAbs 4:6 653- 663 (2012) and Davis et al, Protein Eng Des Sel 2010 23:195-202) which rely on the fact that the C_H3 domains of human IgG and IgA do not bind to each other.

As used herein, the term "protuberance" refers to at least one amino acid which projects from the interface of a first polypeptide and is therefore positionable in a compensatory cavity in an adjacent interface (i.e. the interface of a second polypeptide) so as to stabilize the heterodimer,

and thereby favor heterodimer formation over homodimer formation, for example. The protuberance(s) may exist in the original interface or may be introduced synthetically (e.g., by mutating nucleic acid encoding the interface). Import residues for the formation of a protuberance(s) are generally naturally occurring amino acid residues and can be selected from arginine (R), phenylalanine (F), tyrosine (Y) and tryptophan (W). See, e.g., PCT 2016/144824.

As used herein, the term "cavity" generally refers to at least one amino acid which is recessed from the interface of a second polypeptide and therefore accommodates a corresponding protuberance on an adjacent interface of a first polypeptide. The cavity may exist in the original interface or may be introduced synthetically (e.g., by mutating nucleic acid encoding the interface). Import residues for the formation of a cavity are usually naturally occurring amino acid residues and can be selected from alanine (A), serine (S), threonine (T) and valine (V). See, e.g., PCT 2016/144824.

As used herein, the term “linker” or “polypeptide linker” refers to an amino acid sequence that separates multiple domains in a single protein. Linkers can generally be classified into three groups: flexible, rigid and cleavable. Chen, X., et al., 2013, Adv. Drug Deliv. Rev., 65, 1357– 1369. Linkers can be natural or synthetic. Flexible linkers are typically rich in glycine residues. Klein et al., Protein Engineering, Design & Selection Vol. 27, No. 10, pp. 325–330, 2014; Priyanka et al., Protein Sci., 2013 Feb; 22(2): 153–167. In some embodiments, a bispecific binding agent includes a synthetic linker. A synthetic linker can have a length of from about 10 amino acids to about 200 amino acids, e.g., from 10 to 25 amino acids, from 25 to 50 amino acids, from 50 to 75 amino acids, from 75 to 100 amino acids, from 100 to 125 amino acids, from 125 to 150 amino acids, from 150 to 175 amino acids, or from 175 to 200 amino acids. A synthetic linker can have a length of from 10 to 30 amino acids, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids. A synthetic linker can have a length of from 30 to 50 amino acids, e.g., from 30 to 35 amino acids, from 35 to 40 amino acids, from 40 to 45 amino acids, or from 45 to 50 amino acids. In some embodiments, the linker is a flexible linker. In some embodiments, the linker is rich in glycine (Gly or G) residues. In some embodiments, the linker is rich in serine (Ser or S) residues. In some embodiments, the linker is rich in glycine and serine residues. In some embodiments, the linker has one or more glycine-serine residue pairs (GS), e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GS pairs. In some embodiments, the linker has one or more Gly-Gly-Gly-Ser (GGGS, SEQ ID NO:

1) sequences, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGGS sequences. In some embodiments, the linker has one or more Gly-Gly-Gly-Gly-Ser (GGGGS, SEQ ID NO: 2) sequences, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGGGS sequences. In some embodiments, the linker has one or more Gly-Gly-Ser-Gly (GGSG, SEQ ID NO: 3) sequences, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more GGSG sequences. In some embodiments, the linker is or comprises GSAAAGGSGGSGGS (SEQ ID NO: 4). In some embodiments, the linker is or comprises GGGSGGGS (SEQ ID NO: 5). In some embodiments, the linker is or comprises GSGGSSGSGSGSTGTSSSGTGTSAGTTGTSASTSGSGSG (SEQ ID NO: 13).

The terms "polypeptide," "peptide," and "protein,", used interchangeably herein, refer to a polymeric form of amino acids of any length, which can include genetically coded and non-genetically coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones. The term includes fusion proteins, including, but not limited to, fusion proteins with a heterologous amino acid sequence, fusion proteins with heterologous and homologous leader sequences, with or without N-terminal methionine residues, immunologically tagged proteins, and the like.

As used herein, a "portion" of a polypeptide or protein refers at least 10 amino acids of the reference sequence, e.g., 10 to 200, 25 to 300, 50 to 400, 100 to 500, 200 to 600, 300 to 700, 400 to 800, 500 to 900, or 600 to 1000 or more amino acids of the reference sequence. In some embodiments, the portion of a polypeptide or protein is functional.

As used herein "single-chain Fab fragment," "single-chain Fab segment," or "single-chain Fab," generally refers to a polypeptide comprising a V_H domain or portion thereof, a C_H1 domain or portion thereof, a V_L domain or portion thereof, a C_L domain or portion thereof, and a linker. Single-chain Fab can also refer to an expression technique, wherein the domains of the antibody can be combined in different ways and optionally with linker sequences and other domains and or alterations, e.g. substitutions, to produce an antibody. In some embodiments, single-chain Fab fragments have a sequence from the N-terminus to the C-terminus comprising, a V_H domain or portion thereof, a linker, a V_L domain or portion thereof, C_L domain or portion thereof, a V_H domain or portion thereof, a C_H1 domain or portion thereof, a C_H2 domain or portion thereof, and a C_H3 domain or portion thereof.

As used herein, "single-chain variable fragment-IgG fusion" (scFv-IgG) generally refers to bispecific antibody format that tethers a scFv of distinct specificity e.g. a second antibody, to a full IgG antibody thus creating a bispecific antibody.

I. Bispecific Binding Agents

Bispecific antibodies, which can simultaneously engage two different binding sites, demonstrate great potential to overcome the limitations of monoclonal antibodies via dual specificity. (Yang F, Wen W, Qin W. Bispecific Antibodies as a Development Platform for New Concepts and Treatment Strategies. Int J Mol Sci. 2016 Dec 28;18(1). PMCID: PMC5297683; Garber K. Bispecific antibodies rise again. Nat Rev Drug Discov. 2014 Nov;13(11):799–801). Bispecific antibodies can also increase the affinity, avidity, potency, and selectivity of protein-based therapies while reducing risk of drug resistance by concurrently blocking two different pathways to create a robust, multi- pronged treatment strategy (Cochran JR. Engineered proteins pull double duty. Sci Transl Med. 2010 Feb 3;2(17):17ps5. PMID: 20371477; Fan G, Wang Z, Hao M, Li J.

Bispecific antibodies and their applications. J Hematol OncolJ Hematol Oncol. 2015 Dec 21;8:130. PMCID: PMC4687327; Kontermann RE. Recombinant bispecific antibodies for cancer therapy. Acta Pharmacol Sin. 2005 Jan;26(1):1–9. PMID: 15659107). Furthermore, their well-defined stoichiometry and unimolecular construction eliminates the need for dosing ratio optimization, which can facilitate clinical development (Kontermann RE. Recombinant bispecific antibodies for cancer therapy. Acta Pharmacol Sin. 2005 Jan;26(1):1–9. PMID: 15659107). Bispecific antibodies can also engage a single target at multiple binding sites (i.e. epitopes).

A wide range of bispecific antibody formats have been explored which broadly fall into the categories of non-Fc-fused (FIG. 1A) and Fc-fused constructs (FIGs. 1B and 1C). Inclusion of the Fc region significantly increases the construct's serum half-life through neonatal Fc receptor (FcRN recycling) (Kontermann RE. Recombinant bispecific antibodies for cancer therapy. Acta Pharmacol Sin. 2005 Jan;26(1):1–9. PMID: 15659107). Within the class of Fc-fused constructs, bispecific antibodies may contain a full IgG heavy chain and light chain (FIG. 1B), or just an Fc region (FIG. 1C). Development of an assembly strategy known as a "knobs-in-holes," enabled the creation of (scFV)-Fc fusions (FIG. 1C).

While the knobs-in-holes assembly strategy results in >95% heterodimerization (Merchant AM, Zhu Z, Yuan JQ, Goddard A, Adams CW, Presta LG, Carter P. An efficient route to human bispecific IgG. Nat Biotechnol. 1998 Jul;16(7):677), mispairing of the V_H domain or portion thereof, and the V_L domain or portion thereof between the two specific antibodies still occurs in the conventional IgG format since the first antibody heavy chain or portion thereof and the first antibody light chain or portion thereof and the second antibody heavy chain or portion thereof and the second antibody light chain or portion thereof are secreted separately in recombinant expression schemes and assembled in vitro.

In certain embodiments, provided herein are knob-in-holes assembly strategies that are combined with single-chain Fab expression approaches to ensure proper variable domain pairing (e.g., proper variable domain pairing of an anti-IL-6Rα antibody, or an anti-IL-8R antibody). The single-chain Fab expression approach can connect the C- terminus of the light chain constant domain C_L to the N-terminus of the variable heavy chain V_H using a long flexible linker (Koerber JT, Hornsby MJ, Wells JA. An improved single-chain Fab platform for efficient display and recombinant expression. J Mol Biol. 2015 Jan 30;427(2):576–586. PMCID: PMC4297586). The generic format of bispecific binding agents provided herein is shown in FIG. 1D.

In one exemplary embodiment, a bispecific binding agent (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) includes: 1) a first polypeptide having in the N-terminal to C-terminal direction: a first antibody V_L domain or portion thereof, a first antibody C_L domain or portion thereof, a first linker, a first antibody V_H domain or portion thereof, a first antibody C_H1 domain or portion thereof, a first antibody C_H2 domain or portion thereof, and a first antibody C_H3 domain portion thereof, and 2) a second polypeptide having in the N-terminal to C-terminal direction, a second antibody V_L domain or portion thereof, a second antibody C_L domain or portion thereof, a second linker, a second antibody V_H domain or portion thereof, a second antibody C_H1 domain or portion thereof, a second antibody C_H2 domain or portion thereof, a the second antibody C_H3 domain, wherein the first antibody C_H3 domain or portion thereof, comprises one or more amino acid substitutions, the second antibody C_H3 domain or portion thereof comprises one or more amino acid substitutions, or both, such that the first polypeptide antibody heavy chain or portion thereof, and the second polypeptide antibody heavy chain or portion thereof, preferentially associate with each other to form the bispecific binding agent.

In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) includes at least two polypeptides, wherein each of the two polypeptides includes an antibody heavy chain constant domain, and wherein one or both of the antibody heavy chain constant domains includes one of more amino acid substitutions such that the two polypeptides bind to each other with an increased affinity as compared to two polypeptides that include corresponding antibody heavy chain constant domains that lack the one or more amino acid substitutions. In some embodiments, the one or more amino acid substitutions in the antibody heavy chain constant domain(s) are present in a C_H3 domain(s).

In some embodiments, the one or more amino acid substitutions in the first antibody heavy chain constant domain include substitutions at one or more of amino acid positions in the CH_3 domain (e.g., amino acid positions 249, 251, and 290 of SEQ ID NO: 6, corresponding to amino acid positions 366, 368, and 407 of Ridgway et al. and Merchant et al.). In some embodiments, the one or more amino acid substitutions in the first antibody heavy chain constant domain include substitutions at each of amino acid positions in the CH_3 domain (e.g., amino acid positions 249, 251, and 290 of SEQ ID NO: 6, corresponding to amino acid positions 366, 368, and 407 of Ridgway et al. and Merchant et al.). In some embodiments, the one or more amino acid substitutions in the first antibody heavy chain constant domain include one or more of a T249S substitution, an L251A substitution, and a Y290V substitution in the CH_3 domain (e.g., a T249S substitution, an L251A substitution, and/or a Y290V substitution at amino acid positions 249, 251, and 290 of SEQ ID NO: 6, corresponding to amino acid positions 366, 368, and 407 of Ridgway et al. and Merchant et al.). In some embodiments, the one or more amino acid substitutions in the first antibody heavy chain constant domain include substitutions at each of a T249S substitution, an L251A substitution, and a Y290V substitution in the CH_3 domain (e.g., a T249S substitution, an L251A substitution, and a Y290V substitution at amino acid positions 249, 251, and 290 of SEQ ID NO: 6, corresponding to amino acid positions 366, 368, and 407 of Ridgway et al. and Merchant et al.). In some embodiments, the first antibody heavy chain constant region includes the amino acid sequence of SEQ ID NO: 7.

In some embodiments, the one or more amino acid substitutions in the second antibody heavy chain constant domain include a substitution in the CH_3 domain (e.g., amino acid position 249 of SEQ ID NO: 6, corresponding to amino acid positions 366 of Ridgway et al. and

Merchant et al.). In some embodiments, the one or more amino acid substitutions in the second antibody heavy chain constant domain include a T249W substitution in the CH_3 domain (e.g., a T249W substitution at amino acid position 249 of SEQ ID NO: 6, corresponding to amino acid position 366 of Ridgway et al. and Merchant et al.).In some embodiments, the second antibody heavy chain constant region includes the amino acid sequence of SEQ ID NO: 8.

In some embodiments, a bispecific binding agent provide herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) includes an antibody heavy chain constant domain that is an IgG1, IgG2, IgG3, or IgG4 heavy chain constant domain. In some embodiments, an antibody heavy chain constant domain is an IgG1 heavy chain constant domain. In some embodiments, an antibody heavy chain constant domain is an IgG4 heavy chain constant domain. In some embodiments, a bispecific binding agent provide herein includes an IgG1 antibody heavy chain constant domain and an IgG4 antibody heavy chain constant domain. See, e.g., Spiess et al., J Biol Chem. 2013 Sept. 13;288(37):26583-93, incorporated by reference herein in its entirety).

In some embodiments, a bispecific binding agent provide herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) includes one or more modifications (e.g., amino acid substitutions as compared to a wild type sequence) in one or more domains (e.g., modifications in one or more CH_2 domains). In some embodiments, such modifications may serve to enhance expression, modify glycosylation, or both. Those of ordinary skill in the art will be aware of suitable modifications and will be able to employ such modifications in the context of bispecific binding agents provide herein.

In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) includes at least two polypeptides, wherein each of the two polypeptides includes an antibody heavy chain and an antibody light chain that are connected by a linker (e.g., any of the variety of "linkers" or "polypeptide linkers" described herein). In some embodiments, the two polypeptides include a linker having the same sequence. In some embodiments, the two polypeptides include a linker having a different sequence. In some embodiments, a linker can be about 10 to about 100 amino acids in length. For example a linker can be about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids in length, or any number of amino acids in between. In some embodiments, a

linker includes the amino acid sequence
GGSGGSSGSGSGSTGTSSSGTGTSAGTTGTSASTSGSGSG (SEQ ID NO: 13).

In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) includes a first polypeptide having a first V_H domain present or portion thereof and a first V_L domain or portion thereof, wherein the first VH domain or portion thereof and the first VL domain or portion thereof form a first antigen binding site. In some embodiments, a bispecific binding agent provided herein includes a second polypeptide having a second VH domain present or portion thereof and a second VL domain or portion thereof, wherein the second VH domain or portion thereof and the second VL domain or portion thereof form a second antigen binding site. In some embodiments, a bispecific binding agent provided herein binds a first target (e.g., IL-6Rα, e.g., via a first antigen binding site) and a second target (e.g., IL-8R, e.g., via a second antigen binding site).

In some embodiments, bispecific binding agents provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) bind one of their cognate antigens via antigen-specific variable regions and/or CDRs with an affinity and/or specificity that approximates the affinity and/or specificity of a monoclonal antibody that has corresponding antigen-specific variable regions and/or CDRs. For example, a bispecific binding agent provided herein can bind one of its cognate antigens (e.g., IL-6Rα and/or IL-8R) via antigen-specific variable regions and/or CDRs with an affinity that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% of the affinity of a monoclonal antibody having corresponding antigen-specific variable regions and/or CDRs. In some embodiments, a bispecific binding agent provided herein can bind one of its cognate antigens (e.g., IL-6Rα and/or IL-8R) via antigen-specific variable regions and/or CDRs with an affinity that is greater than the affinity of a monoclonal antibody having corresponding antigen-specific variable regions and/or CDRs. In some embodiments, a bispecific binding agent provided herein can bind one of its cognate antigens (e.g., IL-6Rα and/or IL-8R) via antigen-specific variable regions and/or CDRs with a specificity that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% of the specificity of a monoclonal antibody having corresponding antigen-specific variable regions and/or CDRs. In some embodiments, a bispecific binding agent provided herein can bind one of its cognate antigens (e.g., IL-6Rα and/or IL-8R) via antigen-

specific variable regions and/or CDRs with a specificity that is greater than the specificity of a monoclonal antibody having corresponding antigen-specific variable regions and/or CDRs. In some embodiments, a bispecific binding agent provided herein can bind one of its cognate antigens (e.g., IL-6Rα and/or IL-8R) via antigen-specific variable regions and/or CDRs with both an affinity and a specificity that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% of the affinity and specificity of a monoclonal antibody having corresponding antigen-specific variable regions and/or CDRs. In some embodiments, a bispecific binding agent provided herein can bind one of its cognate antigens (e.g., IL-6Rα and/or IL-8R) via antigen-specific variable regions and/or CDRs with both an affinity and a specificity that is greater than the affinity and specificity of a monoclonal antibody having corresponding antigen-specific variable regions and/or CDRs.

Therapeutic Uses

In some embodiments, bispecific binding agents provided herein (e.g., e.g., bispecific binding agents that bind IL-6Rα and IL-8R) are used in the prevention, treatment, and/or amelioration of one or more diseases or conditions in a subject (e.g., a human subject). In some embodiments, bispecific binding agents provided herein provide improved pharmacological effects and outcomes as compared to two separate agents, each having one of the two binding specificities of the bispecific binding agent.

For example, administration of a bispecific binding agent provided herein to a subject can result in greater clinical effectiveness (e.g., improved prevention, treatment, and/or amelioration of a disease or condition in a subject) as compared to administration (e.g., simultaneous or sequential administration) of two separate agents, each having one of the two binding specificities of the bispecific binding agent. In some embodiments, administration of a bispecific binding agent provided herein to a subject can result in fewer side effects (e.g., as a result of off-target binding) as compared to administration (e.g., simultaneous or sequential administration) of two separate agents, each having one of the two binding specificities of the bispecific binding agent. As will be appreciated in the art, mixed-agent dosing presents certain problems. In some embodiments, a dosing regimen (e.g., dosing amount, dosing frequency, and/or length of dosing) of a bispecific binding agent provided is easier to optimize as compared to a dosing regimen that

includes of two separate agents, each having one of the two binding specificities of the bispecific binding agent.

In some embodiments, bispecific binding agents provided herein (e.g., bispecific binding agents that binds IL-6Rα and IL-8R) are used in the treatment of cancer in a subject (e.g., a human subject). For example, bispecific binding agents provided herein e.g., a bispecific binding agent that binds IL-6Rα and IL-8R can be used to inhibit or prevent metastasis of a primary tumor in a subject. In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) is used in the treatment of cancer in a subject (e.g., a human subject) in combination with one or more "therapeutic interventions". In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) is administered to a subject (e.g., a human subject) simultaneously with the administration of one or more therapeutic interventions. In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) is not administered to a subject (e.g., a human subject) simultaneously with the administration of one or more therapeutic interventions. For example, a bispecific binding agent and a therapeutic intervention can be administered sequentially (e.g., there can be a period of time between administration of the bispecific binding agent and administration of the therapeutic intervention such as, without limitation, 1 minute, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5, hours, 6 hours, 7 hours, 8 hours, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 1 month, 2 months, 3 months, or more).

Examples of therapeutic interventions include, without limitation, adjuvant chemotherapy, neoadjuvant chemotherapy, radiation therapy, hormone therapy, cytotoxic therapy, immunotherapy, adoptive T cell therapy (e.g., chimeric antigen receptors and/or T cells having wild-type or modified T cell receptors), targeted therapy such as administration of kinase inhibitors (e.g., kinase inhibitors that target a particular genetic lesion, such as a translocation or mutation), (e.g. a kinase inhibitor, an antibody, a bispecific antibody), signal transduction inhibitors, bispecific antibodies or antibody fragments (e.g., BiTEs), monoclonal antibodies, immune checkpoint inhibitors, surgery (e.g., surgical resection), or any combination of the above. In some embodiments, a therapeutic intervention can reduce the severity of the cancer,

reduce a symptom of the cancer, and/or to reduce the number of cancer cells present within the subject.

In some embodiments, a therapeutic intervention can include an immune checkpoint inhibitor. Non-limiting examples of immune checkpoint inhibitors include nivolumab (Opdivo), pembrolizumab (Keytruda), atezolizumab (tecentriq), avelumab (bavencio), durvalumab (imfinzi), ipilimumab (yervoy). See, e.g., Pardoll (2012) Nat. Rev Cancer 12: 252-264; Sun et al. (2017) Eur Rev Med Pharmacol Sci 21(6): 1198-1205; Hamanishi et al. (2015) J. Clin. Oncol. 33(34): 4015-22; Brahmer et al. (2012) N Engl J Med 366(26): 2455-65; Ricciuti et al. (2017) J. Thorac Oncol. 12(5): e51-e55; Ellis et al. (2017) Clin Lung Cancer pii: S1525-7304(17)30043-8; Zou and Awad (2017) Ann Oncol 28(4): 685-687; Sorscher (2017) N Engl J Med 376(10: 996-7; Hui et al. (2017) Ann Oncol 28(4): 874-881; Vansteenkiste et al. (2017) Expert Opin Biol Ther 17(6): 781-789; Hellmann et al. (2017) Lancet Oncol. 18(1): 31-41; Chen (2017) J. Chin Med Assoc 80(1): 7-14.

In some embodiments, a therapeutic intervention is adoptive T cell therapy (e.g., chimeric antigen receptors and/or T cells having wild-type or modified T cell receptors). See, e.g., Rosenberg and Restifo (2015) Science 348(6230): 62-68; Chang and Chen (2017) Trends Mol Med 23(5): 430-450; Yee and Lizee (2016) Cancer J. 23(2): 144-148; Chen et al. (2016) Oncoimmunology 6(2): e1273302; US 2016/0194404; US 2014/0050788; US 2014/0271635; US 9,233,125; incorporated by reference in their entirety herein.

In some embodiments, a therapeutic intervention is a chemotherapeutic agent. Non-limiting examples of chemotherapeutic agents include: amsacrine, azacitidine, axathioprine, bevacizumab (or an antigen-binding fragment thereof), bleomycin, busulfan, carboplatin , capecitabine, chlorambucil, cisplatin, cyclophosphamide, cytarabine, dacarbazine, daunorubicin, docetaxel, doxifluridine, doxorubicin, epirubicin, erlotinib hydrochlorides, etoposide, fiudarabine, floxuridine, fludarabine, fluorouracil, gemcitabine, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine, mechlorethamine, melphalan, mercaptopurine, methotrxate, mitomycin, mitoxantrone, oxaliplatin, paclitaxel, pemetrexed, procarbazine, all-trans retinoic acid, streptozocin, tafluposide, temozolomide, teniposide, tioguanine, topotecan, uramustine, valrubicin, vinblastine, vincristine, vindesine, vinorelbine, and combinations thereof. Additional examples of anti-cancer therapies are known in the art; see, e.g. the guidelines for therapy from

the American Society of Clinical Oncology (ASCO), European Society for Medical Oncology (ESMO), or National Comprehensive Cancer Network (NCCN).

In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) and one or more therapeutic interventions (e.g., a chemotherapy or any of the other appropriate therapeutic interventions discloses herein) can be administered to a subject once or multiple times over a period of time ranging from days to weeks (separately or in combination). In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) and one or more therapeutic interventions can be formulated into a pharmaceutically acceptable composition for administration to a subject having cancer (separately or in combination). For example, a therapeutically effective amount of a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) and a therapeutic intervention (e.g. a chemotherapeutic or immunotherapeutic agent) can be formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. A pharmaceutical composition can be formulated for administration in solid or liquid form including, without limitation, sterile solutions, suspensions, sustained-release formulations, tablets, capsules, pills, powders, and granules.

Pharmaceutically acceptable carriers, fillers, and vehicles that may be used in a pharmaceutical composition described herein include, without limitation, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

A pharmaceutical composition containing one or more therapeutic interventions can be designed for oral or parenteral (including subcutaneous, intramuscular, intravenous, and intradermal) administration. When being administered orally, a pharmaceutical composition can be in the form of a pill, tablet, or capsule. Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions that can contain anti-oxidants,

buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient. The formulations can be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets.

In some embodiments, a pharmaceutically acceptable composition including one or more therapeutic interventions can be administered locally or systemically. For example, a composition provided herein can be administered locally by injection into tumors. In some embodiments, a composition provided herein can be administered systemically, orally, or by injection to a subject (e.g., a human).

Effective doses can vary depending on the severity of the cancer, the route of administration, the age and general health condition of the subject, excipient usage, the possibility of co-usage with other therapeutic treatments such as use of other agents, and the judgment of the treating physician.

An effective amount of a composition containing a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R), optionally in combination with one or more therapeutic interventions, can be any amount that reduces the extent of metastasis (e.g., prevents metastasis) of cancer cells present within the subject without producing significant toxicity to the subject. If a particular subject fails to respond to a particular amount, then the amount of a bispecific binding agent can be increased by, for example, two fold. After receiving this higher amount, the subject can be monitored for both responsiveness to the treatment and toxicity symptoms, and adjustments made accordingly. The effective amount can remain constant or can be adjusted as a sliding scale or variable dose depending on the subject response to treatment. Various factors can influence the actual effective amount used for a particular application. For example, the frequency of administration, duration of treatment, use of multiple treatment agents, route of administration, and severity of the condition (e.g., cancer) may require an increase or decrease in the actual effective amount administered.

The frequency of administration of a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) can be any frequency that reduces the extent of metastasis (e.g., prevents metastasis) within the subject without producing significant

toxicity to the subject. For example, the frequency of administration of a bispecific binding agent can be from about two to about three times a week to about two to about three times a month. The frequency of administration of a bispecific binding agent can remain constant or can be variable during the duration of treatment. A course of treatment with a composition containing a bispecific binding agent can include rest periods. For example, a composition containing a bispecific binding agent can be administered daily over a two-week period followed by a two week rest period, and such a regimen can be repeated multiple times. As with the effective amount, various factors can influence the actual frequency of administration used for a particular application. For example, the effective amount, duration of treatment, use of multiple treatment agents, route of administration, and severity of the condition (e.g., cancer) may require an increase or decrease in administration frequency.

An effective duration for administering a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) can be any duration that reduces the extent of metastasis (e.g., prevents metastasis) within the subject without producing significant toxicity to the subject. In some embodiments, the effective duration can vary from several days to several weeks. In general, the effective duration for reducing or preventing metastasis of cancer cells present within the subject can range in duration from about one week to about four weeks. Multiple factors can influence the actual effective duration used for a particular treatment. For example, an effective duration can vary with the frequency of administration, effective amount, use of multiple treatment agents, route of administration, and severity of the condition being treated.

In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) can reduce the extent of metastasis of cancer cells present in a subject. For example, a bispecific binding agent can reduce the extent of metastasis in a subject by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more. In some embodiments, a bispecific binding agent can reduce the extent of metastasis in a subject such that no metastatic cancer cells are observable. In some embodiments, a bispecific binding agent can reduce the number of observable tumors present in a subject.

In some embodiments, a bispecific binding agent provided herein (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) is used to treat one or more of the following cancer types: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), adrenal cancer,

adrenocortical carcinoma, AIDS-related cancers, AIDS-related lymphoma, amyotrophic lateral sclerosis or ALS, anal cancer, appendix cancer, astrocytoma, astrocytoma, childhood cerebellar or cerebral, atypical teratoid/rhabdoid tumor, basal cell carcinoma, bile duct cancer, bile duct cancer, extrahepatic (see cholangiocarcinoma), bladder cancer, bone cancer, bone tumor, osteosarcoma/malignant fibrous histiocytoma, brain cancer, brain stem glioma, brain tumor, brain tumor, cerebellar astrocytoma, brain tumor, cerebral astrocytoma/malignant glioma, brain tumor, ependymoma, brain tumor, medulloblastoma, brain tumor, supratentorial primitive neuroectodermal tumors, brain tumor, visual pathway and hypothalamic glioma, brainstem glioma, breast cancer, bronchial adenomas/carcinoids, bronchial tumor, bronchioles lung cell carcinoma, Burkitt lymphoma, cancer in adolescents, carcinoid tumor, carcinoid tumor, childhood, carcinoid tumor, gastrointestinal, carcinoma of unknown primary, cardiac tumors, central nervous system lymphoma, primary, cerebellar astrocytoma, childhood, cerebral astrocytoma/malignant glioma, childhood, cervical cancer, childhood cancers, chondrosarcoma, chordoma, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), chronic myeloproliferative disorders, chronic myeloproliferative neoplasms, colon cancer, colorectal cancer, colorectal cancer (e.g., metastatic colorectal cancer), craniopharyngioma, cutaneous t-cell lymphoma, desmoplastic small round cell tumor, differentiated thyroid cancer, ductal carcinoma in situ, embryonal tumors, endometrial cancer, ependymoma, epithelioid hemangioendothelioma (EHE), esophageal cancer, esthesioneuroblastoma, Ewing's sarcoma in the Ewing family of tumors, extracranial germ cell tumor, extracranial germ cell tumor, childhood, extragonadal germ cell tumor, extrahepatic bile duct cancer, eye cancer, eye cancer, intraocular melanoma, eye cancer, retinoblastoma, fallopian tube cancer, fibrous histiocytoma of bone, gallbladder cancer, ganglioneuromatosis of the gastroenteric mucosa, gastric (stomach) cancer, gastric (stomach) cancer, gastric carcinoid, gastrointestinal carcinoid tumor, gastrointestinal stromal tumors (GIST), germ cell tumor, germ cell tumor: extracranial, extragonadal, or ovarian, gestational trophoblastic disease, gestational trophoblastic tumor, glioma, glioma of the brain stem, glioma, childhood cerebral astrocytoma, glioma, childhood visual pathway and hypothalamic, hairy cell leukemia, hairy cell tumor, head and neck cancer, heart cancer, hepatocellular (liver) cancer, histiocytosis, Hodgkin's lymphoma, hypopharyngeal cancer, hypothalamic and visual pathway glioma, childhood, inflammatory myofibroblastic tumor, intraocular melanoma, intraocular melanoma, Islet cell carcinoma (endocrine pancreas),

islet cell tumors, Kaposi sarcoma, kidney cancer (renal cell cancer), Langerhans cell histiocytosis, laryngeal cancer, leukaemia, acute lymphoblastic (also called acute lymphocytic leukaemia), leukaemia, acute myeloid (also called acute myelogenous leukemia), leukaemia, chronic lymphocytic (also called chronic lymphocytic leukemia), leukemia, leukemia, chronic myelogenous (also called chronic myeloid leukemia), leukemia, hairy cell, lip and oral cavity cancer, liposarcoma, liver cancer (e.g., primary), lung adenocarcinoma, lung cancer, lung cancer (e.g., small cell lung carcinoma or non-small cell lung carcinoma), lymphoma, lymphoma, AIDS-related, lymphoma, Burkitt, lymphoma, cutaneous T-Cell, lymphoma, Hodgkin, lymphoma, primary central nervous system, lymphomas, Non-Hodgkin (an old classification of all lymphomas except Hodgkin's), macroglobulinemia, male breast cancer, malignant fibrous histiocytoma of bone, malignant fibrous histiocytoma of bone/osteosarcoma, medullary thyroid cancer, medulloblastoma, childhood, melanoma, melanoma, intraocular (eye), melanoma, intraocular (eye), Merkel cell cancer, Merkel cell carcinoma, mesothelioma, mesothelioma, adult malignant, mesothelioma, childhood, metastatic squamous neck cancer, metastatic squamous neck cancer with occult primary, midline tract carcinoma, mouth cancer, multiple endocrine neoplasia syndrome, childhood, multiple endocrine neoplasia syndromes, multiple endocrine neoplasia type 2A or 2B (MEN2A or MEN2B, respectively), multiple myeloma, multiple myeloma/plasma cell neoplasm, mycosis fungoides, myelodysplastic syndromes, myelodysplastic/myeloproliferative diseases, myelodysplastic/myeloproliferative neoplasms, myelogenous leukemia, myelogenous leukemia, chronic, myeloid leukemia, myeloid leukemia, adult acute, myeloid leukemia, childhood acute, myeloma, multiple (cancer of the bone-marrow), myeloproliferative disorders, chronic, myeloproliferative neoplasms, myxoma, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, nasopharyngeal carcinoma, neuroblastoma, oligodendroglioma, oral cancer, oral cavity cancer, oropharyngeal cancer, osteocarcinoma, osteosarcoma, osteosarcoma/malignant fibrous histiocytoma of bone, ovarian cancer, ovarian epithelial cancer (surface epithelial-stromal tumor), ovarian germ cell tumor, ovarian low malignant potential tumor, pancreatic cancer, pancreatic cancer, islet cell, pancreatic neuroendocrine tumors, papillary renal cell carcinoma, papillary thyroid cancer, papillomatosis, paraganglioma, paranasal sinus and nasal cavity cancer, parathyroid cancer, parathyroid hyperplasia, penile cancer, pharyngeal cancer, pheochromocytoma, Phyllodes breast tumors, pineal astrocytoma, pineal germinoma, pineoblastoma and supratentorial primitive

neuroectodermal tumors, childhood, pituitary adenoma, pituitary cancer, plasma cell neoplasia/multiple myeloma, plasma cell neoplasm, pleuropulmonary blastoma, pregnancy and breast cancer, primary central nervous system lymphoma, primary peritoneal cancer, prostate cancer, rectal cancer, recurrent thyroid cancer, refractory differentiated thyroid cancer, renal cell cancer, renal cell carcinoma (kidney cancer), renal pelvis and ureter, transitional cell cancer, retinoblastoma, rhabdomyosarcoma, rhabdomyosarcoma, childhood, salivary gland cancer, sarcoma, sarcoma, Ewing family of tumors, Sarcoma, Kaposi, Sezary syndrome, skin cancer, skin cancer (melanoma), skin cancer (non-melanoma), skin carcinoma, Merkel cell, small intestine cancer, soft tissue sarcoma, squamous cell carcinoma, squamous cell carcinoma - see skin cancer (non-melanoma), squamous neck cancer, squamous neck cancer with occult primary, metastatic, stomach cancer, supratentorial primitive neuroectodermal tumor, childhood, T-cell lymphoma, T-cell lymphoma, cutaneous , testicular cancer, throat cancer, thymoma and thymic carcinoma, Thymoma, childhood, thyroid cancer, thyroid cancer, childhood, transitional cell cancer of the renal pelvis and ureter, trophoblastic tumor, gestational, unknown primary carcinoma, unknown primary site, cancer of, childhood, unknown primary site, carcinoma of, adult, ureter and renal pelvis, transitional cell cancer, urethral cancer, uterine cancer, uterine cancer, endometrial, uterine sarcoma, vaginal cancer, visual pathway and hypothalamic glioma, childhood, vulvar cancer, Waldenstrom macroglobulinemia, and Wilms tumor (kidney cancer).

Construction and Expression of a Knobs-in-Holes Single Chain Bispecific Binding Agent

Vector Construction

In certain embodiments, provided herein are methods of constructing and expressing a bispecific binding agent (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) comprising a first polynucleotide sequence comprising a first segment that encodes a first antibody heavy chain or portion thereof; a second segment that encodes a first linker, and a third segment that encodes a first antibody light chain or portion thereof; a second polynucleotide sequence comprising a first segment that encodes a second antibody heavy chain or portion thereof, a second segment that encodes a second linker, and a third segment that encodes a second antibody light chain or portion thereof; generating a first polypeptide from the first polynucleotide sequence wherein the first polypeptide comprises the first antibody heavy chain or portion thereof, the first linker, and the first antibody light chain or portion thereof, generating

a second polypeptide from the second polynucleotide sequence, wherein the second polypeptide comprises the second antibody heavy chain or portion thereof, the second linker, and the second antibody light chain or portion thereof; wherein the first antibody heavy chain or portion thereof, the second antibody heavy chain or portion thereof, or both, comprises one or more amino acid substitutions (e.g., as compared to a wild type antibody heavy chain) such that the first antibody heavy chain or portion thereof and the second antibody heavy chain or portion thereof preferentially associate with each other to form the bispecific binding agent.

In some embodiments, the first polypeptide is generated by providing an expression vector comprising the first polynucleotide sequence operably linked to a promoter, and expressing the first polypeptide from the first polynucleotide sequence. In some embodiments, the second polypeptide is generated by providing an expression vector comprising the second polynucleotide sequence operably linked to a promoter, and expressing the second polypeptide from the second polynucleotide sequence.

In some embodiments of any of the vectors described herein, the nucleic acid encoding any of the bispecific binding agents described herein is operably linked to one or both of a promoter and an enhancer. In some embodiments of any of the vectors described herein, the promoter is an inducible promoter.

Generally, bispecific binding agents provided herein (e.g., bispecific binding agents that binds IL-6Rα and IL-8R) may be produced using techniques from any of the variety of methods known to those skilled in the art. For example, a nucleic acid sequence coding for a first polypeptide of the bispecific binding agent (e.g., a single chain polypeptide having both an antibody light chain and an antibody heavy chain), a second polypeptide of the bispecific binding agent (e.g., a single chain polypeptide having both an antibody light chain and an antibody heavy chain), or both can be inserted into an expression vector according to conventional techniques. In some embodiments, a nucleic acid sequence coding for a first polypeptide of the bispecific binding agent is inserted into a first expression vector. In some embodiments, a nucleic acid sequence coding for a second polypeptide of the bispecific binding agent is inserted into a second expression vector. In some embodiments, a nucleic acid sequence coding for a first polypeptide of the bispecific binding agent and a nucleic acid sequence coding for the a second polypeptide of the bispecific binding agent are inserted into a first expression vector. In some embodiments, a nucleic acid sequence coding for a first polypeptide of the bispecific binding agent, a nucleic

acid sequence coding for a second polypeptide of the bispecific binding agent, or both are expressed under control of an expression control region, for example, an enhancer and/or a promoter. In some embodiments, a host cell is transfected with an expression vector(s) having a nucleic acid sequence coding for a first polypeptide of the bispecific binding agent, an expression vector having a nucleic acid sequence coding for the a second polypeptide of the bispecific binding agent, or both. In some embodiments, both the first polypeptide and the second polypeptide of a bispecific binding agent are expressed in the same host cell. In some embodiments, the first polypeptide and the second polypeptide of a bispecific binding agent are expressed in different host cells.

Nucleic acids, vector constructs, and expression systems

Nucleic acid (e.g., DNA) sequences coding for any of the polypeptides present in bispecific binding agents provided herein (e.g., bispecific binding agents that bind IL- 6Rα and IL-8R) are also within the scope of the present invention as are methods of making the engineered bispecific binding agents. For example, variable regions can be constructed using PCR mutagenesis methods to alter DNA sequences encoding an immunoglobulin chain, e.g., using methods employed to generate humanized immunoglobulins (see e.g., Kanunan, et al, Nucl. Acids Res. 12:5404,1989; Sato, et al, Cancer Research 53:851-856, 1993; Daugherty, et al, Nucleic Acids Res. 19(9):2471- 2476,1991; and Lewis and Crowe, Gene 101:297-302, 1991). Using these or other suitable methods, variants can also be readily produced. In some embodiments, cloned constant regions can be mutagenized, and sequences encoding variants with the desired specificity can be selected (e.g., a constant region present in a first polypeptide of a bispecific binding agent, a constant region present in a second polypeptide of a bispecific binding agent, or both).

Expression vectors are useful for the purpose of antibody production. Examples of suitable expression vectors include, without limitation, M13 vector, pUC vector, pBR322, pBluescript, pCR-Script, and gWiz. For subcloning and separation of cDNA, for example, pGEM-T, pDIRECT and pT7 may also be used.

Suitable host cells for cloning or expressing the DNA in the vectors herein include, without limitation, prokaryotic cells, yeast cells, or higher eukaryote cells described herein. Suitable prokaryotes for this purpose include, without limitation, eubacteria, such as Gram-negative or

Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans, and Shigella, as well as Bacilli such as B. subtilis and B. licheniformis (e.g., B. licheniformis 41 P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as P. aeruginosa, and Streptomyces. In some embodiments, host cell is E. coli 294 (ATCC 31,446). Other strains such as E. coli B, E. coli X1776 (ATCC 31,537), and E. coli W3110 (ATCC 27,325) are also suitable.

In embodiments in which E. coli such as JM109, DH5α, HB101 or XL1-Blue is used as a host, the expression vector includes a promoter that drives efficient expression of a polypeptide (e.g., a polypeptide of a bispecific binding agent) in E. coli, for example, lacZ promoter (Ward et al., Nature (1989) 341, 544-546; FASEB J. (1992) 6, 2422-2427, hereby incorporated by reference in its entirety), araB promoter (Better et al., Science (1988) 240, 1041-1043, hereby incorporated by reference in its entirety) or T7 promoter. A vector of this type can also include pGEX-5X-1 (Pharmacia), QIA express system (QIAGEN), pEGFP, and pET (in some cases, the host is a T7 RNA polymerase- expressing BL21).

In some embodiments, eukaryotic microbes such as, without limitation, filamentous fungi or yeast can be used as cloning and/or expression hosts for vectors encoding any of the variety of bispecific binding agents provided herein. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and can be used in methods provided herein, such as, without limitation, Schizosaccharomyces pombe; Kluyveromyces hosts such as, e.g., K. lactis, K. fragilis (ATCC 12,424), K. bulgaricus (ATCC 16,045), K. wickeramii (ATCC 24,178), K. waltii (ATCC 56,500), K. drosophilarum (ATCC 36,906), K. thermotolerans, and K. marxianus; yarrowia (EP 402,226); Pichia pastors (EP 183,070); Candida; Trichoderma reesia (EP 244,234); Neurospora crassa; Schwanniomyces such as Schwanniomyces occidentalis; and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as A. nidulans and A. niger.

Suitable host cells for the expression of glycosylated proteins (e.g., bispecific binding agents) can be derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (caterpillar), Aedes aegypti (mosquito), Aedes

albopictus (mosquito), Drosophila melanogaster (fruitfly), and Bombyx mori have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV, and such viruses may be used as the virus herein according to the present disclosure, particularly for transfection of Spodoptera frugiperda cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, tobacco, lemna, and other plant cells can also be utilized as hosts.

In some embodiments, a vector used for polypeptide (e.g., bispecific binding agent) production can be a mammal-derived expression vector (e.g., pcDNA3 (Invitrogen), pEGF-BOS (Nucleic acids, Res., 1990, 18(17), p. 5322, hereby incorporated by reference in its entirety), pEF, pCDM8); insect cell-derived expression vectors (e.g., Bac-toBAC baculovairus expression system (GIBCO BRL), pBacPAK8); vegetable-derived expression vectors (e.g., pMH1, pMH2); animal virus-derived expression vectors (e.g., pHSV, pMV, pAdexLcw), retrovirus-derived expression vectors (e.g., pZIPneo), yeast-derived expression vectors (e.g., Pichia Expression Kit (Invitrogen), pNV11, SP- Q01), Bacillus subtilis-derived expression vectors (e.g., pPL608, pKTH50).

For expression in hosts, (e.g., animal cells such as CHO cells, COS cells or NIH3T3 cells), a vector can have a promoter that drives intracellular expression, for example, SV40 promoter (Mulligan et al., Nature (1979) 277, 108, hereby incorporated by reference in its entirety), MMTV-LTR promoter, EF1α promoter (Mizushima et al., Nucleic Acids Res. (1990) 18, 5322, hereby incorporated by reference in its entirety), CAG promoter (Gene (1991) 108, 193, hereby incorporated by reference in its entirety), or CMV promoter. Examples of useful mammalian host cell lines include, without limitation, Chinese hamster ovary cells, including CHOK1 cells (ATCC CCL61), DXB- 11, DG-44, and Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77: 4216 (1980)); monkey kidney CV1 line transformed by SV40 (COS- 7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, (Graham et al., J. Gen Virol. 36: 59, 1977); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, (Biol. Reprod. 23: 243-251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y Acad. Sci. 383: 44-68 (1982)); MRC 5 cells;

FS4 cells; and a human hepatoma line (Hep G2). In certain embodiments, two mammalian expression plasmids (e.g., a gWiz backbone) can be used, wherein encoding each mammalian expression plasmids encodes at least one polypeptide of a bispecfic binding agent. The aforementioned list of cells are illustrative and non-limiting.

In some embodiments, the vector includes a gene for screening of the transformed cells (e.g., drug-resistant gene capable of being differentiated by drug (e.g., neomycin, G418)). The vector having such characteristics includes, for example, pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, pOP13. In an exemplary embodiment, expression vectors can be co-transfected into human embryonic kidney (HEK 293) cells for soluble expression. In some embodiments, host cells are transformed or transfected with any of the above-described expression or cloning vectors for production of a bispecific binding agent and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, and/or amplifying the genes encoding the desired sequences. In some embodiments, novel vectors and transfected cell lines with multiple copies of transcription units separated by a selective marker can be used for the expression of antibodies that bind target.

When using recombinant techniques, the bispecific binding agent can be produced intracellularly, in the periplasmic space, or directly secreted into the medium, including from microbial cultures. If the bispecific binding agent is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, can be removed, for example, by centrifugation or ultrafiltration. Better et al. (Science 240:1041-43, 1988; ICSU Short Reports 10:105 (1990); and Proc. Natl. Acad. Sci. USA 90:457-461 (1993) describe a procedure for isolating antibodies which are secreted to the periplasmic space of E. coli. [See also, (Carter et al., Bio/Technology 10:163-167 (1992)].

A bispecific binding agent (e.g., a bispecific binding agent that binds IL-6Rα and IL-8R) prepared from microbial or mammalian cells can be purified using, for example, hydroxylapatite chromatography cation or avian exchange chromatography, and affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the bispecific binding agent. Protein A can be used to purify bispecific binding agents that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., J. Immunol. Meth. 62: 1-13, 1983). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., EMBO J. 5:15671575 (1986)). The matrix to which the affinity ligand

is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the bispecific binding agent comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE® chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the bispecific binding agent to be recovered.

The vector may include a signal sequence for polypeptide secretion. For example, pelB signal sequence (Lei, S. P. et al., Bacteriol. (1987) 169, 4397, hereby incorporated by reference in its entirety) may be used for production in periplasm of E. coli. The introduction of the vector into a host cell may be effected, for example, according to a calcium chloride method or an electroporation method. Other suitable methods to introduce an expression vector into a host cell are well known the art. Nucleic acid sequences can be validated by sequencing. Sequencing is carried out using standard techniques (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, and Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. USA 74: 5463-5467, which is incorporated herein by reference) and sequencing methods are well known to a person of ordinary skill in the art.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLE 1: Methods for generating Novel Bispecific Binding Agents

Plasmid Construction

To validate the bispecific binding agent provided herein, the heavy and light antibody chains of a first binding agent, the anti-IL-6Rα antibody tocilizumab (see, e.g., (US 8,562,991), were cloned into an expression vector with the amino acid sequence shown in SEQ ID NO: 9 (the nucleotide sequence encoding SEQ ID NO: 9 is shown in SEQ ID NO: 10). The heavy and light antibody chains of a second binding agent, the anti-IL-8RB antibody 10H2 (see, e.g.,

Chuntharapai A, Lee J, Hébert CA, Kim KJ. Monoclonal antibodies detect different distribution patterns of IL-8 receptor A and IL-8 receptor B on human peripheral blood leukocytes. J Immunol. 1994 Dec 15;153(12):5682–5688. PMID: 7527448) were determined via RACE, performed by Genscript (2 Frohman MA, Dush MK, Martin GR. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci U S A. 1988 Dec;85(23):8998–9002. PMCID: PMC282649) and were cloned into an expression vector with the amino acid sequence shown in SEQ ID NO: 11 (the nucleotide sequence encoding SEQ ID NO: 11 is shown in SEQ ID NO: 12). The sequences of the variable domains of the anti-IL-6Rα (tocilizumab) and anti-IL-8RB (10H2) antibodies were cloned into two human IgG1-based bispecific binding agent formats (FIGs. 2, 3) (SEQ ID NOs: 9, 11, 14, and 15, respectively).

The fully constructed bispecific binding agent comprising the tocilizumab and 10H2 antibody heavy and light chains is shown in FIG. 2, and is denoted as "BS1" in various places herein. The BS1 format combines a knobs-in-holes strategy with a single- chain Fab expression (FIG. 2). As described herein, the full BS1 antibody construction was facilitated by the knobs-in-holes dimerization strategy. Amino acid substitutions were introduced into the tocilizumab C_H3 domain at positions 645, 647, and 686 of SEQ ID NO: 9 creating cavities (holes) in the polypeptide interface. The tocilizumab C_H3 domain amino acid substitutions included serine, alanine, and valine substitutions at positions 645, 647, and 686 (T645S, L647A, and Y686V) (SEQ ID NO: 9). Further, amino acid substitutions were introduced into the 10H2 C_H3 domain at position 642 of SEQ ID NO: 11 creating a protuberance (knob) in the peptide interface. The 10H2 C_H3 domain amino acid substitution included a tryptophan substitution at position 642, (T642W) (SEQ ID NO: 11). A knobs-in-holes strategy enforces proper heterodimerization and single-chain Fab expression ensures appropriate pairing of the heavy and light chains from each antibody. Complementary sets of mutations were introduced into the third constant domains of the tocilizumab and 10H2 antibody heavy chains to favor heterodimerization over homodimerization, and a long flexible linker connecting the C-terminus of the constant light chain to the N-terminus of the variable heavy chain was used (FIG. 2).

For control and comparison purposes, a previously validated bispecific binding agent was also generated with tocilizumab and 10H2 antibodies (FIG. 3). This bispecific binding agent format is a validated scFv-IgG fusion that tethers a scFv specific for IL-6Rα (based on

tocilizumab) to a full IgG antibody specific for IL-8RB (based on the variable regions of 10H2), and is denoted "BS2" (FIG. 3). The scFv included the tocilizumab V_H and V_L domains connected by a flexible $(G4S)_3$ linker. The heavy and light chain DNA constructs (SEQ ID NOs: 14 and 15, respectively) were constructed as previously described (Orcutt KD, Ackerman ME, Cieslewicz M, Quiroz E, Slusarczyk AL, Frangioni JV, Wittrup KD. A modular IgG-scFv bispecific antibody topology. Protein Eng Des Sel. 2010 Apr 1;23(4):221–228). The heavy and light chain DNA construct were co-transfected to produce the BS2 bispecific antibody. Again, small scale co-transfections were used to optimize the DNA plasmid ratio for large-scale expression. Additionally, the full tocilizumab and 10H2 monoclonal hIgG1 antibodies were also expressed via co-transfection of their respective heavy and light chains as controls according to the methods provided herein.

Protein Expression and Purification

HEK 293F cells were cultivated in Freestyle 293 Expression Medium (Thermo Fisher Scientific) supplemented with 2 U/mL penicillin-streptomycin (Gibco). Mammalian expression plasmids (gWiz backbone) encoding the tocilizumab and 10H2 antibody domain fusions (BS1), which contained complementary heavy chain constant domain 3 (C_H3) mutations, were co-transfected into HEK 293F cells for soluble expression. Similarly, BS2 was co-transfected for soluble expression to produce the bispecific antibody. Polyethylenimene was used as a transfection reagent (Spangler JB, Manzari MT, Rosalia EK, Chen TF, Wittrup KD. Triepitopic Antibody Fusions Inhibit Cetuximab-Resistant BRAF and KRAS Mutant Tumors via EGFR Signal Repression. J Mol Biol. 2012 Sep 28;422(4):532–544). The tocilizumab and 10H2 fusion DNA plasmids were titrated using a small-scale expression assay to determine the optimal ratio for large-scale transfections.

Both bispecific binding agent formats, BS1 and BS2, were expressed with anti- IL-6Rα (tocilizumab) and anti-IL-8RB (10H2) variable domains. BS1 and BS2 were purified from transfected HEK 293F cell supernatants via protein G chromatography followed by size exclusion chromatography using a Superdex 200 column on a fast protein liquid chromatography (FPLC) system (GE Healthcare).

The human IL-6Rα extracellular domain (ECD), residues 89-303 of the mature protein, was cloned into the gWiz mammalian expression plasmid with a C-terminal biotin acceptor

peptide (BAP)-LNDIFEAQKIEWHE and a C-terminalhexahistidine sequence. Transient expression in HEK 293F cells was achieved using polyethylenimene as a transfection reagent (Spangler JB, Manzari MT, Rosalia EK, Chen TF, Wittrup KD. Triepitopic Antibody Fusions Inhibit Cetuximab-Resistant BRAF and KRAS Mutant Tumors via EGFR Signal Repression. J Mol Biol. 2012 Sep 28;422(4):532–544). IL-6Rα was extracted from transfected HEK 293 cell supernatants via nickel-nitrilotriacetic acid (Ni-NTA) chromatography and biotinylated using the soluble BirA ligase enzyme in 0.5 mM Bicine pH 8.3, 100 mM ATP, 100 mM magnesium acetate, and 500 mM biotin (Sigma). Biotinylated IL-6Rα ECD was further purified by size exclusion chromatography using a Superdex 200 column on an FPLC instrument (GE Healthcare). Purity (>99%) was confirmed via SDS-PAGE analysis. All proteins were stored in HEPES-buffered saline (HBS, 150 mM NaCl in 10 mM HEPES pH 7.3)

Bio-layer interferometry binding studies

To demonstrate functionality of the BS1 and BS2 bispecific binding agent formats, binding of these agents to human IL-6Rα ECD was quantified via bio-layer interferometry (BLI). Biotinylated human IL-6Rα ECD was immobilized to streptavidin-coated tips for analysis on an Octet® Red96 BLI instrument (ForteBio). Less than 5 signal units (nm) of receptor was immobilized to minimize mass transfer effects. Tips were exposed to serial dilutions of the anti-IL-6R antibody tocilizumab, the anti-IL-8R antibody 10H2, BS1, or BS2 in a 96-well plate for 300 s and dissociation was measured for 600 s. Surface regeneration for all interactions was conducted using 15 s exposure to 0.1 M glycine pH 3.0. Experiments were carried out in PBSA (phosphate-buffered saline [PBS] pH 7.3 plus 0.1% bovine serum albumin [BSA, Thermo]) at 25°C. Data was visualized and processed using the Octet® Data Analysis software version 7.1 (ForteBio). Equilibrium titration curve fitting and equilibrium dissociation constant (K_D) value determination was implemented in GraphPad Prism software using a first-order logistic model. Experiments were reproduced two times with similar results.

Yeast cell surface affinity titrations

Human IL-6 (residues 3-185) was cloned into the pCT302 vector and presented on the surface of yeast, as described previously. (*See* Boder ET, Wittrup KD. Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol. 1997 Jun;15(6):553–557. PMID:

9181578). Yeast displaying human IL-6 were incubated in PBSA containing serial dilutions of recombinant IL-6Rα ECD for 2 hours at room temperature. Cells were then washed and stained with a 1:200 dilution of Alexa647- conjugated streptavidin (Thermo) in PBSA for 15 min at 4°C. After a final wash, cells were analyzed for antibody binding using a CytoFLEX flow cytometer (Beckman Coulter). Background-subtracted and normalized binding curves were fitted to a first-order logistic model and K_D values were determined using GraphPad Prism.

Yeast cell surface competition studies

Approximately $1x10^5$ human IL-6-displaying yeast per well were plated in a 96- well plate and washed with PBSA. Yeast cells were incubated with saturating concentrations of biotinylated human IL-6Rα (300 nM) and serial dilutions of unlabeled competitor antibody (either the anti-IL-6R antibody tocilizumab, the anti-IL-8R antibody 10H2, BS1, or BS2) in PBSA for 2 hours at room temperature. Cells were then washed and stained with a 1:200 dilution of Alexa647-conjugated streptavidin (Thermo) in PBSA for 15 min at 4°C. Cells were washed again and assessed for human IL-6Rα binding on a CytoFLEX flow cytometer (Beckman Coulter). Background-subtracted fluorescent signal as a fraction of receptor subunit binding in the absence of competitor antibody was plotted. Curves were fitted to a first order logistic model and half maximal inhibitory concentrations (IC_{50}s) were computed using GraphPad Prism. Assays were performed in triplicate and repeated two times with consistent results.

Generation of IL-6R- and IL-8R-expressing cell lines

The IL-6Rα and IL-8RB genes were cloned into a lentiviral expression plasmid, and viruses were prepared following manufacturer instructions (pPACKH1 HIV Lentivector Packaging Kit, cat# LV500A-1, System Bioscience). Briefly, $3x10^6$ HEK 293T cells were plated on 10 cm dishes and cultured in Iscove's Modified Dulbecco's Media (IMDM, Thermo Fisher) supplemented with 10% FBS (Hyclone), 2mM L-glutamine and 100 U/mL penicillin-streptomycin (Gibco) overnight. 2 μg of lentivirus-transducing plasmids (pCDH backbone) encoding the IL-6Rα or IL-8R was used to transfect HEK 293T cells with pPACK packaging plasmid mix. GeneJuice (Sigma Aldrich) was used as the transfection reagent. IL-6R and IL-8R lentivirus were collected from media after two days and were filtered through 0.45 μm filters. Approximately 0.1 $x10^6$ HEK 293T cells cultured in a 24-well plate were transduced with IL-6R

or IL-8R or the combination of the IL-6R and IL-8R lentiviruses with 8 µg/mL of polybrene (Sigma Aldrich) in 500 µl of IMDM. Immediately after transduction, HEK 293T cells were centrifuged at 800xg for 30min at 32°C and incubated overnight at 37°C in a humidified 5% CO_2 incubator. The culture media was replaced with fresh complete IMDM culture media on the day after transduction and transduced cells were harvested for testing IL-6R and IL-8R expression via flow cytometry 10 days after transduction.

IL-6R and IL-8R cell surface binding assays

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% (Hyclone), 2mM L-glutamine and 100 U/mL penicillin-streptomycin (Gibco). For surface binding assays, IL-6Rα^+/IL-8R$^-$, IL-6Rα^-/IL-8R$^+$, IL-6Rα^+/IL-8R$^+$, and IL-6Rα^-/IL-8R$^+$ HEK 293T cells were trypsinized for detachment, resuspended in PBSA, and then aliquoted into 96-well plates ($1x10^5$ cells per well). Cells were incubated with titrations of various monoclonal or bispecific antibodies for 2 hr at 4°C with rotation. Cells were then washed and incubated with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-human IgG1 antibody (Sigma-Aldrich, Clone 8c/6-39) in PBSA for 15 min at 4°C. After a final wash, cells were resuspended in PBSA and analyzed on a CytoFLEX flow cytometer (Beckman Coulter). Binding isotherms were fitted to a first-order logistic model and K_D values were calculated using GraphPad Prism data analysis software. Mean fluorescence intensity (MFI) of unstimulated cells was subtracted. Experiments were conducted in triplicate and performed twice with similar results.

IL-6 and IL-8 cell surface binding inhibition assays

IL-6Rα^+/IL-8R$^-$ and IL-6Rα^-/IL-8R$^+$ HEK 293T cells were cultured as previously described, and were trypsinized, resuspended in PBSA, and aliquoted into 96-well plates ($2x10^5$ cells per well). IL-6Rα^+/IL-8R$^-$ 293T cells were incubated with titrations of various monoclonal or bispecific antibodies in the presence of saturating concentration of biotinlyated IL-6 (100 nM) (Acro Biosystem, cat#: IL6-H8218-25UG) for 2 hr at 4°C with rotation. Cells were then washed and incubated with 1:200 dilution of Alexa Fluor 647-conjugated streptavidin (Fisher Scientific, cat#: S21374) in PBSA for 15 min at 4°C. After a final wash, cells were resuspended in PBSA and analyzed on a CytoFLEX flow cytometer (Beckman Coulter). Curves were fitted to a first-

order logistic model and IC_{50} values were calculated using GraphPad Prism data analysis software. Mean fluorescence intensity (MFI) of unstimulated cells was subtracted. Similarly, IL-6Rα^-/IL-8R$^+$ 293T cells were incubated with titrations of various monoclonal or bispecific antibodies with saturating concentration of His-tagged IL-8 (400 nM) (Sino Biological, cat#: 10098-H08Y-100) for 2 hr at 4°C with rotation. The cells were then washed and incubated with a 1:50 dilution of Alexa Fluor 647-conjugated anti-penta His antibody (Qiagen, cat#: 35370) in PBSA for 15 min at 4°C. After a final wash, cells were resuspended in PBSA and analyzed on a CytoFLEX flow cytometer (Beckman Coulter). Curves were fitted to a first-order logistic model and IC_{50} values were calculated using GraphPad Prism data analysis software. Experiments were conducted in triplicate and performed twice with similar results.

EXAMPLE 2: Methods for Testing Generated Bispecific Binding Agents

IL-6 signaling inhibition assay

HepG2 cells were cultured in Minimum Essential Medium (MEM, Thermo Fisher) supplemented with 10% FBS (Hyclone), 2mM L-glutamine, 100 U/mL penicillin- streptomycin (Gibco). For the signaling inhibition assay, HepG2 cells were trypsinized for detachment, resuspended in PBSA and then aliquoted into 96-well plates ($2x10^5$ cells per well). Cells were incubated with titrations of various monoclonal or bispecific antibodies with saturating concentration of IL-6 (10 nM) (R&D Systems, cat#:206-IL-010) for 20 min at 37°C with rotation. HepG2 cells were then fixed with 1.6% PFA, permeabilized with methanol and incubated with 1:50 dilution of Alexa Fluor 647 conjugated anti-pSTAT3 antibody (BD Biosciences, clone 4/P-STAT3) in PBSA for 2h at room temperature. After two washes, cells were resuspended in PBSA and analyzed on a CytoFLEX flow cytometer (Beckman Coulter). IC_{50} values were calculated using a first order logistic fitting models in GraphPad Prism data analysis software. Mean fluorescence intensity (MFI) of unstimulated cells was subtracted. Experiments were conducted in triplicate and performed twice times with similar results.

3-Dimensional Migration Assays

MDA-MB-231 human triple negative breast cancer cells (ATCC) and HT-1080 human fibrosarcoma cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Corning), with

high glucose (4.5 g/L), L-glutamine & sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) (Corning). MDA-MB-231 medium contained 1% Pen/Strep (Gibco), and HT-1080 medium contained .05 mg/ml of Gentamycin (VWR). Cells were maintained at 37 °C with 5% CO_2. Cells were incubated with trypsin-EDTA (Sigma) for less than 5 minutes for detachment from the dish. Cells were diluted in culture medium and pelleted. Cells were resuspended in fresh medium and counted using TrypanBlue (Invitrogen) to exclude dead cells. The 3D migration matrix was then prepared by diluting rat tail high concentration collagen type 1 (Corning) to 2 mg/ml concentration using an equal volumetric ratio of ice-cold cell medium and buffering agents HEPES (Acros Organics) & sodium bicarbonate (Gibco). Cells were added to the collagen for a final concentration of 100 cells/ul, and then the solution was neutralized using NaOH (EMD Millipore). The soluble collagen solution was plated in a cell-culture treated polystyrene 24-well plate (Falcon) on a heat block set to 37°C and allowed to partially set for 5 minutes. The plate was then placed in a 37 °C with 5% CO_2 incubator for an hour until the gel is fully set, then additional medium is added. Cells were allowed to incubate in the gels until accustomed to the new environment, which required 48 hours while for MDA-MB-231 cells and 24 hours for HT-1080 cells. The treatment conditions included a negative control containing only fresh medium, tocilizumab (Genentech) plus reparixin (MedChem Express) (T+R), recombinant tocilizumab plus recombinant 10H2 antibodies (anti-IL-6R+anti-IL-8R), or a bispecific antibody construct (BS1 or BS2). The doses used were 150 nM for commercial tocilizumab, recombinant tocilizumab, BS1, and BS2, with reparixin and 10H2 added in a 1:1 (w/w) ratio with the tocilizumab for the two combination treatments.

Phase contrast images of the single cell collagen matrices were taken with a 10x objective every 10 minutes for 16 h using an ORCA-ER digital camera (Hamamatsu) mounted on a Nikon TE2000 microscope. At least 50 cells were tracked for each condition in each experiment with a minimum of 3 independent biological repeats per condition. Cells were tracked using Metamorph (Molecular Devices), and the x- and y-coordinates were used to calculate the mean squared displacement (MSD). Anisotropic persistent random walk model (APRW), a custom model designed for analyzing 3-dimensional migration, was run using MATLAB to process the x,y coordinates and generate additional information, such as the diffusivity and persistence of individual cells.

3-Dimensional Proliferation Assays

MDA-MB-231 and HT-1080 cells were cultured and counted as described previously. The 3D soluble collagen was prepared in the same manner as for the migration studies. The single cell suspensions were added to a cell culture-treated polystyrene 96-well plate (Falcon) on a heat block set to 37°C and allowed to partially set for 5 minutes. The plate was then placed in a 37 °C with 5% CO_2 incubator for an hour until the gel was fully set. Additional medium was then added. The treatment conditions included a negative control containing only fresh medium, tocilizumab (Genentech) plus reparixin (MedChem Express) (T+R), recombinant tocilizumab plus recombinant 10H2 antibodies (anti-IL-6R+anti-IL-8R), or a bispecific antibody construct (BS1 or BS2). The doses used were 150 nM for commercial tocilizumab, recombinant tocilizumab, BS1, and BS2, with reparixin and 10H2 added in a 1:1 (w/w) ratio with the tocilizumab for the two combination treatments. Treatments were added 24 hours after gels were set. Approximately 45 hours post-treatment, an equal volume of 2x PrestoBlue (Invitrogen) was added to each well in addition to several wells of fresh medium for background readings and incubation proceeded at 37°C for 3 hours to allow for complete dispersion of the dye. 100 ul of media from each well was then transferred to a cell culture-treated polystyrene black bottomed 96-well plate (Costar). Absorbance was measured at 570 nm, with 600 nm reference wavelength using a SpectraMax M3 Multi-Mode Mircoplate Reader (Molecular Devices). The average of the background well absorbance values were then subtracted from the experimental values and all conditions were normalized to the control condition. At least 4 technical repeats were performed per condition per plate, with a minimum of 3 independent biological repeats per condition.

Mouse orthotopic breast cancer tumor xenograft models

All procedures conducted were approved through the Johns Hopkins University Animal Care and Use Committee, in accordance with the NIH Guide for the Care and Use of Laboratory Animals. For all studies, 5-7 week old female NOD scid gamma (NSG) mice were obtained through an internal core facility at the Johns Hopkins Medical Institution and were maintained in housing with a 12-hr dark/light cycle. MDA-MB-231 triple negative breast cancer cells confirmed to be mycoplasma free and then expanded. After trypsinization and neutralization with FBS-containing medium, the cells were washed twice with Dulbecco's PBS (DPBS) (Gibco) and then

resuspended in a 1:1 mixture of ice-cold DPBS and Matrigel (Corning). Syringes prepped with $1x10^6$ MDA-MB-231 cells in 100 ul of the 1:1 solution were used to introduce tumors into the mammary fat pad. Mice were monitored but untouched for a week to allow the cell pellets time to establish solid tumors. Tumor sizes were calculated using two measurements taken by calipers, the first measurement taken of the longest dimension and the second measurement taken perpendicular to the first. The volume was then estimated either as a sphere (if the two measurements were only a millimeter or less apart) or as an ellipsoid. For all pilots and for the full-length study, mice receiving treatment were injected intraperitoneally every 3 days, starting 10 days after the cell injection. All mice were weighed and tumors were measured on the same schedule as the treatments. Each group in the 3 pilot studies included only 1 mouse. For the first pilot study, the mice were not given any treatment as the goal was to determine the earliest timepoint at which micro-metastases were detectable. A healthy control mouse did not receive an injection of MDA-MB-231 cells and the other 4 mice received tumors. Various timepoints were selected as endpoints, and mice were sacrificed to measure metastatic burden in the lung via quantitative PCR. Timepoints greater than 35 days was determined to be ideal for evaluation of lung metastases. For the second pilot study, a dose titration of BS1 was used to determine the effective range. All mice were sacrificed at a single endpoint for metastatic burden analysis. For the third pilot study, another dose titration of BS1 was conducted to verify the results in the second pilot study. All mice were sacrificed at a single endpoint for analysis of metastatic burden. Based on these results, a dose of 1 mg/kg of the bispecific antibodies was chosen for the full study (containing appropriately sized cohorts to achieve statistical significance). The dose of the individual monoclonal antibodies was also set to 1 mg/kg each, whereas tocilizumab and reparixin were used at the dose shown to be effective previously, 30 mg/kg Jayatilaka H, Tyle P, Chen JJ, Kwak M, Ju J, Kim HJ, Lee JSH, Wu P-H, Gilkes DM, Fan R, Wirtz D. Synergistic IL-6 and IL-8 paracrine signalling pathway infers a strategy to inhibit tumour cell migration. Nat Commun. 2017 26;8:15584. PMCID: PMC5458548).

For the full study, one week after the injection of MDA-MB-231 cells, mice were weighed and tumor size measured, before being randomly sorted into 5 groups of 5 mice each representing the following conditions: PBS (control), tocilizumab (Genentech) plus reparixin (MedChem Express) (T+R), recombinant tocilizumab plus recombinant 10H2 antibodies (anti-IL-6R+anti-IL-8R), or a bispecific antibody construct (BS1 or BS2). Mice were treated for 3.5 weeks. Tumors

and lungs were extracted for testing. The lungs were inflated with 2% agarose (Boston BioProducts), with one lobe of lung preserved in 10% formalin (VWR) sent to an internal core at Johns Hopkins Medical Institute for sectioning and H&E staining. The remaining lung tissue was flash frozen with liquid nitrogen and stored at -80°C.

Primer	SEQ ID NO:	Forward	SEQ ID NO:	Reverse
HK2 Human	38	CCAGTTCATTCACATCATCAG	39	CTTACACGAGGTCACATAGC
α-Tubulin Human	29	AGGAGTCCAGATCGGCAATG	40	GTCCCCACCACCAATGGTTT
α-Tubulin Mouse	30	CACACAAGCTCACTCACCCT	41	CTGTTATTAGGGATGTGACTCCA
β-Actin Human	31	CATGTACGTTGCTATCCAGGC	42	CTCCTTAATGTCACGCACGAT
β-Actin Mouse	32	ATGAGCTGCCTGACGGCCAGGTCATC	43	TGGTACCACCAGACAGCACTGTGTTG
GAPDH Human	33	GCACCGTCAAGGCTGAGAAC	44	GCCTTCTCCATGGTGGTGAA
GAPDH Mouse	34	ACCACAGTCCATGCCATCAC	45	CACCACCCTGTTGCTGTAGCC
RPL13A Human	35	AGCCTCATCTGCAATGTAGGG	46	TCAGACTCCTCGGATTCTTCTTT
RPL13A Mouse	35	AGGGGTTGGTATTCATCCGC	47	ATGCCTGCTGAGGCTTTGTT
18s Human	37	GAGGATGAGGTGGAACGTGT	48	AGAAGTGACGCAGCCCTCTA
18s Mouse	38	CGGCGACGACCCATTCGAAC	49	GAATCGAACCCTGATTCCCCGT

Assessment of metastatic burden to the lung

A portion of the dissected lung tissue from each mouse (~20 mg) was digested to extract DNA using PureLink Genomic DNA Mini Kit (Invitrogen). qPCR was performed utilizing iTaq Univer SYBR Green Supermix (Bio-Rad) and primers synthesized by IDT. Human HK2 was used to determine the relative number of human cells that had reached the secondary organs, while the remaining primers were used as reference genes to correct for overall DNA content, with a minimum of 4 reference genes used in each analysis. For the 3 pilot studies, a minimum of 2 independent qPCR runs with 3 technical repeats was conducted for each sample, while the results for the subsequent study represent the average of three independent qPCR runs containing 3 technical repeats.

EXAMPLE 3: Expression and Biophysical Validation of Bispecific Binding Agents

Bispecific binding agents were successfully expressed and purified from mammalian cells

BS1 and BS2 were expressed and purified to >99% homogeneity from HEK 293F cells via transient transfection. Representative FPLC traces and SDS-PAGE analyses are shown in FIG. 4. Both bispecific binding agents appeared as distinct, monodisperse peaks by FPLC analysis with minimal aggregation, and migrated at the expected molecular weights in non-reducing and reducing SDS-PAGE analyses.

Bispecific binding agents bind to IL-6Rα

To confirm functionality of our bispecific agents, antibodies were titrated against recombinant IL-6Rα via BLI. The anti-IL-6Rα antibody tocilizumab bound its target receptor with an apparent bivalent affinity of 28 nM, whereas the anti-IL-8RB antibody 10H2 did not engage IL-6Rα. Moreover, both tocilizumab-containing engineered bispecific binding agents also bound IL-6Rα (FIG. 5). As expected, BS1 had a weaker binding affinity (K_D=120 nM) compared with tocilizumab due to its monovalent interaction with IL-6Rα. In addition, BS2 had an intermediate binding affinity, since it engages IL-8R bivalently, but the engagement topology differs from that of a conventional monoclonal IgG, as the two scFv moieties are fused to the C-terminus of the light chain constant domain (FIGs. 2 and 3).

Bispecific binding agents block IL-6Rα binding to the IL-6 cytokine

Tocilizumab is known to compete with IL-6 for IL-6Rα engagement. Thus, bispecific binding agents containing the tocilizumab variable regions (BS1 and BS2) were also expected to obstruct cytokine binding. Human IL-6 was displayed on the surface of yeast (*See* Boder ET, Wittrup KD. Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol. 1997 Jun;15(6):553–557. PMID: 9181578) and the K_D of soluble IL-6Rα ECD was determined to be 88 nM (FIG. 6A). To demonstrate IL-6 inhibition, IL-6-displaying yeast were incubated with a fixed saturating concentration of biotinylated IL-6Rα ECD (300 nM), and titrations of antibody competitor, (either the anti-IL-6R antibody tocilizumab, the anti-IL-8R antibody 10H2, BS1, or BS2) were added to assess disruption of the IL-6/IL-6Rα interaction

(FIG. 6B). 10H2 did not compete with the IL-6/IL-6Rα interaction. However, tocilizumab (IC_{50}=59 nM), BS1 (IC_{50}=190 nM), and BS2 (IC_{50}=78 nM) all inhibited IL-6/IL-6Rα binding. Notably, BS1 was found to be less competitive than tocilizumab due to its monovalent engagement of IL-6Rα, and BS2 was found to be less efficient at blocking the IL-6/IL-6Rα interaction than tocilizumab, but more efficient than BS1, since it binds bivalently to IL-6Rα, but in a different topology compared to a conventional monoclonal antibody (FIG. 3).

Bispecific antibodies specifically bind to IL-6Rα and IL-8R on 293T cells

To determine whether engineered bispecific binding agents BS1 and BS2 specifically engage target antigens in a physiologically relevant context, antibody binding to IL-6Rα$^+$/IL-8R$^-$, IL-6Rα$^-$/IL-8R$^+$, IL-6Rα$^+$/IL-8R$^+$, and IL-6Rα$^-$/IL-8R$^-$ lentivirally transduced HEK 293T cells was measured via flow cytometry. BS1 and BS2 bound to both IL-6Rα (FIG. 7A, K_D=24 and 19 nM, respectively) and IL-8R (FIG. 7B, K_D=10 and 3.5 nM, respectively). In contrast, the constituent anti-IL-6R monoclonal antibody tocilizumab only recognized IL-6Rα$^+$ cells and the constituent anti-IL-8R monoclonal antibody 10H2 only recognized IL-8R$^+$ cells. None of the antibodies bound to IL-6Rα$^-$/IL-8R$^-$ 293T cells, demonstrating target specificity. BS2 bound to IL-6Rα$^+$/IL-8R$^+$ 293T cells with similar affinity (K_D=3.4 nM) compared to tocilizumab (K_D=3.1 nM) and 10H2 (K_D=4.1 nM), whereas BS1 bound to IL-6Rα$^+$/IL-8R$^+$ 293T cells with weaker affinity than BS2 (K_D=14 nM) due to its monovalent engagement of each target (FIG. 7C).

Bispecific binding agents block IL-6/IL-6Rα and IL-8/IL-8R interactions

After confirmed the dual targeting of both bispecific binding agents (BS1 and BS2), the competitive binding properties of these antibodies were also characterized on IL-6Rα- or IL-8R-expressing HEK 293T cells. Both BS1 and BS2 competed with IL-6 binding to IL-6Rα on IL-6Rα$^+$/IL-8R$^-$ 293T cells (IC_{50}=120 nM and 96 nM, respectively) (FIG. 8A). The constituent anti-IL-6R monoclonal antibody tocilizumab also inhibited IL-6 binding (IC_{50}=30 nM), whereas 10H2 had no inhibitory effects. Aligning with trends observed in the binding assay, tocilizumab was a more potent competitor compared to bispecific antibodies due to its conventional bivalent format. BS2 was slightly more potent than BS1 due to its bivalency, albeit in an alternate topology compared to standard antibody construction. Similarly, both BS1 and BS2 competed

with IL-8 binding to IL-8R on IL-6Rα$^-$/IL-8R$^+$ HEK 293T cells (IC_{50}=99 nM and 22 nM, respectively) (FIG. 8B). The constituent anti-IL-8R monoclonal antibody 10H2 also inhibited IL-8 binding (IC_{50}=22 nM), whereas tocilizumab did not exert any inhibitory effects. BS2 and 10H2 inhibited with equal potency since they both comprise the full IL-8R-targeting hIgG antibody. BS1 exhibited weaker potency of inhibition compared to BS2 and 10H2 due to its monovalency.

EXAMPLE 4: Functional Validation of Bispecific Binding Agents

Bispecific antibodies inhibit STAT3 signaling in HepG2 cells

To demonstrate that inhibition of the IL-6/IL-6R interactions corresponds with inhibition of IL-6-mediated signaling, we performed competitive signaling studies on IL-6-responsive HepG2 cells. Both BS1 and BS2 inhibited STAT3 phosphorylation (a downstream signaling event following IL-6 stimulation) in HepG2 cells (IC_{50}=110 nM and 220 nM, respectively) (FIG. 9). The anti-IL-6R antibody tocilizumab also inhibited IL-6 signaling (IC_{50}=76 nM), whereas the anti-IL-8R antibody 10H2 did not effect inhibition. As observed for binding inhibition assays, tocilizumab inhibited signaling more potently than bispecific antibodies due to its standard bivalent antibody format.

Bispecific antibodies inhibit cancer cell migration more effectively than monoclonal antibody and antibody/small molecule combination treatments

In solid tumor cancers, metastases are formed by cancer cells that travel from the primary tumor through the extracellular matrix (ECM), sometimes traversing blood or lymphatic vessels, to establish a secondary site of disease. For *in vitro* migration studies, collagen (type 1) gels were used as a surrogate for the ECM, in which cancer cells (MDA-MB-231 or HT-1080) were dispersed into single cell suspensions for accurate tracking of their movement over time. BS1 and BS2 were both able to reduce cancer cell migration as effectively as tocilizumab plus reparixin (T+R) combination treatment (FIG. 10A,B), and more effectively than tocilizumab plus 10H2 (anti-IL-6R+anti-IL-8R) combination treatment. These results were quantitatively assessed by processing the x- and y- coordinates of each cell to get the mean squared displacement (MSD), as well as the diffusivity and persistence using the anisotropic persistent random walk (APRW) model (FIG. 11A-F). BS1 and BS2 significantly reduced the mean squared

displacement of MDA-MB-231 and HT-1080 cells ($P < 0.0001$). The reduction in MSD seen with BS2 was significantly less than that induced by the T+R and BS1 conditions in both MDA-MB-231 cells ($P = 0.0005$ and $P = 0.0003$, respectively) and HT-1080 cells ($P = 0.0093$ and $P = 0.0015$, respectively). Similarly, BS1 and BS2 significantly reduced the diffusivity of MDA-MB-231 and HT-1080 cells ($P < 0.0001$). The reduction in diffusivity induced by BS2 (although not BS1) compared to T+R was significant in both MDA-MB-231 cells ($P = 0.0087$) and HT-1080 cells ($P = 0.0069$). Notably, the effect of BS2 was greater than that of BS1 ($P = 0.0055$) in HT-1080 although not MDA-MB-231 cells. BS1 and BS2 also reduced the persistence of cell migration, with lower persistence correlating to a more circular trajectory and high persistence corresponding to a more linear trajectory. For MDA-MB-231 cells, BS1 reduced persistence by 67% ($P < 0.0001$) and BS2 by 61% ($P = 0.0002$) compared to untreated control cells. The persistence of HT-1080 cells was reduced 31% by BS1 ($P = 0.0037$), and 53% by BS2 ($P < 0.0001$) compared to untreated control cells. The superior anti-migratory effects of BS2 compared to BS1 may be due to its higher valency (BS2 is tetravalent whereas BS1 is bivalent), which results in a higher apparent affinity.

Cancer cell proliferation unaffected by treatment with bispecific antibodies

The overwhelming majority of anti-cancer treatments shown to have anti-metastatic potential also impact cell proliferation. One unique feature of bispecific binding agents targeting IL-6R and IL-8R is that at the dose required to inhibit cell migration there is no effect on cell proliferation (FIG. 12A,B) compared to control cells. Consistent with this finding, tocilizumab plus reparixin (T+R) and tocilizumab+10H2 (anti-IL-6R+anti-IL-8R) combination treatments did not influence cell proliferation.

In vivo pilots reveal the optimal timeline and dose for orthotopic breast tumor xenograft models in mice

To evaluate the therapeutic potential for bispecific binding agents, we interrogated its capacity to inhibit metastasis in an orthotopic breast tumor xenograft model in mice. The first *in vivo* pilot study was designed to determine the optimal timepoint for detection of micro-metastases in the lung in our orthotopic triple negative breast cancer model. Mice were inoculated with tumor cells and sacrificed at 4 different time points for evaluation of metastatic

burden in the lung. One mouse was not injected with cancer cells and that was used as a healthy control. The standard comparison analysis used for qPCR results exaggerated the data due to the large difference in cycle threshold seen for each sample. As such, the raw cycle threshold values are shown with the higher values representing smaller representation of the gene (FIG. 13A). Based on this initial pilot, a study length of greater than 35 days was determined to be optimal for metastatic burden quantification. Since BS2 performed as well or better than BS1 in cell migration inhibition studies, BS1 was used for the pilot dose titration studies. Past *in vivo* studies with commercial tocilizumab plus reparixin (T+R) had been conducted using a 30 mg/kg does of the antibody (Jayatilaka H, Tyle P, Chen JJ, Kwak M, Ju J, Kim HJ, Lee JSH, Wu P-H, Gilkes DM, Fan R, Wirtz D. Synergistic IL-6 and IL-8 paracrine signalling pathway infers a strategy to inhibit tumour cell migration. Nat Commun. 2017 26;8:15584. PMCID: PMC5458548). Thus the second pilot included 4 doses with a maximum of 30 mg/kg as well a PBS-treated control mouse. Lung tissue from a non-tumor bearing mouse was used as a healthy control. The results from the second pilot study showed that BS1 effectively inhibited metastasis at doses as low as 1 mg/kg. (FIG. 13B). To finalize the dose, a third pilot study was conducted focused on lower doses. These results showed strong agreement with the second pilot, and revealed that the bispecific was effective in blocking metastasis at a dose as low as 0.1 mg/kg (FIG. 13C), 300-fold lower than the effective dose observed for T+R combination treatment (Jayatilaka H, Tyle P, Chen JJ, Kwak M, Ju J, Kim HJ, Lee JSH, Wu P-H, Gilkes DM, Fan R, Wirtz D. Synergistic IL-6 and IL-8 paracrine signalling pathway infers a strategy to inhibit tumour cell migration. Nat Commun. 2017 26;8:15584. PMCID: PMC5458548). (FIG. 13C). Since the 1 mg/kg dose of BS1 was extremely effective in both pilots, that dose was selected for both bispecific binding agents in the full study (with appropriately sized groups to achieve statistical significance).

Bispecific antibodies inhibit metastasis in an orthotopic breast tumor xenograft model in mice more effectively than monoclonal antibody and antibody/small molecule combination treatments

Guided by pilot studies, a full orthotopic MDA-MB-231 triple negative breast cancer orthotopic xenograft model in mice was designed. Treatment groups (n=5 mice per cohort) included PBS, tocilizumab plus reparixin (T+R), tocilizumab+10H2 (anti-IL-6R+anti-IL-8R), BS1, and BS2. Tumor growth was monitored for 5 weeks, and showed similar trends in all groups (FIG. 14). On day 35, the study was terminated and the tumors and lungs were excised.

As expected, no difference was seen in the final tumor weight between any of the cohorts (FIG. 15), corroborating *in vitro* findings that none of the treatments impacted proliferation. With respect to metastatic burden in the lungs, bispecific antibodies performed better than T+R combination treatment and anti-IL-6R+anti-IL-8R combination treatment, and both BS1 and BS2 reduced tumor cell DNA in the lungs by nearly 50% (FIG. 16) compared to PBS-treated control mice. This promising finding was confirmed by H&E staining of mouse lung tissue (FIG. 17).

SEQ ID NO: 1

Exemplary Linker
GGGS

SEQ ID NO: 2

Exemplary Linker
GGGGS

SEQ ID NO: 3

Exemplary Linker
GGSG

SEQ ID NO: 4

Exemplary Linker
GSAAAGGSGGSGGS

SEQ ID NO: 5

Exemplary Linker

GGGSGGGS

SEQ ID NO: 6

Human IgG1 CH_1, CH_2, and CH_3 Domains, Wild-Type
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG

PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEGLHNHYTQKSLSLSPGK

SEQ ID NO: 7

Human IgG1 CH_1, CH_2, and CH_3 Domains with "Holes" Substitutions (Exemplary holes substitutions at positions 249, 251, and 290 are bolded and underlined)

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
EEMTKNQVSL**S**C**A**VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDK
SRWQQGNVFSCSVMHEGLHNHYTQKSLSLSPGK

SEQ ID NO: 8

Human IgG1 CH_1, CH_2, and CH_3 Domains with "Knobs" Substitution (Exemplary knob substitution at position 249 is bolded and underlined)

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
EEMTKNQVSL**W**CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
KSRWQQGNVFSCSVMHEGLHNHYTQKSLSLSPGK

SEQ ID NO: 9

BS1 Antibody Chain 1 with Tociliuzmab Light and Heavy Chains and Human IgG1 CH_1, CH_2, and CH_3 Domains with "Holes" Substitutions

Relevant domains are shown below (described in an N- to C-terminal direction)

- Signal Sequence is shown in dark shading
- Tocilizumab light chain variable region is underlined
- Tocilizumab light chain constant region is italicized
- The linker region is shown in light shading
- Tocilizumab heavy chain variable region is in bold text
- Exemplary holes substitutions at positions 645, 647, and 686 are in bold and underlined text

MRVPAQLLGLLLLWLPGARCAGSDIQMTQSPSSLSASVGDRVTITCRASQDISSYLNWY
QQKPGKAPKLLIYYTSRLHSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQGNTLPYT
FGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS*

*QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*GGSGGSSG
SGSGSTGTSSSGTGTSAGTTGTSASTSGSGSG**EVQLQESGPGLVRPSQTLSLTCTVSGY**
SITSDHAWSWVRQPPGRGLEWIGYISYSGITTYNPSLKSRVTMLRDTSKNQFSLRLS
SVTAADTAVYYCARSLARTTAMDYWGQGSLVTVSSASTKGPSVFPLAPSSKSTSGGT
AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI
CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEV
TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**S**C**A**VKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFL**V**SKLTVDKSRWQQGNVFSCSVMHEGLH
NHYTQKSLSLSPGK

SEQ ID NO: 10

Nucleic Acid Sequence Encoding BS1 Antibody Chain 1 with Tocilizumab Light and Heavy Chains and Human IgG1 CH1, CH2, and CH3 Domains with "Holes" Substitutions

atgagggtccccgctcagctcctggggctcctgctgctctggctcccaggtgcacgatgtgccggatccgacatccagatgacccagagc
cccagcagcctgagcgccagcgtgggcgaccgcgtgaccatcacctgccgcgccagccaggacatcagcagctacctgaactggtacc
agcagaagcccggcaaggcccccaagctgctgatctactacaccagccgcctgcacagcggcgtgcccagccgcttcagcggcagcgg
cagcggcaccgacttcaccttcaccatcagcagcctgcagcccgaggacatcgccacctactactgccagcagggcaacaccctgcccta
caccttcggccagggcaccaaggtggagatcaagcgtacggtggctgcaccatctgtcttcatcttcccgccatctgatgagcagttgaaatc
tggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaagtacagtggaaggtggataacgccctccaat
cgggtaactcccaggagagtgtcacagagcaggacagcaaggacagcacctacagcctcagcagcaccctgacgctgagcaaagcag
actacgagaaacacaaagtctacgcctgcgaagtcacccatcagggcctgagctcgcccgtcacaaagagcttcaacaggggtgagtgc
ggtggttctgggggatctagcggatcggggtctgggtcgactggtacctcgtcaagcgggacgggtactagtgctggtactacgggcactt
cagctagcacctctggctcggggtccggcgaggtgcagctgcaggagagcggccccggcctggtgcgccccagccagaccctgagcc
tgacctgcaccgtgagcggctacagcatcaccagcgaccacgcctggagctgggtgcgccagccacctggtcgcggactggagtggat
cggctacatcagctacagcggcatcaccacctacaaccccagcctgaagagccgcgtgaccatgctgcgcgacaccagcaagaaccagt
tcagcctgcgcctgagcagcgtgaccgccgccgacaccgccgtgtactactgcgcccgcagcctggcccgcaccaccgccatggacta
ctggggccagggcagcctggtgaccgtgagcagcgctagcaccaagggcccatcggtcttcccctggcaccctcctccaagagcacct
ctgggggcacagcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagc
ggcgtgcacaccttcccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgaccgtgccctccagcagcttgggcaccc
agacctacatctgcaacgtgaatcacaagcccagcaacaccaaggtggacaagaaagttgagcccaaatcttgtgacaaaactcacacat
gcccaccgtgcccagcacctgaactcctgggggggaccgtcagtcttcctcttcccccaaaacccaaggacaccctcatgatctcccggac
ccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggaggtgcataa
tgccaagacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatg
gcaaggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggcagccccgaga
accacaggtgtacaccctgcccccatcccgggaggagatgaccaagaaccaggtcagcctgtcctgcgctgtcaaaggcttctatcccag
cgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgacggctccttct
tcctcgtgagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatgcatgagggtctgcacaacc
actacacgcagaagagcctctccctgtctccgggtaaa

SEQ ID NO: 11

BS1 Antibody Chain 2 with 10H2 Light and Heavy Chains and Human IgG1 CH_1, CH_2, and CH_3 Domains with "Knobs" Substitutions

Relevant domains are shown below (described in an N- to C-terminal direction)

- Signal Sequence is shows in dark shading
- 10H2 light chain variable region is underlined
- 10H2 light chain constant region is italicized
- The linker region is shown in light shading
- 10H2 heavy chain variable region is in bold text
- Exemplary knobs substitution at positions 642 is in bold and underlined text

MRVPAQLLGLLLLWLPGARCAGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYAN
WVQEKPDHLFTGLIGGTNNRPPGVPARFSGSLIGDKAALTITGAQIEDEAIYFCALWYSN
HLVFGGGTKLTVL*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS*
*GNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*GGSGG
SSGSGSGSTGTSSSGTGTSAGTTGTSASTSGSGSG**EVQLVETGGRLVQPKGSLKLSCAV**
SGITFKTNAMNWVRQAPGKGLEWVARIRTKSYNYATYYADSVKDRFTISRDDSQSI
LYLQMNNLKTEDTAMYHCVREGRWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGGT
AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI
CNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEV
TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL**W**CLVKGFYP
SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEGL
HNHYTQKSLSLSPGK

SEQ ID NO: 12

Nucleic Acid Sequence Encoding BS1 Antibody Chain 2 with 10H2 Light and Heavy Chains and Human IgG1 CH1, CH2, and CH3 Domains with "Knobs" Substitution

atgagggtccccgctcagctcctggggctcctgctgctctggctcccaggtgcacgatgtgccggatcccaggctgttgtgactcaggaatct
gcactcaccacatcacctggtgaaacagtcacactcacttgtcgctcaagtactggggctgttacaactagtaactatgccaactgggtccaag
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SEQ ID NO: 13

Exemplary Linker
GGSGGSSGSGSGSTGTSSSGTGTSAGTTGTSASTSGSGSG

SEQ ID NO: 14

BS2 10H2 Heavy Chain
Relevant domains are shown below (described in an N- to C-terminal direction)

- Signal Sequence is show in dark shading
- 10H2 variable heavy chain region is italicized

MRVPAQLLGLLLLWLPGARCAGS*EVQLVETGGRLVQPKGSLKLSCAVSGITFKTNAMNWV
RQAPGKGLEWVARIRTKSYNYATYYADSVKDRFTISRDDSQSILYLQMNNLKTEDTAMYHCVR
EGRWGQGTLVTVSA*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD
SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEGLHNHYTQKSLSLSPGK

SEQ ID NO: 15

BS2 10H2+Tocilizumab scFv Light Chain
Relevant domains are shown below (described in an N- to C-terminal direction)

- Signal Sequence is show in dark shading
- 10H2 variable light chain region is underlined
- Kappa constant light chain region is italicized
- The linker region is shown in light shading
- Tocilizumab variable heavy chain region is in bold text
- scFv linker region is shown in light shading with underlined text
- Tocilizumab variable light chain region is in bold and underlined text

MRVPAQLLGLLLLWLPGARCAGSQAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYAN
WVQEKPDHLFTGLIGGTNNRPPGVPARFSGSLIGDKAALTITGAQIEDEAIYFCALWYSN
HLVFGGGTKLTVL*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS
GNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*GGGGS

GGGGSEVQLQESGPGLVRPSQTLSLTCTVSGYSITSDHAWSWVRQPPGRGLEWIGY ISYSGITTYNPSLKSRVTMLRDTSKNQFSLRLSSVTAADTAVYYCARSLARTTAMDY WGQGSLVTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDISS YLNWYQQKPGKAPKLLIYYTSRLHSGVPSRFSGSGSGTDFTFTISSLQPEDIATYYC QQGNTLPYTFGQGTKVEIK

SEQ ID NO: 16

Tocilizumab Heavy Chain Variable Region CDR1
SDHAWS

SEQ ID NO: 17

Tocilizumab Heavy Chain Variable Region CDR2
YISYSGITTYNPSLKS

SEQ ID NO: 18

Tocilizumab Heavy Chain Variable Region CDR3
SLARTTAMDY

SEQ ID NO: 19

Tocilizumab Light Chain Variable Region CDR1
RASQDISSYLN

SEQ ID NO: 20

Tocilizumab Light Chain Variable Region CDR2
YTSRLHS

SEQ ID NO: 21

Tocilizumab Light Chain Variable Region CDR3
QQGNTLPYT

SEQ ID NO: 22

10H2 Heavy Chain Variable Region CDR1
GITFKTNA

SEQ ID NO: 23

10H2 Heavy Chain Variable Region CDR2
IRTKSYNYAT

SEQ ID NO: 24

10H2 Heavy Chain Variable Region CDR3
VREGR

SEQ ID NO: 25

10H2 Light Chain Variable Region CDR1
TGAVTTSNY

SEQ ID NO: 26

10H2 Light Chain Variable Region CDR2
GTN

SEQ ID NO: 27

10H2 Light Chain Variable Region CDR3
ALWYSNHLV

SEQ ID NO: 28

Forward HK2 Human

CCAGTTCATTCACATCATCAG

SEQ ID NO: 29

Forward α-Tubulin Human

AGGAGTCCAGATCGGCAATG

SEQ ID NO: 30

Forward α-Tubulin Mouse

CACACAAGCTCACTCACCCT

SEQ ID NO: 31

Forward β-Actin Human

CATGTACGTTGCTATCCAGGC

SEQ ID NO: 32

Forward β-Actin Mouse

ATGAGCTGCCTGACGGCCAGGTCATC

SEQ ID NO: 33

Forward GAPDH Human

GCACCGTCAAGGCTGAGAAC

SEQ ID NO: 34

Forward GAPDH Mouse

ACCACAGTCCATGCCATCAC

SEQ ID NO: 35

Forward RPL13A Human

AGCCTCATCTGCAATGTAGGG

SEQ ID NO: 36

Forward RPL13A Mouse

AGGGGTTGGTATTCATCCGC

SEQ ID NO: 37

Forward 18s Human

GAGGATGAGGTGGAACGTGT

SEQ ID NO: 38

Forward 18s Mouse

CGGCGACGACCCATTCGAAC

SEQ ID NO: 39

Reverse HK2 Human

CTTACACGAGGTCACATAGC

SEQ ID NO: 40

Reverse α-Tubulin Human

GTCCCCACCACCAATGGTTT

SEQ ID NO: 41

Reverse α-Tubulin Mouse

CTGTTATTAGGGATGTGACTCCA

SEQ ID NO: 42

Reverse β-Actin Human

CTCCTTAATGTCACGCACGAT

SEQ ID NO: 43

Reverse β-Actin Mouse

TGGTACCACCAGACAGCACTGTGTTG

SEQ ID NO: 44

Reverse GAPDH Human

GCCTTCTCCATGGTGGTGAA

SEQ ID NO: 45

Reverse GAPDH Mouse

CACCACCCTGTTGCTGTAGCC

SEQ ID NO: 46

Reverse RPL13A Human

TCAGACTCCTCGGATTCTTCTTT

SEQ ID NO: 47

Reverse RPL13A Mouse

ATGCCTGCTGAGGCTTTGTT

SEQ ID NO: 48

Reverse 18s Human

AGAAGTGACGCAGCCCTCTA

SEQ ID NO: 49

Reverse 18s Mouse

GAATCGAACCCTGATTCCCCGT

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS

1. A bispecific binding agent comprising:

first polypeptide comprising a first antibody heavy chain or portion thereof, a linker, and a first antibody light chain or portion thereof, wherein the first linker connects the first antibody heavy chain or portion thereof and the first antibody light chain or portion thereof, and wherein the first antibody heavy chain or portion thereof and the first antibody light chain or portion thereof form a first binding site specific for IL-6Rα;

a second polypeptide comprising a second polypeptide antibody heavy chain or portion thereof, a second linker, and a second polypeptide antibody light chain or portion thereof, wherein the second linker connects the second antibody heavy chain or portion thereof and the second antibody light chain or portion thereof, and wherein the second antibody heavy chain or portion thereof and the second antibody light chain or portion thereof form a second binding site specific for IL-8R,

wherein the first antibody heavy chain or portion thereof comprises one or more amino acid substitutions, the second antibody heavy chain or portion thereof comprises one or more amino acid substitutions, or both, such that the first polypeptide antibody heavy chain or portion thereof and the second polypeptide antibody heavy chain or portion thereof preferentially associate with each other to form the bispecific binding agent.

2. The bispecific binding agent of claim 1, wherein the first antibody heavy chain or portion thereof comprises a C_H1 domain or portion thereof, a C_H2 domain or portion thereof, a C_H3 domain or portion thereof, and a V_H domain or portion thereof.

3. The bispecific binding agent of claim 1, wherein the second antibody heavy chain or portion thereof, comprises a C_H1 domain or portion thereof, a C_H2 domain or portion thereof, a C_H3 domain or portion thereof, and a V_H domain or portion thereof.

4. The bispecific binding agent of claim 1, wherein the first polypeptide and the second polypeptide preferentially associate with each other as compared to a corresponding first

polypeptide comprising an antibody heavy chain that lacks the one or more amino acid substitutions, a corresponding second polypeptide comprising a second antibody heavy chain that lacks the one or more amino acid substitutions, or both.

5. The bispecific binding agent of claim 1, wherein the first antibody light chain comprises a C_L domain or portion thereof and a V_L domain or portion thereof.

6. The bispecific binding agent of claim 1, wherein the second antibody light chain comprises a C_L domain or portion thereof and a V_L domain or portion thereof.

7. The bispecific binding agent of claim 1, wherein the first polypeptide linker connects a C_L domain of the first antibody light chain to a V_H domain of the first antibody heavy chain.

8. The bispecific binding agent of claim 1, wherein the second polypeptide linker connects a C_L domain of the second antibody light chain to a V_H domain of the second antibody heavy chain.

9. The bispecific binding agent of claim 1, wherein the first polypeptide linker comprises a polypeptide having at least 80% sequence identity to SEQ ID NO. 13.

10. The bispecific binding agent of claim 1, wherein the second polypeptide linker comprises a polypeptide having at least 80% sequence identity to SEQ ID NO. 13.

11. The bispecific binding agent of claim 1, wherein the one or more amino acid substitutions in the first antibody heavy chain or portion thereof comprises an amino acid substitution at a one or more of positions 645, 647, and 686 of SEQ ID NO. 9.

12. The bispecific binding agent of claim 1, wherein the one or more amino acid substitutions in the second antibody heavy chain or portion thereof comprises an amino acid substitution at a one or more of positions 642 of SEQ ID NO. 11.

13. The bispecific binding agent of claim 1, wherein the first antibody heavy chain or portion thereof comprises a V_H domain comprising:

a heavy chain CDR1 domain comprising SEQ ID NO. 16,

a heavy chain CDR2 domain comprising SEQ ID NO. 17, and

a heavy chain CDR3 domain comprising SEQ ID NO. 18; and

wherein the first antibody light chain or portion thereof comprises a V_L domain comprising:

a light chain CDR1 domain comprising SEQ ID NO. 19,

a light chain CDR2 domain comprising SEQ ID NO. 20, and

a light chain CDR3 domain comprising SEQ ID NO. 21.

14. The bispecific binding agent of claim 1,

wherein the second antibody heavy chain or portion thereof comprises a V_H domain comprising:

a heavy chain CDR1 domain comprising SEQ ID NO. 22,

a heavy chain CDR2 domain comprising SEQ ID NO. 23, and

a heavy chain CDR3 domain comprising SEQ ID NO. 24; and

wherein the second antibody light chain or portion thereof comprises a V_L domain comprising:

a light chain CDR1 domain comprising SEQ ID NO. 25,

a light chain CDR2 domain comprising SEQ ID NO. 26, and

a light chain CDR3 domain comprising SEQ ID NO. 27.

15. The bispecific binding agent of claim 1,

wherein the first antibody heavy chain or portion thereof comprises a V_H domain comprising: a first heavy chain CDR1 domain comprising SEQ ID NO. 16,

a first heavy chain CDR2 domain comprising SEQ ID NO. 17, and

a first heavy chain CDR3 domain comprising SEQ ID NO. 18;

wherein the first antibody light chain or portion thereof comprises a V_L domain comprising:

a first light chain CDR1 domain comprising SEQ ID NO. 19,

a first light chain CDR2 domain comprising SEQ ID NO. 20, and
a first light chain CDR3 domain comprising SEQ ID NO. 21;
wherein the second antibody heavy chain or portion thereof comprises a V_H domain comprising:
a second heavy chain CDR1 domain comprising SEQ ID NO. 22,
a second heavy chain CDR2 domain comprising SEQ ID NO. 23, and
a second heavy chain CDR3 domain comprising SEQ ID NO. 24; and
wherein the second antibody light chain or portion thereof comprises a V_L domain comprising: a second light chain CDR1 domain comprising SEQ ID NO. 25,
a second light chainCDR2 domain comprising SEQ ID NO. 26, and
a second light chain CDR3 domain comprising SEQ ID NO. 27.

16. The bispecific binding agent of claim 1, wherein the first binding site comprises: the V_H domain comprising residues 278-396 of SEQ ID NO. 9, and the V_L domain comprising residues 24-130 of SEQ ID NO. 9.

17. The bispecific binding agent of claim 1, wherein the second binding site comprises: the V_H domain comprising residues 280-393 of SEQ ID NO. 11, and the V_L domain comprising residues 24-132 of SEQ ID NO. 11.

18. A pharmaceutical composition comprising the bispecific binding agent of any one of claims 1-17 and a pharmaceutically acceptable carrier.

19. A method of treating a disease in a subject in need thereof, the method comprising administering a therapeutically effective amount the bispecific binding agent of any one of claims 1-17 or the pharmaceutical composition in claim 18.

20. The method of claim 19, wherein the disease is cancer.

21. The method of claim 20, wherein the method inhibits metastatic cell migration of the cancer.

22. The method of claim 20, wherein the cancer is a breast cancer.

23. The method of claim 20, wherein the cancer is triple negative breast cancer.

24. The method of claim 20, wherein the cancer is a pancreatic cancer.

25. The method of claims 20, wherein the cancer is a pancreatic ductal adenocarcinoma.

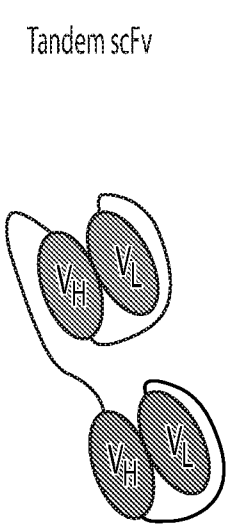


Figure 1A.

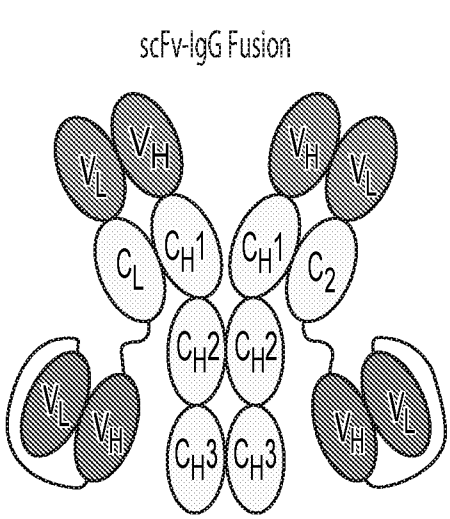


Figure 1B.

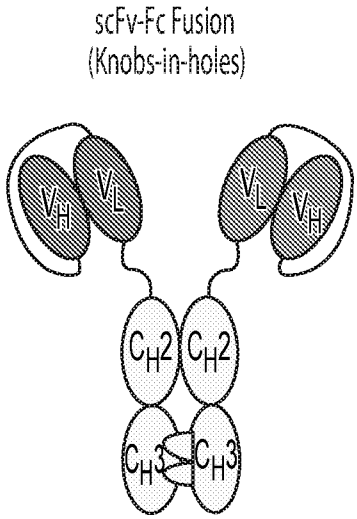


Figure 1C.

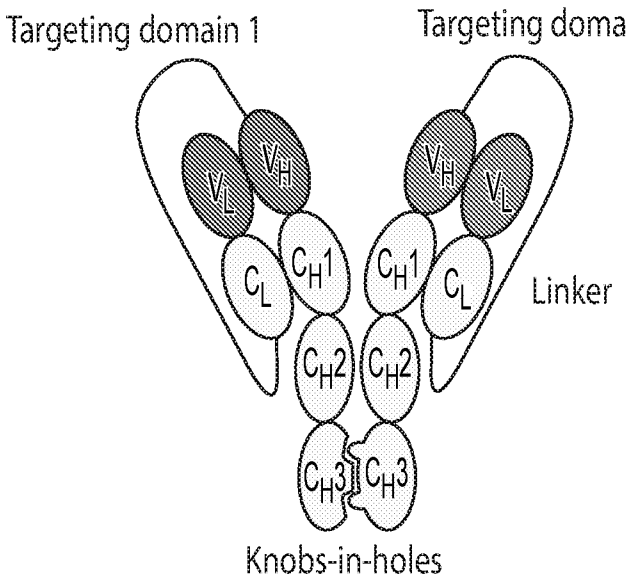


Figure 1D.

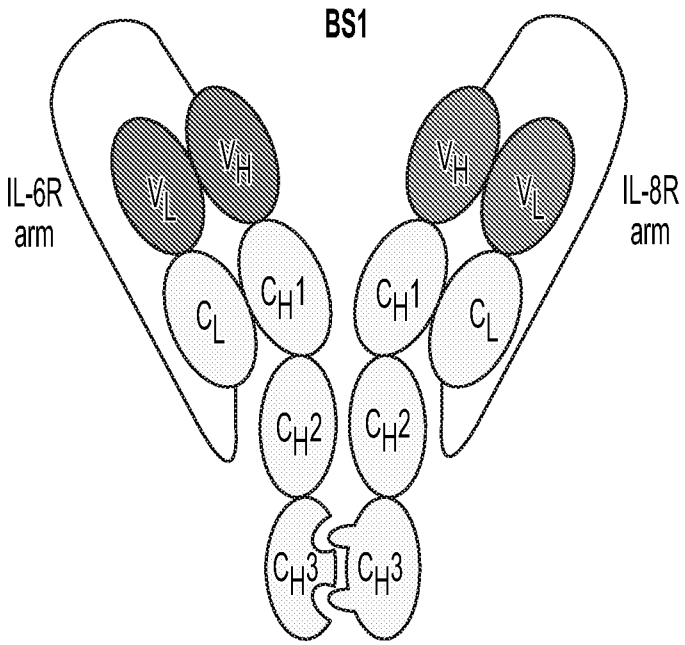
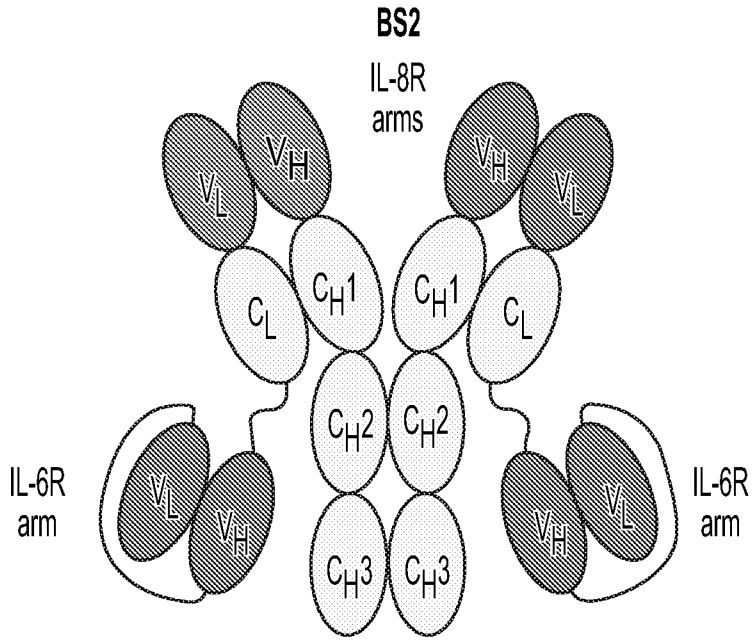


Figure 2.



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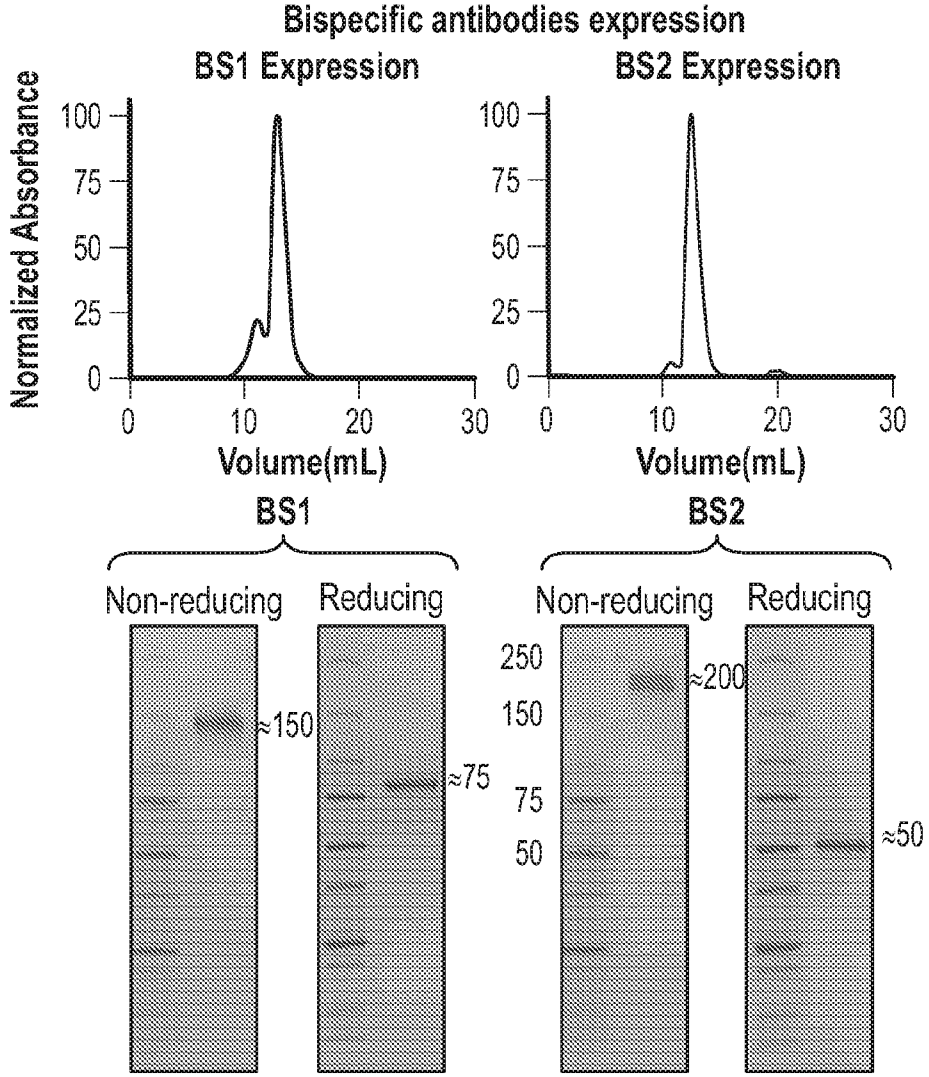
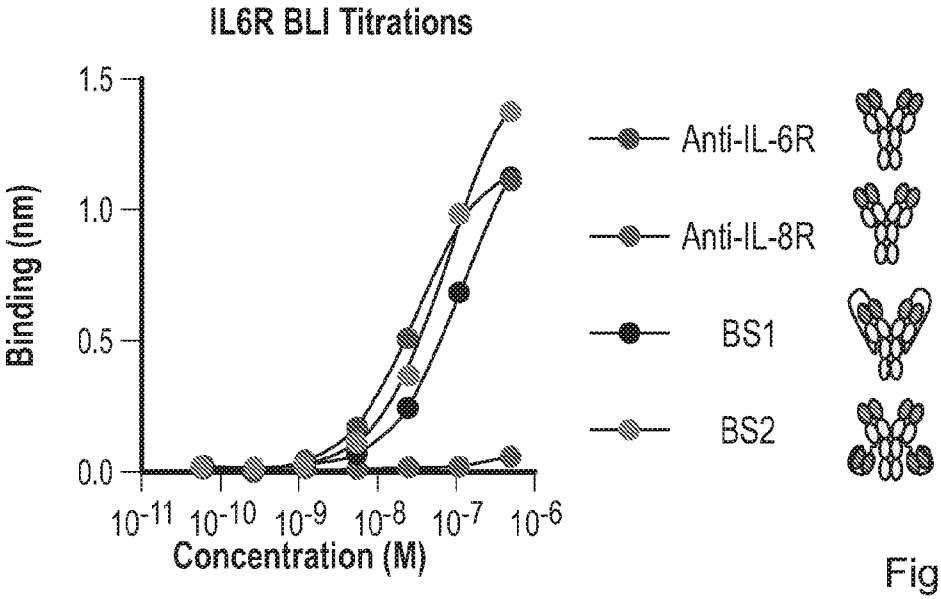


Figure 4.



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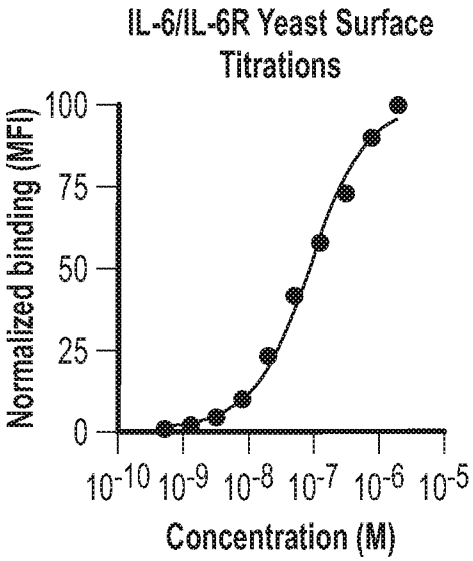

Figure 6A.

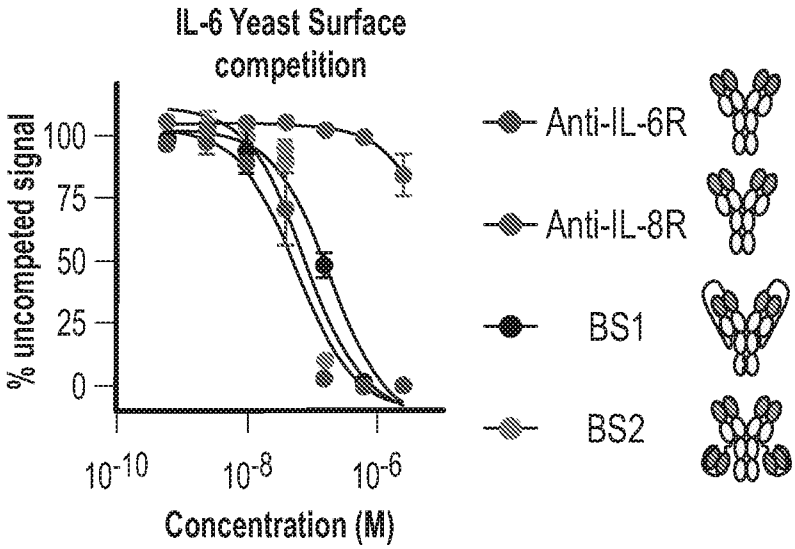

Figure 6B.

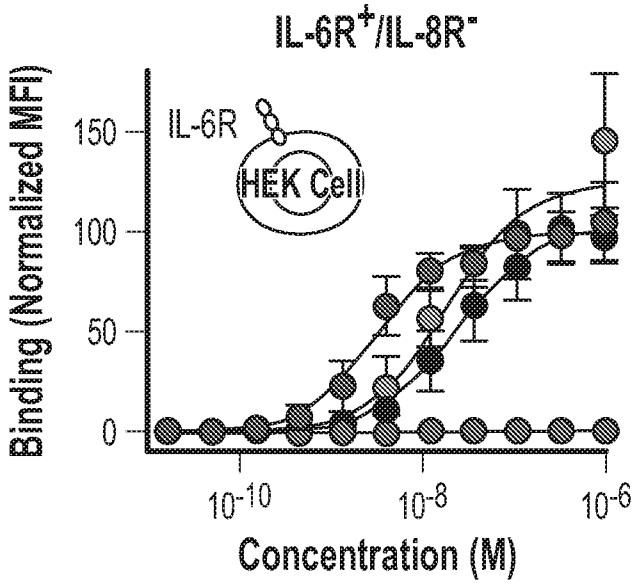

Figure 7A.

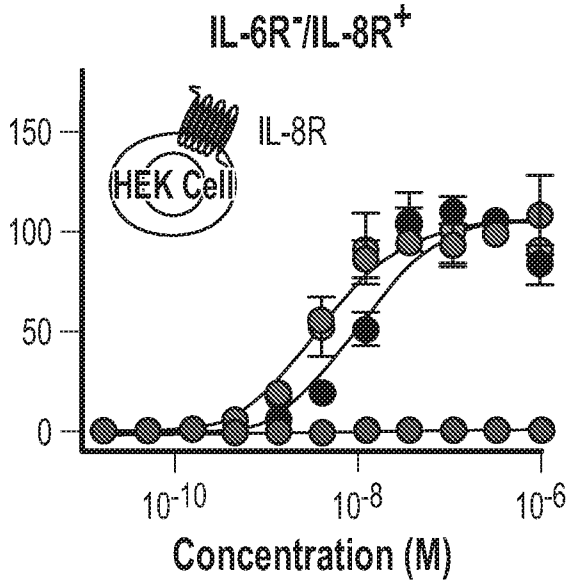

Figure 7B.

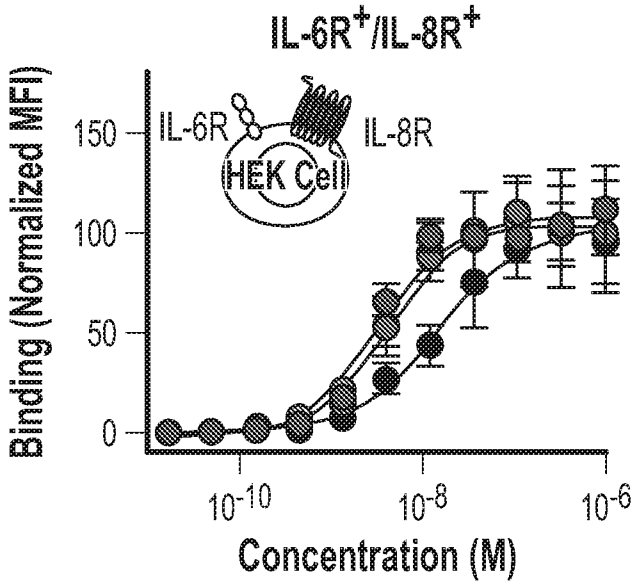

Figure 7C.

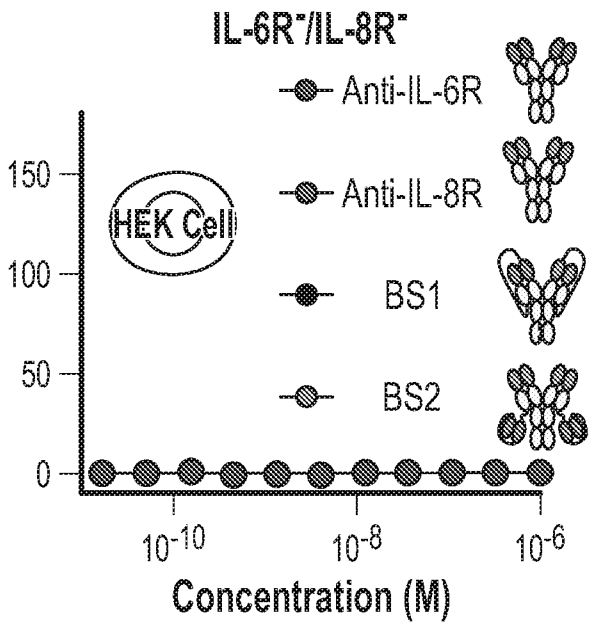

Figure 7D.

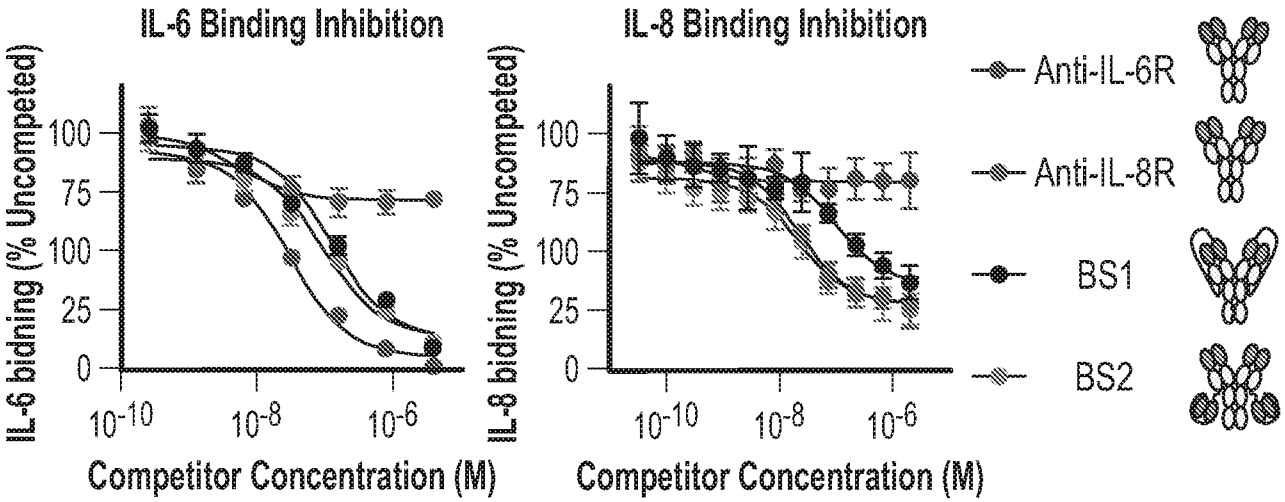

Figure 8A.

Figure 8B.

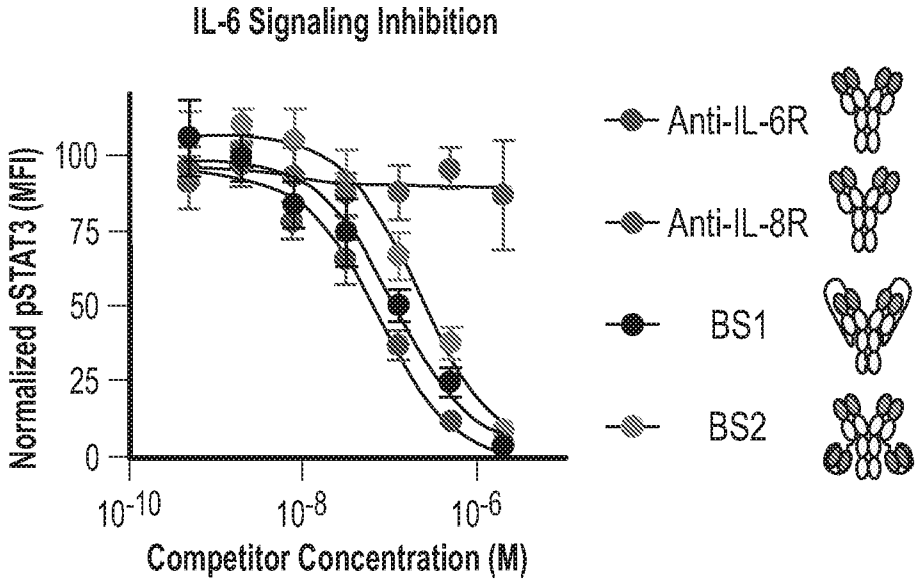

Figure 9.

MDA-MB-231

Figure 10A.

HT-1080

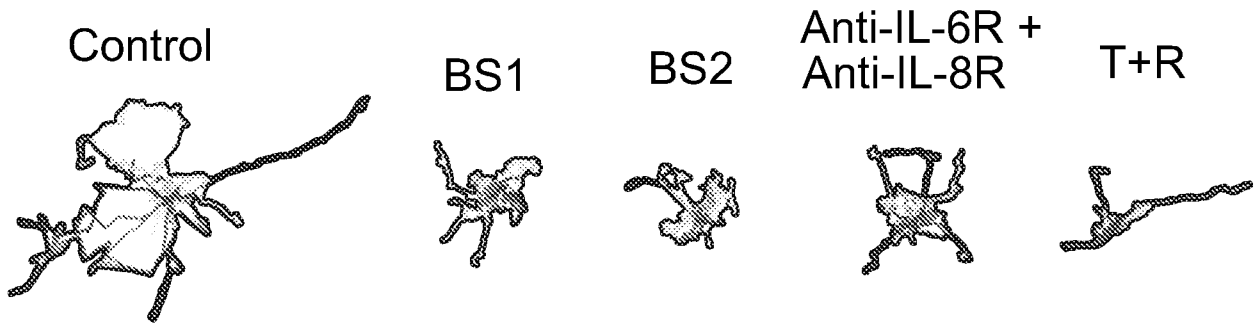

Figure 10B.

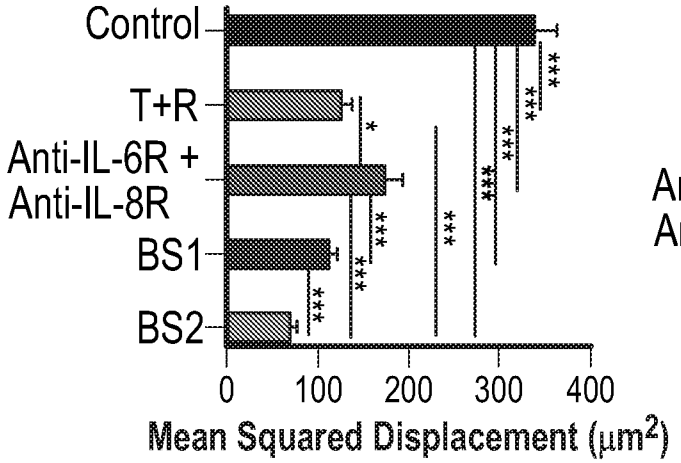

Figure 11A.

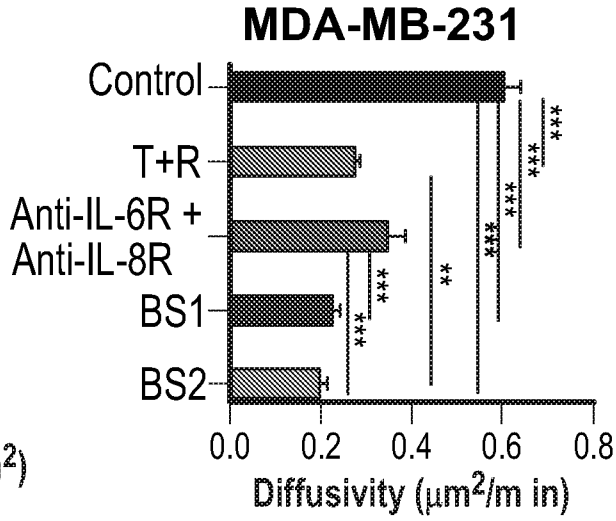

Figure 11B.

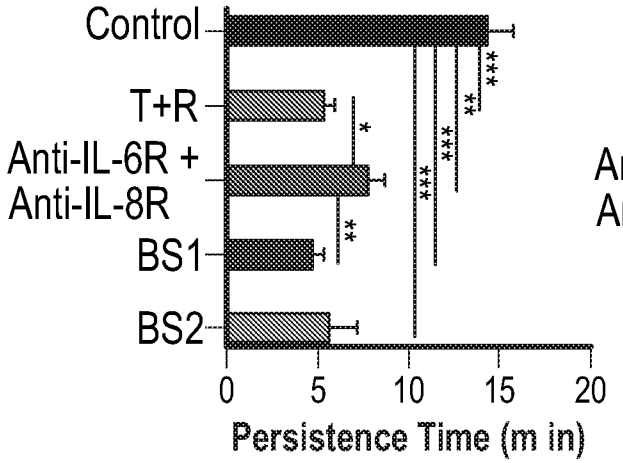

Figure 11C.

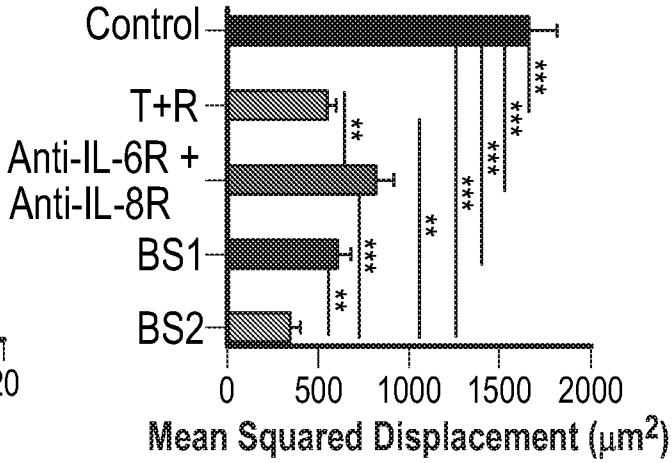

Figure 11D.

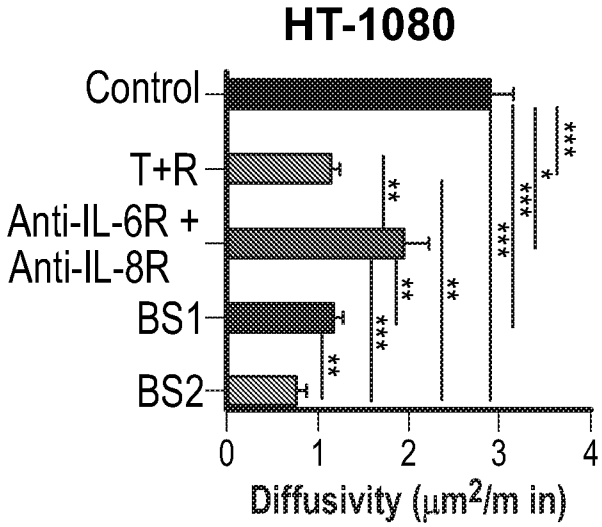

Figure 11E.

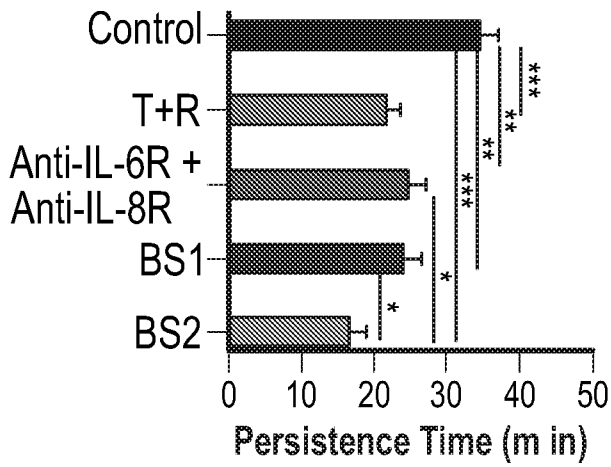

Figure 11F.

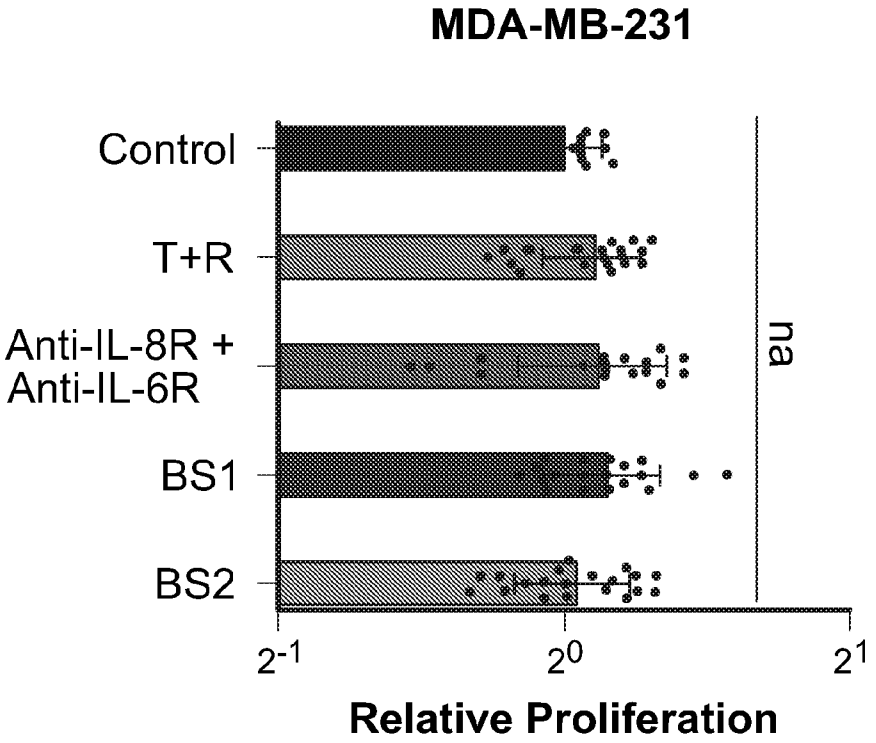

Figure 12A.

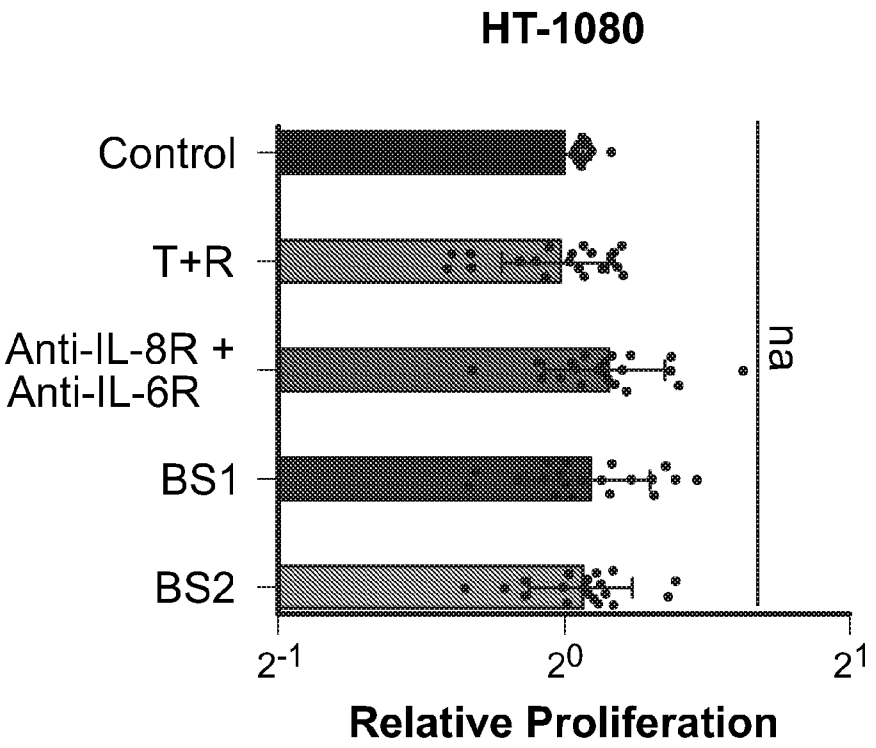

Figure 12B.

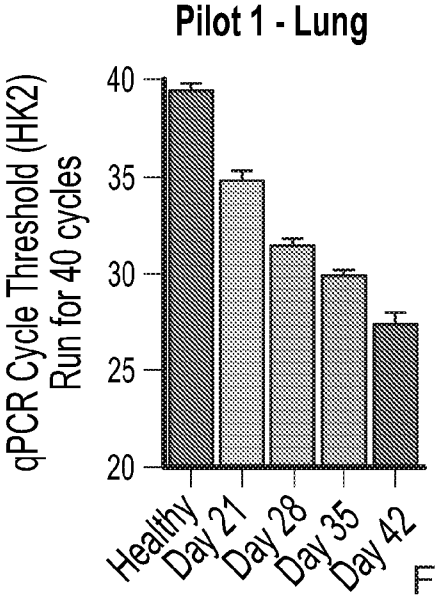

Figure 13A.

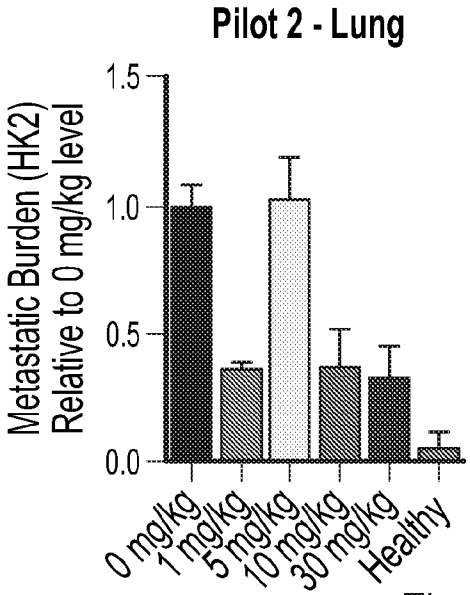

Figure 13B.

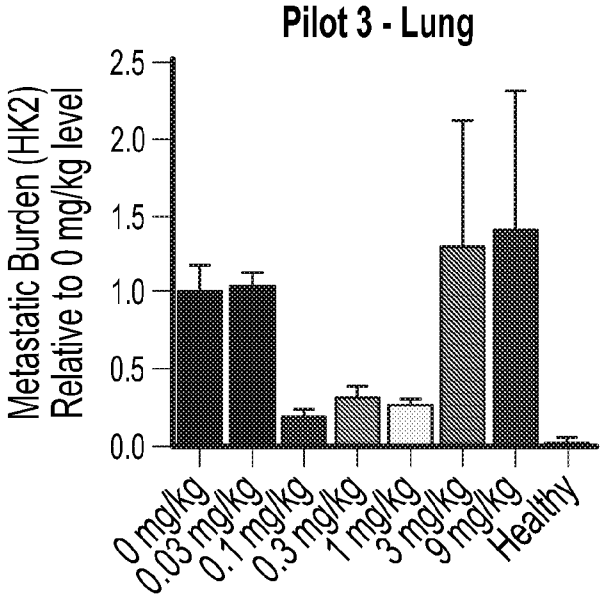

Figure 13C.

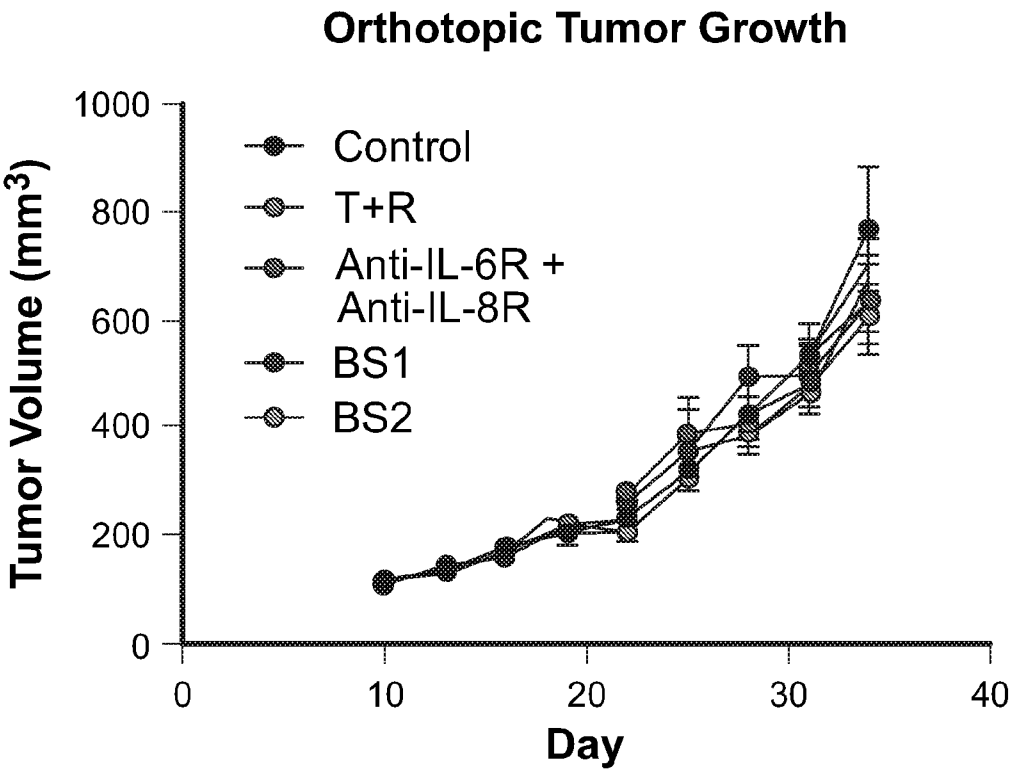

Figure 14.

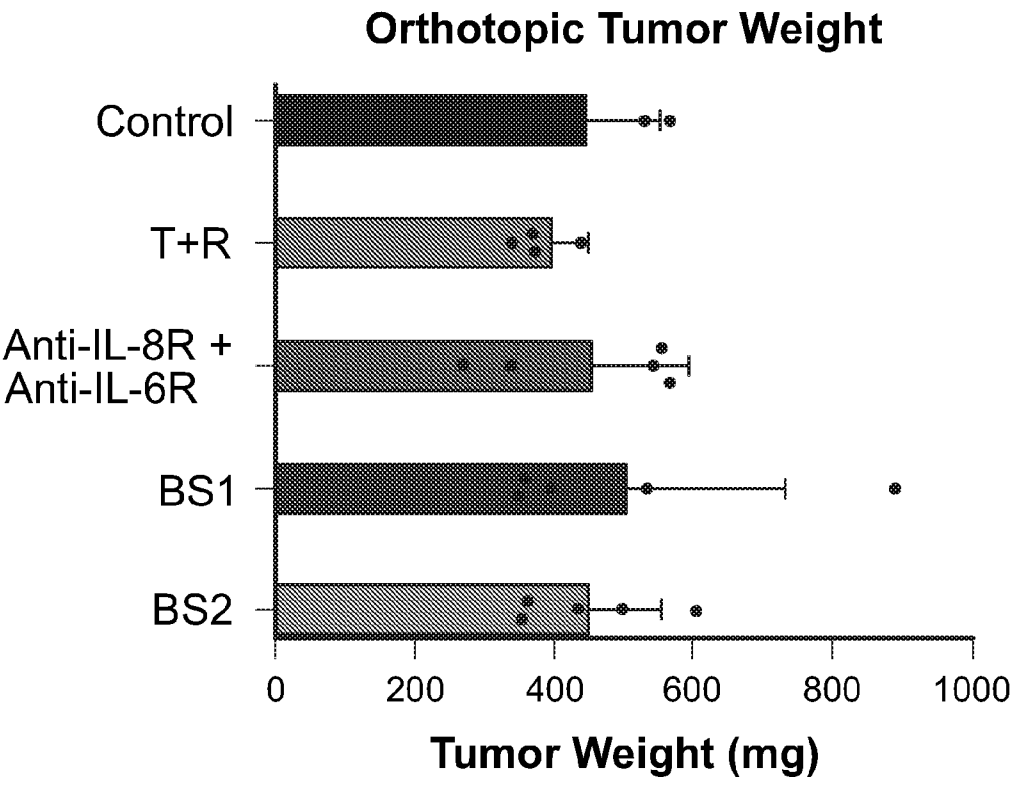

Figure 15.

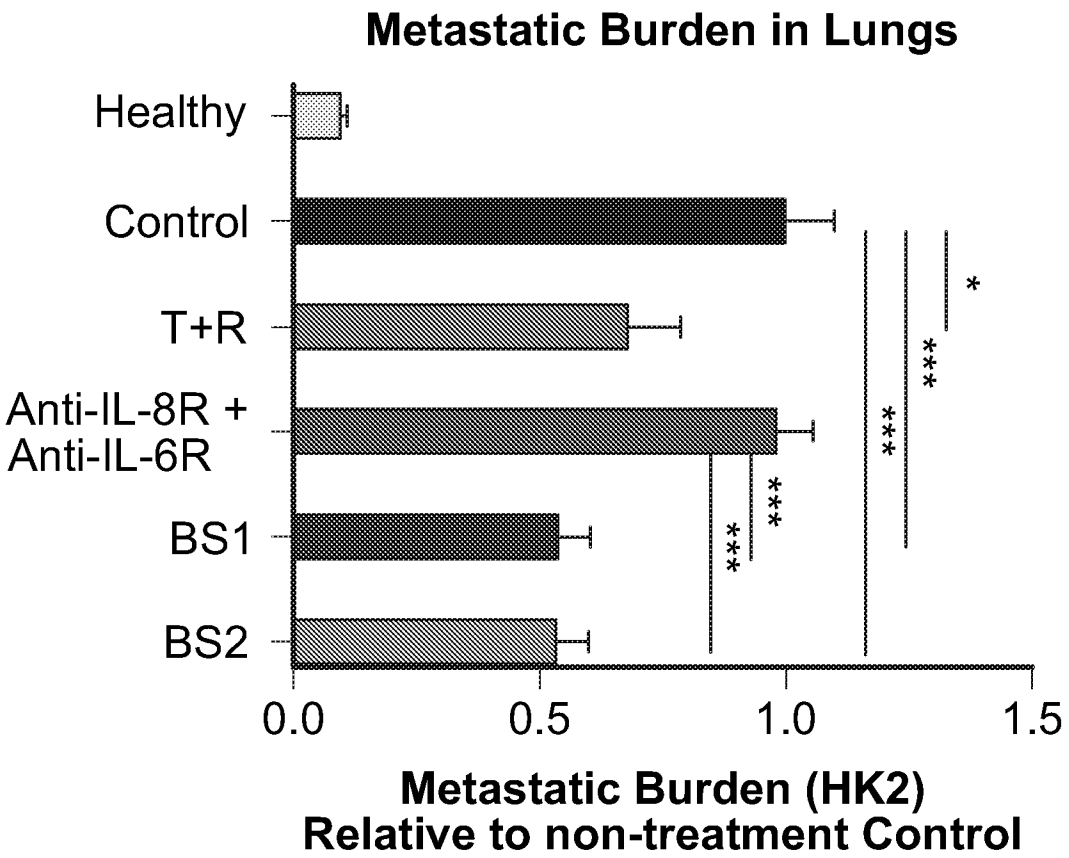

Figure 16.

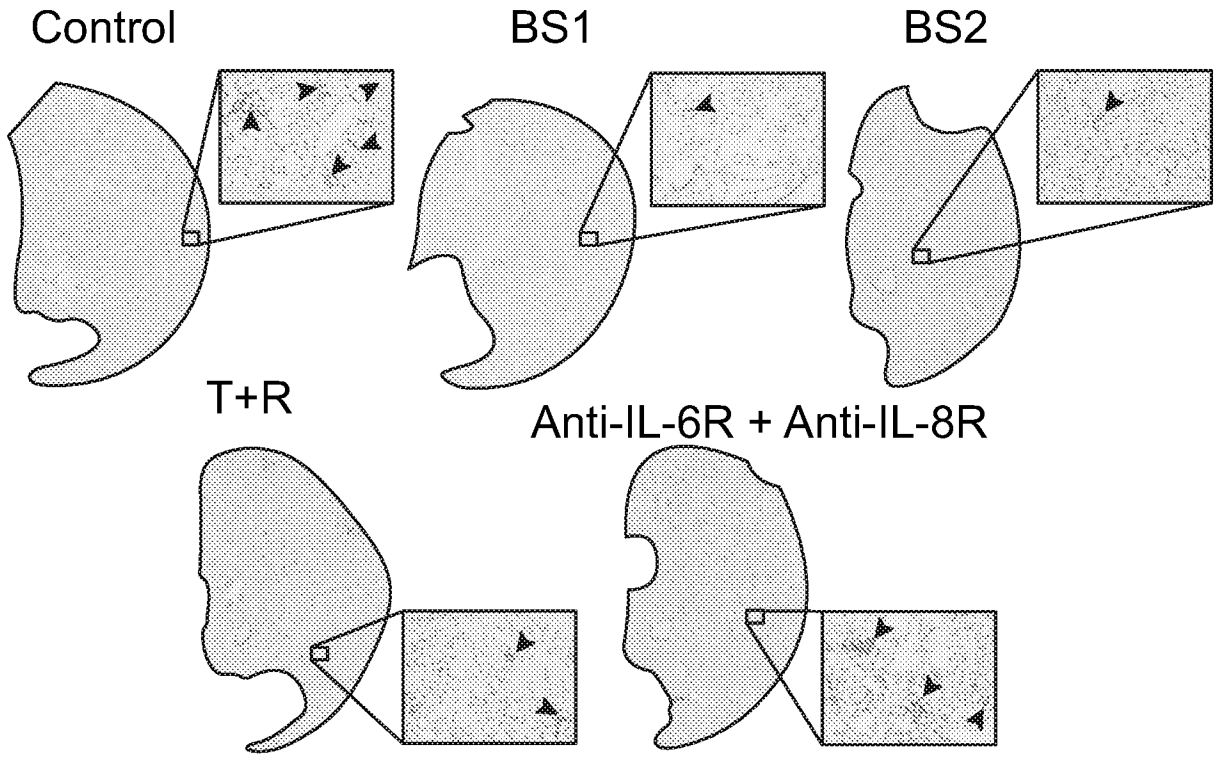

Figure 17.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2020/035211

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/24 *(2006.01)*
C07K 16/46 *(2006.01)*
A61K 39/395 *(2006.01)*
A61P 35/00 *(2006.01)*

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 16/24, 16/46, A61K 39/395, A61P 35/00

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

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

PatSearch (RUPTO Internal), USPTO, PAJ, Espacenet, Information Retrieval System of FIPS, Google

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2013/055958 A1 (GENENTECH, INC. et al.) 18.04.2013, claims, paragraphs [33], [34], [82], [84], [131], [156], [160], [166] - [168], [200], [223] - [224], example 1, 2	1-13, 16, 18-25
A		14, 15, 17
Y	CULSHAW S. et al. What can the periodontal community learn from the pathophysiology of rheumatoid arthritis? J Clin Periodontol, 2011, Volume 38, Issue s11, pp.106-113, p. 110, left col.	1-13, 16, 18-25
A		14, 15, 17
Y	US 2016/0333077 A1 (INTEGRATED BIOTHERAPEUTICS INC) 17.11.2016, paragraph [0050], fig. 4	9-10
Y	US 9856319 B2 (ABBVIE INC.) 02.01.2018, tables 6, 29, SEQ ID NO: 41, 42, 43	11-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

"E" earlier document but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 August 2020 (24.08.2020)	03 September 2020 (03.09.2020)

Name and mailing address of the ISA/RU:	Authorized officer
Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	O. Tulinova Telephone No. 8(495) 531-65-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2020/035211

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	IGAWA T. et al. Antibody recycling by engineered pH-dependent antigen binding improves the duration of antigen neutralization. Nat Biotechnol., 2010, vol.28(11), pp.1203-1207, p. 1207, GenBank: AB591055, AB591056, retrieved GENPEPT [database online] [retrieved on 18.08.2020], Retrieved from <Accession No. BAJ21229.1, 2017-11-17>, <Accession No. BAJ21230.1, 2017-11-17>	13, 16 14, 15, 17
Y	US 9168300 B2 (ONCOMED PHARMACEUTICAL INC) 27.10.2015, claims	18-25
Y A	SHENGLING FU et al. Blocking Interleukin-6 and Interleukin-8 Signaling Inhibits Cell Viability, Colony-forming Activity, and Cell Migration in Human Triple-negative Breast Cancer and Pancreatic Cancer Cells, ANTICANCER RESEARCH, 2018, vol.38, pp.6271-6279, p. 6271, abstract, p. 6275, paragraph 2, fig. 1-3	20-25 14, 15, 17