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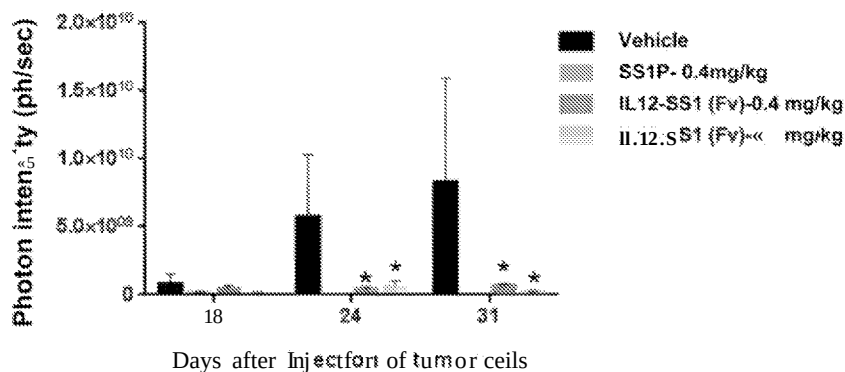
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(54) Title: MESOTHELIN-SPECIFIC IMMUNOCYTOKINE AND USE THEREOF

FIG. 4A



(57) Abstract: Described herein is the development of an immunocytokine based on interleukin-12 (IL12) and the mesothelin-specific SSI Fv. The IL12-SS1 (Fv) immunocytokine was produced in insect cells using a baculovirus expression system. The SSI single-chain Fv was fused to the C terminus of the p35 subunit of IL12 through a short linker. The single-chain IL12-SS1 (Fv) immunocytokine binds native mesothelin proteins on mesothelioma and ovarian cancer cells, as well as recombinant mesothelin. The recombinant immunocytokine retains sufficient bioactivity of IL12 and significantly inhibits human malignant mesothelioma in an animal model.

MESOTHELIN-SPECIFIC IMMUNOCYTOKINE AND USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/820,543, filed
5 May 7, 2013, which is herein incorporated by reference in its entirety.

FIELD

This disclosure concerns an immunocytokine comprising interleukin-12 (IL12) fused to a
mesothelin-specific antibody fragment, and its use for stimulating an immune response against
10 mesothelin-expressing tumor cells.

BACKGROUND

Mesothelin has been suggested as a therapeutic target because it is highly expressed in
malignant mesotheliomas (Chang *et al*, *Cancer Res* 52:181-186, 1992; Chang and Pastan, *Proc*
15 *Natl Acad Sci USA* 93:136-140, 1996) and other solid tumors such as cholangiocarcinoma, ovarian
cancer, pancreatic cancer and lung adenocarcinoma (Hassan and Ho, *Eur J Cancer* 44:46-53, 2008;
Ho, *Biodrugs* 25:275-284, 2011; Argani *et al*, *Clin Cancer Res* 7:3862-3868, 2001). The
mesothelin gene encodes a -71 kDa precursor protein that is processed to a -31 kDa N-terminal
protein and a - 40 kDa C-terminal membrane-bound mature mesothelin (Hassan and Ho, *Eur J*
20 *Cancer* 44:46-53, 2008).

Over the last two decades, a number of anti-mesothelin monoclonal antibodies (mAbs) have
been developed. One such antibody, SSI, was generated by immunization of mice with a
eukaryotic expression vector coding for mesothelin. When high serum antibody titers were
obtained, a phage display library was made from the spleen mRNA of these mice. A single-chain
25 variable fragment (scFv)-displaying phage (called SS) was selected that specifically bound to
recombinant mesothelin and mesothelin-positive cells (Chowdhury *et al*, *Proc Natl Acad Sci USA*
95:669-674, 1998). The SS Fv was further improved by *in vitro* affinity maturation (Chowdhury
and Pastan, *Nat Biotechnol* 17:568-572, 1999) and fused to a truncated *Pseudomonas* exotoxin to
develop the SS1P immunotoxin (Pastan *et al*, *Nat Rev Cancer* 6:559-565, 2006). However, in a
30 phase I clinical trial of SS1P, many patients produced neutralizing antibodies to *Pseudomonas*
exotoxin, which reduced SS1P efficacy, making further treatment ineffectual (Hassan *et al*, *Clin*
Cancer Res 13:5144-5149, 2007).

Interleukin-12 (IL12) is a disulfide-linked heterodimeric glycoprotein consisting of a 35
(p35) and a 40 (p40) kDa subunit (Yoon *et al*, *EMBO J* 19:3530-3541, 2000). IL12 can enhance

the activation of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), promoting the production of interferon-gamma (IFN- γ), and inducing the differentiation of T helper cells (Nastala *et al.*, *J Immunol* 153:1697-1706, 1994; Amsen *et al.*, *Curr Opin Immunol* 21:153-160, 2009).

IL12 also has potent antitumor, anti-angiogenic and anti-metastatic activities. However, systemic administration of IL12 is thought to be highly toxic, particularly in multiple doses (Car *et al.*, *Toxicol Pathol* 27:58-63, 1999). Fusion of IL12 to tumor-specific antibodies has been previously reported for a number of different targets, including anti-CD30 antibody for Hodgkin's lymphoma (Heuser *et al.*, *Int J Cancer* 106:545-552, 2003), anti-HER2 antibody for HER2-expressing tumors (Helguera *et al.*, *Mol Cancer Ther* 5:1029-1040, 2006), and anti-extra-domain D (ED-B) of fibronectin for targeting tumor vessels (Lo *et al.*, *Cancer Immunol Immunother* 56:447-457, 2007; Gafner *et al.*, *Int J Cancer* 119:2205-2212, 2006).

SUMMARY

Disclosed herein is an immunocytokine comprising IL12 subunits p35 and p40 linked to the scFv of anti-mesothelin antibody SSI. The single-chain IL12-SS1 immunocytokine binds native mesothelin proteins on mesothelioma and ovarian cancer cells, as well as recombinant mesothelin. In addition, the IL12-SS1 immunocytokine retains bioactivity of IL12 and significantly inhibits human malignant mesothelioma in an animal model.

Provided herein is a conjugate comprising IL12 subunits p35 and p40 linked to a mesothelin-specific antibody or antibody fragment comprising a variable heavy (VH) domain and a variable light (VL) domain, wherein the VH and VL domains comprise the CDR sequences of the mesothelin-specific SSI antibody. In some embodiments, the immunocytokine comprises in the N-terminal to C-terminal direction, the p40 subunit, the p35 subunit, the VH domain and the VL domain. In non-limiting examples, the antibody fragment is a scFv.

Also provided are compositions comprising a conjugate disclosed herein, nucleic acid molecules and vectors encoding a disclosed conjugate and isolated host cells comprising said vectors.

Further provided are methods of treating a subject having a mesothelin-expressing cancer, and methods of inhibiting tumor growth or metastasis in a subject having a mesothelin-expressing cancer, by administering a therapeutically effective amount of a conjugate disclosed herein.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1C: Construction of IL12-SS1 (Fv) fusion protein. (FIG. 1A) Map of the baculovirus expression vector. IL12-SS1 (Fv) was inserted at the recombination sites attB1 and attB2 using the gateway cloning system. (FIG. 1B) Cloning strategy and schematic representation of the IL12-SS1 (Fv) fusion protein. The IL12-SS1 (Fv) fusion protein consists of p40 and p35 fused to scFv (SSI). (FIG. 1C) SDS-PAGE analysis of 5 µg of IL12-SS1 (Fv) protein under reducing (R) or non-reducing (NR) conditions confirms protein size. Analysis: SDS-PAGE, 4-20% gradient gel, Coomassie blue staining. M; protein standards, kDa.

FIGS. 2A-2D: Binding of IL12-SS1 (Fv) to mesothelin (MSLN) and cancer cells. (FIG. 2A) The binding specificity of IL12-SS1 (Fv) on MSLN was detected by ELISA. (FIG. 2B) FACS analysis of IL12-SS1 (Fv) on mesothelin-expressing cancer cells. NCI-H226, OVCAR-3, H9, A431, and HEK-293 cells were stained with IL12-SS1 (Fv), which was detected by PE-conjugated anti-histidine antibody (solid line). Mesothelin expression was detected by a mouse anti-MSLN antibody (MN, dotted line) or an irrelevant isotype mAb control (shaded surface). Samples were analyzed by flow cytometry. H9: A431 cell line stably expressing mesothelin; OVCAR-3: human ovarian cancer cell lines; NCI-H226: human mesothelioma cell line; HEK-293: human embryonic kidney cell line. (FIG. 2C) FACS analysis of IL12-SS1 (Fv) on A431 cells. A431 cells were stained with IL12-SS1 (Fv) in the presence (dotted line) or absence (solid line) of an anti-IL12R β2 blocking antibody. The binding of IL12-SS1 (Fv) was detected by FITC-conjugated anti-Flag antibody. (FIG. 2D) SS1P and IL12-SS1 (Fv) bound to H9 cells in a dose-dependent manner. H9 cells were stained with the indicated concentration of IL12-SS1 (Fv) and then detected by flow cytometry. The *K_d* value for SS1P and IL12-SS1 (FV) binding to H9 cells are approximately 2.8 and 60 nM, respectively.

FIGS. 3A-3B: *In vitro* functional analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat and activated by 10 µg/ml of PH-A for 4 days. The IL12 activity assay was performed with PBMCs in the presence of murine recombinant IL12 (mIL12) or IL12-SS1 (Fv) fusion proteins for 4 days. (FIG. 3A) Cell proliferation was monitored by a colorimetric assay (WST-8). (FIG. 3B) INF-γ in the supernatant was detected by ELISA.

FIGS. 4A-4B: Tumor therapy studies with IL12-SS1 (Fv). (FIG. 4A) Treatment of 8-week old female athymic nude mice with IL12-SS1 (Fv) after intraperitoneal injection of LMB-H226-GL. SS1P (0.4 mg/kg), IL12-SS1 (Fv) (0.4 mg/kg), IL12-SS1 (Fv) (1.6 mg/kg), or vehicle (PBS) were intraperitoneally injected 4 times at days 13, 15, 17, and 19 after tumor cell injection. Tumor growth was measured by bioluminescence photometry at day 18, 21, and 31. The

photometry of the *in vivo* imaging was acquired using Living Image 3.1.0 software (Caliper Life Sciences). *Indicates the significant ($p < 0.05$) difference between treatment and control (vehicle) by using the one-way ANOVA statistical test (GraphPad Prism 5.03). (FIG. 4B) Photograph of representative mice treated with IL12-SS1 (Fv), SS1P and vehicle (PBS).

FIGS. 5A-5C: Sequences of the SSI VH, VL and scFv. (FIG. 5A) Shown are the nucleotide (SEQ ID NO: 1) and amino acid (SEQ ID NO: 2) sequences of the SSI VH domain. Kabat amino acid numbering is indicated above the amino acid sequence (the Kabat positions do not directly correspond to the amino acid positions of SEQ ID NO: 2). The location of each CDR is underlined (Kabat) or in bold (IMGT). (FIG. 5B) Shown are the nucleotide (SEQ ID NO: 3) and amino acid (SEQ ID NO: 4) sequences of the SSI VL domain. Kabat amino acid numbering is indicated above the amino acid sequence (the Kabat positions do not directly correspond to the amino acid positions of SEQ ID NO: 4). The location of each CDR is underlined (Kabat) or in bold (IMGT). (FIG. 5C) Shown are the nucleotide (SEQ ID NO: 5) and amino acid (SEQ ID NO: 6) sequences of the SSI scFv. The VH domain is underlined and the VL domain is in bold.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on April 18, 2014, 23.4 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NO: 1 is the nucleotide sequence of the SS1 VH domain.

SEQ ID NO: 2 is the amino acid sequence of the SS1 VH domain.

SEQ ID NO: 3 is the nucleotide sequence of the SS1 VL domain.

SEQ ID NO: 4 is the amino acid sequence of the SS1 VL domain.

SEQ ID NO: 5 is the nucleotide sequence of the SS1 scFv.

SEQ ID NO: 6 is the amino acid sequence of the SS1 scFv.

SEQ ID NO: 7 is the nucleotide sequence of the p35 subunit of mouse IL12.

SEQ ID NO: 8 is the amino acid sequence of the p35 subunit of mouse IL12.

SEQ ID NO: 9 is the nucleotide sequence of the p40 subunit of mouse IL12.

SEQ ID NO: 10 is the amino acid sequence of the p40 subunit of mouse IL12.

SEQ ID NO: 11 is the nucleotide sequence of the p35 subunit of human IL12.

SEQ ID NO: 12 is the amino acid sequence of the p35 subunit of human IL12.

SEQ ID NO: 13 is the nucleotide sequence of the p40 subunit of human IL12.

SEQ ID NO: 14 is the amino acid sequence of the p40 subunit of human IL12.

SEQ ID NOs: 15-23 are primer sequences.

SEQ ID NOs: 24 and 25 are amino acid sequences of peptide linkers.

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DETAILED DESCRIPTION

I. Abbreviations

	ELISA	enzyme-linked immunosorbent assay
	Fv	variable fragment
10	IFN	interferon
	IL12	interleukin 12
	i.p.	intraperitoneal
	MSLN	mesothelin
	NK	natural killer
15	PBMC	peripheral blood mononuclear cells
	rFc	recombinant constant fragment

II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional usage.

20 Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

25 In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Antibody: A polypeptide ligand comprising at least a light chain or heavy chain immunoglobulin variable region which recognizes and binds (such as specifically recognizes and specifically binds) an epitope of an antigen, such as mesothelin, or a fragment thereof.

30 Immunoglobulin molecules are composed of a heavy and a light chain, each of which has a variable region, termed the variable heavy (VH) region and the variable light (VL) region. Together, the VH region and the VL region are responsible for binding the antigen recognized by the antibody.

Antibodies include intact immunoglobulins and the variants and portions of antibodies well known in the art, such as single-domain antibodies (*e.g.* VH domain antibodies), Fab fragments,

Fab' fragments, F(ab)'₂ fragments, single chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker, while in dsFvs, the chains have been mutated to introduce a disulfide bond to stabilize the

association of the chains. The term "antibody" also includes genetically engineered forms such as chimeric antibodies (for example, humanized murine antibodies) and heteroconjugate antibodies (such as bispecific antibodies). See also, *Pierce Catalog and Handbook*, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, J., *Immunology*, 3rd Ed., W. H. Freeman & Co., New York, 1997.

Typically, a naturally occurring immunoglobulin has heavy (H) chains and light (L) chains interconnected by disulfide bonds. There are two types of light chain, lambda (λ) and kappa (κ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE.

Each heavy and light chain contains a constant region and a variable region, (the regions are also known as "domains"). In combination, the heavy and the light chain variable regions specifically bind the antigen. Light and heavy chain variable regions contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs." The extent of the framework region and CDRs has been defined according to Kabat *et al.* (see, Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, U.S. Department of Health and Human Services, 1991) and the ImMunoGeneTics database (IMGT) (see, Lefranc, *Nucleic Acids Res* 29:207-9, 2001). The Kabat and IMGT databases are maintained online. The sequences of the framework regions of different light or heavy chains are relatively conserved within a species, such as humans. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three-dimensional space.

The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are often identified by the chain in which the particular CDR is located. Thus, a VHCDR3 (or H-CDR3) is located in the variable domain of the heavy chain of the antibody in which it is found, whereas a VLCDR1 (or L-CDR1) is the CDR1 from the variable domain of the light chain of the antibody in which it is found. An antibody that binds mesothelin, for example, will have specific VHregion and VLregion sequences, and thus specific CDR sequences. Antibodies with different specificities (*i.e.* different combining sites for different antigens) have different CDRs. Although it is the CDRs that vary from antibody to antibody, only a limited

number of amino acid positions within the CDRs are directly involved in antigen binding. These positions within the CDRs are called specificity determining residues (SDRs).

References to " v_H " or "VH" refer to the variable region of an immunoglobulin heavy chain, including that of an Fv, scFv, dsFv or Fab. References to " v_L " or "VL" refer to the variable region of an immunoglobulin light chain, including that of an Fv, scFv, dsFv or Fab.

A "monoclonal antibody" is an antibody produced by a single clone of B-lymphocytes or by a cell into which the light and/or heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. Monoclonal antibodies include humanized monoclonal antibodies.

A "chimeric antibody" has framework residues from one species, such as human, and CDRs (which generally confer antigen binding) from another species, such as a murine antibody that specifically binds mesothelin.

A "human" antibody (also called a "fully human" antibody) is an antibody that includes human framework regions and all of the CDRs from a human immunoglobulin. In one example, the framework and the CDRs are from the same originating human heavy and/or light chain amino acid sequence. However, frameworks from one human antibody can be engineered to include CDRs from a different human antibody. A "humanized" immunoglobulin is an immunoglobulin including a human framework region and one or more CDRs from a non-human (for example a mouse, rabbit, rat, or synthetic) immunoglobulin. The non-human immunoglobulin providing the CDRs is termed a "donor," and the human immunoglobulin providing the framework is termed an "acceptor." In one embodiment, all the CDRs are from the donor immunoglobulin in a humanized immunoglobulin. Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, *i.e.*, at least about 85-90%, such as about 95% or more identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. A humanized antibody binds to the same antigen as the donor antibody that provides the CDRs. The acceptor framework of a humanized immunoglobulin or antibody may have a limited number of substitutions by amino acids taken from the donor framework. Humanized or other monoclonal antibodies can have additional conservative amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions. Humanized immunoglobulins can be constructed by means of genetic engineering (see for example, U.S. Patent No. 5,585,089).

Binding affinity: Affinity of an antibody for an antigen. In one embodiment, affinity is calculated by a modification of the Scatchard method described by Frankel *et al.* (*Mol. Immunol.*, 16:101-106, 1979). In another embodiment, binding affinity is measured by an antigen/antibody dissociation rate. In another embodiment, a high binding affinity is measured by a competition radioimmunoassay. In another embodiment, binding affinity is measured by ELISA. An antibody that "specifically binds" an antigen (such as mesothelin) is an antibody that binds the antigen with high affinity and does not significantly bind other unrelated antigens.

Breast cancer: A type of cancer that forms in tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk). **Triple negative breast cancer** refers to a type of breast cancer in which the cancer cells do not express estrogen receptors, progesterone receptors or significant levels of HER2/neu protein. Triple negative breast cancer is also called ER-negative PR-negative HER2/neu-negative breast cancer.

Chemotherapeutic agent: Any chemical agent with therapeutic usefulness in the treatment of diseases characterized by abnormal cell growth. Such diseases include tumors, neoplasms, and cancer as well as diseases characterized by hyperplastic growth such as psoriasis. In one embodiment, a chemotherapeutic agent is an agent of use in treating mesothelioma or another tumor, such as stomach cancer, squamous cell carcinomas, prostate cancer, pancreatic cancer, lung cancer, cholangiocarcinoma, breast cancer (such as triple negative breast cancer) or ovarian cancer. In one embodiment, a chemotherapeutic agent is a radioactive compound. One of skill in the art can readily identify a chemotherapeutic agent of use (see for example, Slapak and Kufe, *Principles of Cancer Therapy*, Chapter 86 in Harrison's Principles of Internal Medicine, 14th edition; Perry *et al.*, *Chemotherapy*, Ch. 17 in Abeloff, *Clinical Oncology* 2nd ed., © 2000 Churchill Livingstone, Inc; Baltzer, L., Berkery, R. (eds.): *Oncology Pocket Guide to Chemotherapy*, 2nd ed. St. Louis, Mosby-Year Book, 1995; Fischer, D.S., Knobf, M.F., Durivage, H.J. (eds): *The Cancer Chemotherapy Handbook*, 4th ed. St. Louis, Mosby-Year Book, 1993). Combination chemotherapy is the administration of more than one agent to treat cancer. One example is the administration of an antibody (or immunocytokine) that binds mesothelin used in combination with a radioactive or chemical compound.

Cholangiocarcinoma: A type of cancer that develops in cells that line the bile ducts in the liver.

Conservative variant: "Conservative" amino acid substitutions are those substitutions that do not substantially affect or decrease the affinity of a protein, such as an antibody to mesothelin, or a fusion protein such as an immunocytokine specific for mesothelin. For example, an antibody (or fragment thereof) that specifically binds mesothelin can include at most about 1, at most about

2, at most about 5, at most about 10, or at most about 15 conservative substitutions and specifically bind a mesothelin polypeptide. The term "conservative variant" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid, provided that antibody specifically binds mesothelin. Non-conservative substitutions are those that reduce an activity or binding to mesothelin.

Conservative amino acid substitution tables providing functionally similar amino acids are well known to one of ordinary skill in the art. The following six groups are examples of amino acids that are considered to be conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Complementarity determining region (CDR): Amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native Ig binding site. The light and heavy chains of an Ig each have three CDRs, designated L-CDR1, L-CDR2, L-CDR3 and H-CDR1, H-CDR2, H-CDR3, respectively.

Degenerate variant: In the context of the present disclosure, a "degenerate variant" refers to a polynucleotide encoding a mesothelin-specific antibody or immunocytokine that includes a sequence that is degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included as long as the amino acid sequence of the mesothelin antibody or immunocytokine that binds mesothelin encoded by the nucleotide sequence is unchanged.

Epitope: An antigenic determinant. These are particular chemical groups or peptide sequences on a molecule that are antigenic, *i.e.* that elicit a specific immune response. An antibody specifically binds a particular antigenic epitope on a polypeptide, such as mesothelin.

Framework region: Amino acid sequences interposed between CDRs. Framework regions include variable light and variable heavy framework regions. The framework regions serve to hold the CDRs in an appropriate orientation for antigen binding.

Host cells: Cells in which a vector can be propagated and its DNA expressed. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations

that occur during replication. However, such progeny are included when the term "host cell" is used.

Immune response: A response of a cell of the immune system, such as a B cell, T cell, or monocyte, to a stimulus. In one embodiment, the response is specific for a particular antigen (an "antigen-specific response"). In one embodiment, an immune response is a T cell response, such as a CD4⁺ response or a CD8⁺ response. In another embodiment, the response is a B cell response, and results in the production of specific antibodies.

Immunocytokine: A covalent linkage of a cytokine to an antibody or functional fragment thereof.

Interleukin-12 (IL12): A disulfide-linked heterodimeric cytokine composed of two subunits - p35 and p40. IL12 acts primarily on T cells and natural killer cells and is required for the T-cell-independent induction of IFN- γ . IL12 also plays an important role in the differentiation of both Th1 and Th2 cells. IL12 has potent antitumor, anti-angiogenic and anti-metastatic activities. Nucleotide and amino acid sequences for the p35 subunit of IL12 are publically available, such as under NCBI Gene ID numbers 3592 (human), 16159 (mouse), 84405 (rat), 403977 (dog), 493741 (cat), 703205 (rhesus macaque), 443064 (sheep), 460816 (chimpanzee) and 407090 (chicken). IL12 p40 sequences are also publically available, such as under NCBI Gene ID numbers 3593 (human), 16160 (mouse), 64546 (rat), 403976 (dog), 768273 (cat), 694747 (rhesus macaque), 443472 (sheep), 471723 (chimpanzee) and 404671 (chicken). Exemplary human and mouse p35 and p40 sequences are set forth herein as SEQ ID NOs: 7-14.

Isolated: An "isolated" biological component, such as a nucleic acid, protein (including antibodies), organelle or cell has been substantially separated or purified away from other biological components in the environment (such as a cell or organism) in which the component naturally occurs, *i.e.*, other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Linker: In some cases, a linker is a peptide within an antibody binding fragment (such as an Fv fragment) which serves to indirectly bond the variable heavy chain to the variable light chain. "Linker" can also refer to a peptide serving to link a targeting moiety, such as an antibody, to an effector molecule, such as a cytotoxin or a cytokine.

The terms "conjugating," "joining," "bonding" or "linking" refer to making two polypeptides into one contiguous polypeptide molecule, or to covalently attaching a radionuclide or other molecule to a polypeptide, such as an scFv. In the specific context, the terms include

reference to joining a ligand, such as an antibody moiety, to an effector molecule, such as a cytokine. The linkage can be either by chemical or recombinant means. "Chemical means" refers to a reaction between the antibody moiety and the effector molecule such that there is a covalent bond formed between the two molecules to form one molecule.

5 **Lung cancer:** Cancer that forms in tissues of the lung, usually in the cells lining air passages. The two main types are small cell lung cancer and non-small cell lung cancer (NSCLC). These types are diagnosed based on how the cells look under a microscope.

Mammal: This term includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

10 **Mesothelin:** A 40 kDa cell-surface glycosylphosphatidylinositol (GPI)-linked glycoprotein. The human mesothelin protein is synthesized as a 71 kD precursor which is then proteolytically processed. The 31 kD amino terminus of mesothelin is secreted and is referred to as megakaryocyte potentiating factor (Yamaguchi *et al.*, *J. Biol. Chem.* 269:805-808, 1994). The 40 kD carboxyl terminus remains bound to the membrane as mature mesothelin (Chang *et al.*, *Natl. Acad. Sci. USA* 93:136-140, 1996). Exemplary nucleic acid and amino acid sequences of mesothelin are as described in PCT Publication No. WO 97/25,068; U.S. Patent No. 6,083,502; Chang and Pastan, *Int. J. Cancer* 57:90, 1994; Chang and Pastan, *Proc. Natl. Acad. Sci. USA* 93:136, 1996; Brinkmann *et al.*, *Int. J. Cancer* 71:638, 1997; and Chowdhury *et al.*, *Mol. Immunol.* 34:9, 1997. Mesothelin also refers to mesothelin proteins or polypeptides which remain
15 intracellular as well as secreted and/or isolated extracellular mesothelin protein. A "**mesothelin-expressing cancer**" refers to a cancer in which the cancer cells express higher levels of mesothelin than a control, such as non-cancer cells or a reference value. Expression of mesothelin can be detected using, for example, any immunoassay known in the art, such as by ELISA, Western blot or immunohistochemistry of tumor cells (such as cells obtained by biopsy). Several types of cancer
20 are known in the art to express high levels of mesothelin, thus a mesothelin-expressing cancer can also be identified by diagnosing a subject with a particular type of cancer that is known to express mesothelin, such as mesothelioma, stomach cancer, squamous cell carcinomas, prostate cancer, pancreatic cancer, lung cancer, cholangiocarcinoma, breast cancer or ovarian cancer.

Mesothelioma: A type of neoplasm derived from the lining cells of the pleura and
30 peritoneum which grows as a thick sheet covering the viscera, and is composed of spindle cells or fibrous tissue which may enclose gland-like spaces lined by cuboidal cells. Mesotheliomas often originate in the tissue lining the lung, heart or abdomen. In some cases, mesotheliomas are caused by exposure to asbestos.

Neoplasia, malignancy, cancer or tumor: A neoplasm is an abnormal growth of tissue or cells that results from excessive cell division. Neoplastic growth can produce a tumor. The amount of a tumor in an individual is the "tumor burden" which can be measured as the number, volume, or weight of the tumor. A tumor that does not metastasize is referred to as "benign." A tumor that
5 invades the surrounding tissue and/or can metastasize is referred to as "malignant." In some embodiments of the present disclosure, the cancer is mesothelioma, stomach cancer, squamous cell carcinomas, prostate cancer, pancreatic cancer, lung cancer, cholangiocarcinoma, breast cancer or ovarian cancer.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the
10 second nucleic acid sequence. For instance, a promoter, such as the CMV promoter, is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Ovarian cancer: Cancer that forms in tissues of the ovary (one of a pair of female reproductive glands in which the ova, or eggs, are formed). Most ovarian cancers are either ovarian epithelial carcinomas (cancer that begins in the cells on the surface of the ovary) or malignant germ cell tumors (cancer that begins in egg cells).
15

Pancreatic cancer: A disease in which malignant (cancer) cells are found in the tissues of
20 the pancreas. Pancreatic cancer is also called exocrine cancer.

Pharmaceutical agent: A chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject or a cell.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers of use are conventional. *Remington's Pharmaceutical Sciences*, by E.W. Martin, Mack Publishing Co.,
25 Easton, PA, 15th Edition, 1975, describes compositions and formulations suitable for pharmaceutical delivery of the immunocytokines disclosed herein.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline,
30 balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (such as powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor

amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Preventing, treating or ameliorating a disease: "Preventing" a disease refers to inhibiting the full development of a disease. "Treating" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop, such as a reduction in tumor burden or a decrease in the number or size of metastases. "Ameliorating" refers to the reduction in the number or severity of signs or symptoms of a disease, such as cancer.

Prostate cancer: Cancer that forms in tissues of the prostate (a gland in the male reproductive system found below the bladder and in front of the rectum).

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein is more enriched than the peptide or protein is in its natural environment within a cell. In one embodiment, a preparation is purified such that the protein or peptide represents at least 50% of the total peptide or protein content of the preparation. Substantial purification denotes purification from other proteins or cellular components. A substantially purified protein is at least 60%, 70%, 80%, 90%, 95% or 98% pure. Thus, in one specific, non-limiting example, a substantially purified protein is 90% free of other proteins or cellular components.

Recombinant: A recombinant nucleic acid or protein is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques.

Sample (or biological sample): A biological specimen containing genomic DNA, RNA (including mRNA), protein, or combinations thereof, obtained from a subject. Examples include, but are not limited to, peripheral blood, tissue, cells, urine, saliva, tissue biopsy, fine needle aspirate, surgical specimen, and autopsy material.

Sequence identity: The similarity between amino acid or nucleic acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or variants of a polypeptide or nucleic acid molecule will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988; Higgins and Sharp, *Gene* 73:237, 1988; Higgins and Sharp, *CABIOS* 5:151, 1989; Corpet *et al*, *Nucleic Acids Research* 16:10881, 1988; and Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988. Altschul *et al*, *Nature Genet.* 6:1 19, 1994, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al*, *J. Mol. Biol.* 215:403, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. A description of how to determine sequence identity using this program is available on the NCBI website on the internet.

Homologs and variants of a ν_L or a ν_H of an antibody that specifically binds mesothelin or a fragment thereof are typically characterized by possession of at least about 75%, for example at least about 80%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity counted over the full length alignment with the amino acid sequence of the antibody using the NCBI Blast 2.0, gapped blastp set to default parameters. For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer than around 30 amino acids), the alignment can be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs and variants will typically possess at least 80% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85% or at least 90% or 95% depending on their similarity to the reference sequence. Methods for determining sequence identity over such short windows are available at the NCBI website on the internet. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided.

Squamous cell carcinoma: A malignant neoplasm derived from stratified squamous epithelium, but which may also occur in sites such as bronchial mucosa where glandular or

columnar epithelium is normally present. Squamous cell carcinoma is the most common type of skin cancer.

Stomach cancer: Cancer that forms in tissues lining the stomach; also called gastric cancer.

5 **Subject:** Living multi-cellular vertebrate organisms, a category that includes both human and veterinary subjects, including human and non-human mammals.

10 **Therapeutically effective amount:** A quantity of a specific substance sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to inhibit or suppress growth of a tumor. In one embodiment, a therapeutically effective amount is the amount necessary to eliminate, reduce the size, or prevent metastasis of a tumor. When administered to a subject, a dosage will generally be used that will achieve target tissue concentrations (for example, in tumors) that has been shown to achieve a desired *in vitro* effect.

15 **Vector:** A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

20 Unless otherwise explained, 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 disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. "Comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

30 **III. Introduction**

Mesothelin is a glycosylphosphatidylinositol-anchored glycoprotein that is highly expressed on the cell surface of malignant mesothelioma and other cancers. Monoclonal antibodies against mesothelin are being evaluated for the treatment of mesothelin-expressing cancer.

Immunocytokines represent a new class of armed antibodies. To improve the anti-tumor immune

response in current mesothelin-targeted antibody therapy, described herein is the development of an immunocytokine based on interleukin-12 (IL12), which possesses potent anti-tumor activity in a wide variety of solid tumors, and the mesothelin-specific SSI Fv. The IL12-SSI (Fv) immunocytokine was produced in insect cells using a baculovirus expression system. The SSI single-chain Fv was fused to the C terminus of the p35 subunit of IL12 through a short linker. Disclosed herein is the finding that the single-chain IL12-SSI (Fv) immunocytokine bound native mesothelin proteins on mesothelioma (NCI-H226) and ovarian (OVCAR-3) cells as well as recombinant mesothelin on A431/H9 cells. The recombinant immunocytokine retained bioactivity of IL12 and significantly inhibited human malignant mesothelioma (NCI-H226) grown in the peritoneal cavity of nude mice. IL12-SSI (Fv) is the first reported immunocytokine targeting mesothelin-positive tumors.

IV. Mesothelin-Specific Immunocytokines

Provided herein are mesothelin-specific immunocytokines comprising interleukin-12 (IL12) subunits p35 and p40 linked to a mesothelin-specific antibody or antibody fragment comprising the CDR sequences of the mesothelin-specific antibody SSI. The IL12-SSI immunocytokine disclosed herein has significant advantages compared to other antibody-based therapeutics for mesothelin-expressing cancer, such as the SSIP immunotoxin. *Pseudomonas* exotoxin (PE), which the human body identifies as a foreign antigen, is highly immunogenic and induces neutralizing antibodies in patients treated with SSIP, thus limiting the therapeutic potential of SSIP. In contrast, administration of human IL12, a self-antigen, is not likely to induce an immune response. Even a slight decrease in immunogenicity of an immunocytokine (relative to SSIP) would have a substantial positive impact on therapeutic efficacy. Furthermore, the mesothelin-expressing tumor xenograft data disclosed in the Examples below demonstrates that a low dose of IL12-SSI (0.4 mg/kg) is just as effective as a higher dose of IL12-SSI (1.6 mg/kg), indicating that IL12-SSI can be administered in much smaller doses than SSIP and yet retain efficacy in reducing tumor growth and spread. Moreover, linking IL12 to the SSI scFv is believed to not only minimize (or eliminate) the toxic side effects associated with administering IL12 alone, but it allows for the specific targeting of higher levels of IL12 to tumor cells.

The nucleotide and amino acid sequences of the VH and VL domains of SSI, as well as the positions of the CDRs, are provided below (and shown in FIG. 5A and FIG. 5B):

SSI VH nucleotide sequence (SEP ID NO: 1)

5 CAGGTGCAGCTGCAGCAGTCTGGGCCTGAGCTGGAGAAGCCTGGCGCTTCAGTGAAGA
 TATCCTGCAAGGCTTCTGGTTACTCATTCAGTGGCTACACCATGAACTGGGTGAAGCAG
 AGCCATGGAAAGAGCCTTGAGTGGATTGGACTTATTACTCCTTACAATGGTGCTTCTAG
 CTACAACCAGAAGTTCAGGGGGCAAGGCCACATTAAGTGTAGACAAGTCATCCAGCACA
 GCCTACATGGACCTCCTCAGTCTGACATCTGAAGACTCTGCAGTCTATTTCTGTGCAAG
 10 GGGGGGTTACGACGGGAGGGGTTTTGACTACTGGGGCCAAGGGACCACGGTCACCGT
 CTCCTCA

SSI VH amino acid sequence (SEP ID NO: 2)

15 QVQLQQSGPELEKPGASVKISCKASGYSFTGYTMNWVKQSHGKSLEWIGLITPYNGASSY
 NQKFRGKATLTVDKS SSTAYMDLLSLTSEDS AVYFCARGGYDGRGFD YWGQTTVTVS S

SSI VL nucleotide sequence (SEP ID NP: 3)

20 GACATCGAGCTCACTCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCA
 CCATGACCTGCAGTGCCAGCTCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGTC
 AGGCACCTCCCCCAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCA
 GGTCGCTTCAGTGGCAGTGGGTCTGGAACTCTTACTCTCTCACAATCAGCAGCGTGG
 AGGCTGAAGATGATGCAACTTATTACTGCCAGCAGTGGAGTAAGCACCTCTCACGTT
 CGGTGCTGGGACAAAGTTGGAAATAAAA

SSI VL amino acid sequence (SEP ID NP: 4)

25 DIELTQSPAIMASAPGEKVTMTCSASSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPGRF
 SGSGSGNSYSLTISSVEAEDDATYYCQQWSKHPLTFGAGTKLEIK

Amino acid positions of the SSI VH (SEQ ID NO: 2) and VL (SEQ ID NO: 4) CDRs

	H-CDR1	H-CDR2	H-CDR3	L-CDR1	L-CDR2	L-CDR3
Kabat	31-35	50-66	99-108	24-33	49-55	88-96
IMGT	26-33	51-58	97-108	25-31	49-51	88-96

30 Provided herein is a conjugate comprising IL12 subunits p35 and p40 linked to a
 mesothelin-specific antibody or antibody fragment comprising a variable heavy (VH) domain and a
 variable light (VL) domain. In some embodiments, the VH domain comprises amino acid residues
 31-35, 50-66 and 99-108 of SEQ ID NO: 2 and/or the VL domain comprises amino acid residues
 24-33, 49-55 and 88-96 of SEQ ID NO: 4. In other embodiments, the VH domain comprises amino
 35 acid residues 26-33, 51-58 and 97-108 of SEQ ID NO: 2 and/or the VL domain comprises amino
 acid residues 25-31, 49-51 and 88-96 of SEQ ID NO: 4.

In some examples, the antibody or antibody fragment of the conjugate comprises the CDR
 sequences of the SSI antibody (as determined by Kabat or IMGT) and the amino acid sequence of
 the VH domain is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%,

at least 98% or at least 99% identical to SEQ ID NO: 2. In particular examples, the amino acid sequence of VH domain comprises or consists of SEQ ID NO: 2.

In some examples, the antibody or antibody fragment of the conjugate comprises the CDR sequences of the SSI antibody (as determined by Kabat or IMGT) and the amino acid sequence of the VL domain is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 4. In particular examples, the amino acid sequence of VL domain comprises or consists of SEQ ID NO: 4.

In some embodiments, the conjugate comprises an antibody fragment, such as but not limited to, a Fab fragment, a Fab' fragment, a F(ab)₂ fragment, a single chain variable fragment (scFv), or a disulfide stabilized variable fragment (dsFv). In some examples, the antibody fragment is a scFv. In particular non-limiting examples, the amino acid sequence of the scFv comprises SEQ ID NO: 6 and is encoded by the nucleotide sequence of SEQ ID NO: 5 (see also FIG. 5C):

scFv SSI nucleotide sequence (SEP ID NO: 5)

15 ATGGCCCAGGTGC AGCTGCAGCAGTCTGGGCCTGAGCTGGAGAAGCCTGGCGCTTC AG
TGAAGATATCCTGCAAGGCTTCTGGTTACTCATTCCTGGCTACACCATGAACTGGGTG
AAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGACTTATTACTCCTTACAATGGTG
CTTCTAGCTACAACCAGAAGTTCAGGGGCAAGGCCACATTAAGTGTAGACAAGTCATC
CAGCACAGCCTACATGGACCTCCTCAGTCTGACATCTGAAGACTCTGCAGTCTATTTCT
20 GTGCAAGGGGGGGTTACGACGGGAGGGGTTTTGACTACTGGGGCCAAGGGACCACGG
TCACCGTCTCCTCAGGTGTAGGCGGTTGAGGCGGCGGTGGCTCTGGCGGTGGCGGATC
GGACATCGAGCTCACTCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTC
ACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGT
CAGGCACCTCCCCAAAAGATGGATTTATGACACATCCAACTGGCTTCTGGAGTCCC
25 AGGTCGCTTCAGTGGCAGTGGGTCTGGAACTCTTACTCTCTCACAATCAGCAGCGTG
GAGGCTGAAGATGATGCAACTTATTACTGCCAGCAGTGGAGTAAGCACCTCTCACGT
TCGGTGCTGGGACAAAGTTGGAAATAAAA

scFv SSI amino acid sequence (SEP ID NO: 6)

30 MAQVQLQQSGPELEKPGASVKISCKASGYSTGYTMNWVKQSHGKSLEWIGLITPYNGAS
SYNQKFRGKATLTVDKSSSTAYMDLLSLTSEDSAVYFCARGGYDGRGFDYWGQGTITVTV
SSGVGGSGGGSGGGGSDIELTQSPAISASPGEKVTMTCSASSSVSYMHWYQQKSGTSP
KRWIYDTSKLASGVPRFSGSGSGNSYSLTISSVEAEDDATYYCQQWSKHPLTFGAGTKLE
IK

In other embodiments, the antibody is an immunoglobulin molecule. In particular examples, the antibody is an IgG.

In some embodiments, the IL12 is mammalian IL12, such as but not limited to, human or mouse IL12. In one non-limiting example, the IL12 is human IL12. IL12 sequences from a variety of species are publically available (such as from GenBank™), including human and mouse sequences.

Exemplary mouse and human IL12 sequences of the conjugates provided herein include the following:

Mouse p35 nucleotide sequence (SEP ID NO: 7)

5 AGGGTCATTCCAGTCTCTGGACCTGCCAGGTGTCTTAGCCAGTCCCGAAACCTGCTGA
AGACCACAGATGACATGGTGAAGACGGCCAGAGAAAACTGAAACATTATTCCTGCA
CTGCTGAAGACATCGACCATGAAGACATCACACGGGACCAAACCAGCACATTGAAGA
CCTGTTTACCACTGGAACCTACACAAGAACGAGAGTTGCCTGGCTACTAGAGAGACTTC
TTCCACAACAAGAGGGAGCTGCCTGCCCCACAGAAGACCTCTTTGATGATGACCCTG
10 TGCCTTGGTAGCATCTATGAGGACTTGAAGATGTACCAGACAGAGTTCCAGGCCATCA
ACGCAGCACTTCAGAATCACAACCATCAGCAGATCATTCTGGACAAGGGTATGCTGGT
GGCTATTGATGAGCTGATGCAGTCTCTGAATCATAATGGCGAGACTCTGCGCCAGAAA
CCTCCTGTGGGAGAAGCAGACCCTTACAGAGTGAAAATGAAGCTCTGCATCCTGCTTC
ACGCCTTCAGCACCCGCGTCGTGACCATCAACAGGGTGATGGGCTATCTGAGTTCCGC
15 C

Mouse p35 amino acid sequence (SEP ID NO: 8)

RVIPVSGPARCLSQSRNLLKTTDDMVKTAREKLKHYSCTAEDIDHEDITRDQTSTLKTCLPL
ELHKNESCLATRETSSTRGSCLPPQKTSMLMTLCLGSIYEDLKMYQTEFQAINAALQNHN
HQQIILDKGMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVV TIN
20 RVMGYLSSA

Mouse p40 nucleotide sequence (SEP ID NP: 9)

ATGTGTCCTCAGAAGCTAACCATCTCCTGGTTTGCCATCGTTTTGCTGGTGTCTCCACTC
ATGGCCATGTGGGAGCTGGAGAAAGACGTTTATGTTGTAGAGGTGGACTGGACTCCCG
25 ATGCCCCTGGAGAAACAGTGAACCTCACCTGTGACACGCCTGAAGAAGATGACATCAC
CTGGACCTCAGACCAGAGACATGGAGTCATAGGCTCTGGAAAGACCCTGACCATCACT
GTCAAAGAGTTTCTAGATGCTGGCCAGTACACCTGCCACAAAGGAGGCGAGACTCTGA
GCCACTCACATCTGCTGCTCCACAAGAAGGAAAATGGAATTTGGTCCACTGAAATTTT
AAAAAATTTCAAAAACAAGACTTTCTGAAGTGTGAAGCACCAAATTACTCCGGACGG
30 TTCACGTGCTCATGGCTGGTGCAAAGAAACATGGACTTGAAGTTCAACATCAAGAGCA
GTAGCAGTTCCCTGACTCTCGGGCAGTGACATGTGGAATGGCGTCTCTGTCTGCAGA
GAAGGTCACACTGGACCAAAGGGACTATGAGAAGTATTCAGTGTCTGCCAGGAGGA
TGTCACCTGCCCAACTGCCGAGGAGACCCTGCCATTGAAGTGGCGTTGGAAGCACGG
CAGCAGAATAAATATGAGAACTACAGCACCAGCTTCTTCATCAGGGACATCATCAAAC
35 CAGACCCGCCCAAGAACTTGCAGATGAAGCCTTTGAAGAACTCACAGGTGGAGGTCA
GCTGGGAGTACCCTGACTCCTGGAGCACTCCCCATTCTACTTCTCCCTCAAGTTCTTT
GTTCGAATCCAGCGCAAGAAAGAAAAGATGAAGGAGACAGAGGAGGGGTGTAACCA
GAAAGGTGCGTTCTCGTAGAGAAGACATCTACCGAAGTCCAATGCAAAGGCGGGAA
TGTCTGCGTGCAAGCTCAGGATCGCTATTACAATTCTCATGCAGCAAGTGGGCATGT
40 GTTCCTGCAGGGTCCGATCC

Mouse p40 amino acid sequence (SEP ID NP: 10)

MCPQKLTISWFAIVLLVSPLMAMWELEKDVIYVVEVDWTPDAPGETVNLTCDTPEEDDIT
WTSDQRHGVIGSGKLTITVKEFLDAGQYTCHKGGETLSHSHLLHKKENGIWSTEILKNF
45 KNKTFLLKCEAPNYSGRFTCSWL VQRNMDLKFNIKSSSSSPDSRAVTCGMASLSAEKVTL D
QRDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYSTSFIRDIKPDPPKNLQMK
PLKNSQVEVSWEYPDSWSTPHSYFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVEKTSTE
VQCKGGNVCVQAQDRYYNSSCSKWACVPCRVR S

Human p35 nucleotide sequence (SEQ ID NO: 11)

ATGTGGCCCCCTGGGTCAGCCTCCCAGCCACCGCCCTCACCTGCCGCGGCCACAGGTC
 TGCATCCAGCGGCTCGCCCTGTGTCCCTGCAGTGCCGGCTCAGCATGTGTCCAGCGCGC
 5 AGCCTCCTCCTTGTGGCTACCCTGGTCCTCCTGGACCACCTCAGTTTGGCCAGAAACCT
 CCCC GTGGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCAAAACCTGC
 TGAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTACCCTTG
 CACTTCTGAAGAGATTGATCATGAAGATATCACAAAAGATAAAAACCAGCACAGTGGA
 GGCCTGTTTACCATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACC
 10 TCTTTCATAACTAATGGGAGTTGCCTGGCCTCCAGAAAGACCTCTTTTATGATGGCCCT
 GTGCCTTAGTAGTATTTATGAAGACTTGAAGATGTACCAGGTGGAGTTCAAGACCATG
 AATGCAAAGCTTCTGATGGATCCTAAGAGGCAGATCTTTCTAGATCAAAACATGCTGG
 CAGTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAGTGAGACTGTGCCACAAAA
 ATCCTCCCTTGAAGAACCGGATTTTTATAAAACTAAAATCAAGCTCTGCATACTTCTTC
 15 ATGCTTTCAGAAATTCGGGCAGTGACTATTGATAGAGTGATGAGCTATCTGAATGCTTCC
 TAA

Human p35 amino acid sequence (SEQ ID NO: 12)

MWPPGSASQPPSPAAATGLHPAARPVSLQCRLSMCPARSLLVATLVLLDHL SLARNLPV
 20 ATPDPGMFPC LHHSQNLLRAVS NMLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEACLPL
 ELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIEDLKMYQVEFKTMNAKLLMDP
 KRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDR
 VMSYLNAS

Human p40 nucleotide sequence (SEP ID NO: 13)

ATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCTGGCATCTCCCCTC
 GTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAAATTGGATTGGTATCCGG
 ATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCTGAAGAAGATGGTATCAC
 CTGGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCTGACCATCCAA
 30 GTCAAAGAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAA
 GCCATTCGCTCCTGCTGCTTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATTTA
 AAGGACCAGAAAGAACCCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAATTAT
 TCTGGACGTTTCACCTGCTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGT
 CAAAAGCAGCAGAGGCTCTTCTGACCCCCAAGGGGTGACGTGCGGAGCTGCTACACTC
 35 TCTGCAGAGAGAGTCAGAGGGGACAACAAGGAGTATGAGTACTCAGTGGAGTGCCAG
 GAGGACAGTGCCTGCCAGCTGCTGAGGAGAGTCTGCCCATTTAGGTCATGGTGGATG
 CCGTTCACAAGCTCAAGTATGAAAACCTACACCAGCAGCTTCTTCATCAGGGACATCAT
 CAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAAGAATTCTCGGCAGGTG
 GAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTACTTCTCCCTGAC
 40 ATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAGAAAAGAAAGATAGAGTCTTCAC
 GGACAAGACCTCAGCCACGGTCATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGCC
 CAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGCAGTTAG

Human p40 amino acid sequence (SEQ ID NO: 14)

45 MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGIT
 WTLDSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKD
 QKEPKNKTFLRCEAKNYSGRFTCWLLTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERV
 RGDNKEYEYSVEQCEDSACPAAEESLPIEVMVDAVHKLKYENYTSFFIRDIIKPDPPKNLQ

LKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRK
NASISVRAQDRYYSSSWSEWASVPCS

In some examples in which the IL12 is mouse IL12, the amino acid sequence of the p35 subunit is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 8 and/or the amino acid sequence of the p40 subunit is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 10. In particular non-limiting examples, the amino acid sequence of the p35 subunit comprises or consists of SEQ ID NO: 8 and/or the amino acid sequence of the p40 subunit comprises or consists of SEQ ID NO: 10.

In other examples in which the IL12 is human IL12, the amino acid sequence of the p35 subunit is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 12 and/or the amino acid sequence of the p40 subunit is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 14. In particular non-limiting examples, the amino acid sequence of the p35 subunit comprises or consists of SEQ ID NO: 12 and/or the amino acid sequence of the p40 subunit comprises or consists of SEQ ID NO: 14.

The IL12 p35 and p40 subunits can be linked to the antibody or antibody fragment in any suitable format (*e.g.*, monomeric, homodimeric, heterodimeric) or orientation (*e.g.* to the N-terminus or to the C-terminus) so long as the immunocytokine retains sufficient IL12 biological activity and antigen-binding activity. A variety of different IL12 immunocytokine formats have been described in the art (see, *e.g.*, Gafner *et al*, *Int J Cancer* 119:2205-2212, 2006; U.S. Application Publication No. 2009/0035255; and PCT Publication No. WO 2013/014149).

In some embodiments, the p35 subunit is linked to the VH domain of the antibody or antibody fragment.

In some embodiments, the conjugate comprises in the N-terminal to C-terminal direction the p40 subunit, the p35 subunit, the VH domain and the VL domain. In some examples, the conjugate further includes a linker between the p40 subunit and the p35 subunit and/or a linker between the p35 subunit and the VH domain.

The linkers can be any molecule used to join the separate protein components of the immunocytokine. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. Where the antibody and the effector molecule are polypeptides, the linkers may be joined to the constituent amino acids through their side groups (such as through a disulfide linkage to cysteine) or to the alpha carbon amino and carboxyl groups of the terminal amino acids.

Peptide linkers can vary in size to achieve proper conformation of the fused proteins, as well as to retain biological activity of the linked proteins. In some embodiments, the peptide linker is about 2 to about 40 amino acids in length, about 4 to about 30 amino acids in length, about 4 to about 20 amino acids in length, or about 6 to about 20 amino acids in length. In some examples, the linker is about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids in length. In particular examples, the linker is 6 or 15 amino acid in length.

In some examples, the linker comprises repeating serine and glycine residues, such as one or more repeats of four serine residues and one glycine residue. In specific non-limiting examples, the linker comprises the amino acid sequence (Ser₄Gly)₃ (SEQ ID NO: 24) or the amino acid sequence GSADGG (SEQ ID NO: 25). In one non-limiting example, the linker between the p40 subunit and p35 subunit is (Ser₄Gly)₃ (SEQ ID NO: 24). In another non-limiting example, the linker between the p35 subunit and the VH domain is GSADGG (SEQ ID NO: 25).

Further provided herein are isolated nucleic acid molecules encoding an immunocytokine (conjugate) as described herein. In some embodiments, the nucleic acid molecule comprises the sequence of SEQ ID NO: 5 (the nucleotide sequence of the SSI scFv), SEQ ID NO: 7 (murine p35), or SEQ ID NO: 9 (murine p40), or any combination thereof. In some embodiments, the nucleic acid molecule comprises the sequence of SEQ ID NO: 5, SEQ ID NO: 11 (human p35) or SEQ ID NO: 13 (human p40), or any combination thereof.

Also provided are vectors comprising a nucleic acid molecule encoding an immunocytokine disclosed herein. Isolated cells comprising such vectors are also provided by the present disclosure.

V. Mesothelin-Specific Immunocytokine Compositions

Compositions are provided herein that include a mesothelin-specific immunotoxin as disclosed herein in a pharmaceutically acceptable carrier. The compositions can be prepared in unit dosage forms for administration to a subject. The amount and timing of administration are at the discretion of the treating clinician to achieve the desired outcome. The immunocytokine can be formulated for systemic or local (such as intra-tumor) administration. In one example, the immunocytokine is formulated for parenteral administration, such as intravenous administration.

The compositions for administration can include a solution of the immunocytokine dissolved in a pharmaceutically acceptable carrier, such as an aqueous carrier. A variety of aqueous carriers can be used, for example, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and

buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of immunocytokine in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the subject's needs.

A typical pharmaceutical composition for intravenous administration includes about 0.1 to 10 mg of immunocytokine per subject per day. Dosages from 0.1 up to about 100 mg per subject per day may be used, particularly if the agent is administered to a secluded site and not into the circulatory or lymph system, such as into a body cavity or into a lumen of an organ. Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science*, 19th ed., Mack Publishing Company, Easton, PA (1995).

Immunocytokines may be provided in lyophilized form and rehydrated with sterile water before administration, although they are also provided in sterile solutions of known concentration. The solution can then be added to an infusion bag containing 0.9% sodium chloride, USP, and in some cases administered at a dosage of from 0.5 to 15 mg/kg of body weight. Considerable experience is available in the art in the administration of antibody-based drugs, which have been marketed in the U.S. since the approval of **RITUXAN®** in 1997. Antibodies and immunoconjugates can be administered by slow infusion, rather than in an intravenous push or bolus. In one non-limiting example, a higher loading dose is administered, with subsequent, maintenance doses being administered at a lower level. For example, an initial loading dose of 4 mg/kg may be infused over a period of some 90 minutes, followed by weekly maintenance doses for 4-8 weeks of 2 mg/kg infused over a 30 minute period if the previous dose was well tolerated.

Controlled release parenteral formulations can be made as implants, oily injections, or as particulate systems. For a broad overview of protein delivery systems see, Banga, A.J., *Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems*, Technomic Publishing Company, Inc., Lancaster, PA, (1995). Particulate systems include microspheres, microparticles, microcapsules, nanocapsules, nanospheres, and nanoparticles. Microcapsules contain the therapeutic protein or conjugate as a central core. In microspheres the therapeutic is dispersed throughout the particle. Particles, microspheres, and microcapsules smaller than about 1 μm are generally referred to as nanoparticles, nanospheres, and nanocapsules, respectively. Capillaries have a diameter of approximately 5 μm so that only nanoparticles are administered intravenously. Microparticles are typically around 100 μm in diameter and are administered subcutaneously or intramuscularly. See, for example, Kreuter, J., *Colloidal Drug Delivery Systems*,

J. Kreuter, ed., Marcel Dekker, Inc., New York, NY, pp. 219-342 (1994); and Tice & Tabibi, *Treatise on Controlled Drug Delivery*, A. Kydonieus, ed., Marcel Dekker, Inc. New York, NY, pp. 315-339, (1992).

Polymers can be used for ion-controlled release of the immunocytokine compositions disclosed herein. Various degradable and nondegradable polymeric matrices for use in controlled drug delivery are known in the art (Langer, *Accounts Chem. Res.* 26:537-542, 1993). For example, the block copolymer, polaxamer 407, exists as a viscous yet mobile liquid at low temperatures but forms a semisolid gel at body temperature. It has been shown to be an effective vehicle for formulation and sustained delivery of recombinant interleukin-2 and urease (Johnston *et al*, *Pharm. Res.* 9:425-434, 1992; and Pec *et al.*, *J. Parent. Sci. Tech.* 44(2):58-65, 1990).

Alternatively, hydroxyapatite has been used as a microcarrier for controlled release of proteins (Ijntema *et al*, *Int. J. Pharm.* 112:215-224, 1994). In yet another aspect, liposomes are used for controlled release as well as drug targeting of the lipid-capsulated drug (Betageri *et al*, *Liposome Drug Delivery Systems*, Technomic Publishing Co., Inc., Lancaster, PA (1993)). Numerous additional systems for controlled delivery of therapeutic proteins are known (see U.S. Patent No. 5,055,303; U.S. Patent No. 5,188,837; U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; U.S. Patent No. 4,957,735; U.S. Patent No. 5,019,369; U.S. Patent No. 5,055,303; U.S. Patent No. 5,514,670; U.S. Patent No. 5,413,797; U.S. Patent No. 5,268,164; U.S. Patent No. 5,004,697; U.S. Patent No. 4,902,505; U.S. Patent No. 5,506,206; U.S. Patent No. 5,271,961; U.S. Patent No. 5,254,342 and U.S. Patent No. 5,534,496).

VI. Use of Mesothelin-Specific Immunocytokines

The immunocytokines disclosed herein can be administered to slow or inhibit the growth of tumor cells or inhibit the metastasis of tumor cells, such as mesothelioma, prostate cancer, lung cancer, stomach cancer, squamous cell carcinoma, pancreatic cancer, cholangiocarcinoma, triple negative breast cancer or ovarian cancer. In these applications, a therapeutically effective amount of an immunocytokine is administered to a subject in an amount sufficient to inhibit growth, replication or metastasis of cancer cells, or to inhibit a sign or a symptom of the cancer. Suitable subjects may include those diagnosed with a cancer that expresses mesothelin, such as, but not limited to, mesothelioma, prostate cancer, lung cancer, stomach cancer, squamous cell carcinoma, pancreatic cancer, cholangiocarcinoma, triple negative breast cancer or ovarian cancer.

In some embodiments, provided herein is a method of treating a subject with a mesothelin-expressing cancer by selecting a subject with a cancer that expresses mesothelin and administering to the subject a therapeutically effective amount of an immunocytokine (or conjugate) or

composition disclosed herein. Also provided herein is a method of inhibiting tumor growth or metastasis in a subject having a mesothelin-expressing cancer by selecting a subject having a mesothelin-expressing cancer and administering to the subject a therapeutically effective amount of an immunocytokine (or conjugate) or composition disclosed herein. In some examples, the mesothelin-expressing cancer is mesothelioma, prostate cancer, lung cancer, stomach cancer, squamous cell carcinoma, pancreatic cancer, cholangiocarcinoma, triple negative breast cancer or ovarian cancer.

A therapeutically effective amount of a mesothelin-specific immunocytokine will depend upon the severity of the disease and the general state of the patient's health. A therapeutically effective amount of the immunocytokine is that which provides either subjective relief of a symptom(s) or an objectively identifiable improvement as noted by the clinician or other qualified observer.

Administration of the immunocytokines disclosed herein can also be accompanied by administration of other anti-cancer agents or therapeutic treatments (such as surgical resection of a tumor). Any suitable anti-cancer agent can be administered in combination with the immunocytokines disclosed herein. Exemplary anti-cancer agents include, but are not limited to, chemotherapeutic agents, such as, for example, mitotic inhibitors, alkylating agents, anti-metabolites, intercalating antibiotics, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, anti-survival agents, biological response modifiers, anti-hormones (*e.g.* anti-androgens) and anti-angiogenesis agents. Other anti-cancer treatments include radiation therapy and other antibodies that specifically target cancer cells.

Non-limiting examples of alkylating agents include nitrogen mustards (such as mechlorethamine, cyclophosphamide, melphalan, uracil mustard or chlorambucil), alkyl sulfonates (such as busulfan), nitrosoureas (such as carmustine, lomustine, semustine, streptozocin, or dacarbazine).

Non-limiting examples of antimetabolites include folic acid analogs (such as methotrexate), pyrimidine analogs (such as 5-FU or cytarabine), and purine analogs, such as mercaptopurine or thioguanine.

Non-limiting examples of natural products include vinca alkaloids (such as vinblastine, vincristine, or vindesine), epipodophyllotoxins (such as etoposide or teniposide), antibiotics (such as dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, or mitomycin C), and enzymes (such as L-asparaginase).

Non-limiting examples of miscellaneous agents include platinum coordination complexes (such as cis-diamine-dichloroplatinum II also known as cisplatin), substituted ureas (such as

hydroxyurea), methyl hydrazine derivatives (such as procarbazine), and adrenocortical suppressants (such as mitotane and aminoglutethimide).

Non-limiting examples of hormones and antagonists include adrenocorticosteroids (such as prednisone), progestins (such as hydroxyprogesterone caproate, medroxyprogesterone acetate, and
5 magestrol acetate), estrogens (such as diethylstilbestrol and ethinyl estradiol), antiestrogens (such as tamoxifen), and androgens (such as testosterone propionate and fluoxymesterone). Examples of the most commonly used chemotherapy drugs include Adriamycin, Alkeran, Ara-C, BiCNU, Busulfan, CCNU, Carboplatinum, Cisplatinum, Cytosan, Daunorubicin, DTIC, 5-FU, Fludarabine, Hydrea, Idarubicin, Ifosfamide, Methotrexate, Mithramycin, Mitomycin, Mitoxantrone, Nitrogen
10 Mustard, Taxol (or other taxanes, such as docetaxel), Velban, Vincristine, VP-16, while some more newer drugs include Gemcitabine (Gemzar), Herceptin, Irinotecan (Camptosar, CPT-11), Leustatin, Navelbine, Rituxan STI-571, Taxotere, Topotecan (Hycamtin), Xeloda (Capecitabine), Zevelin and calcitriol.

Non-limiting examples of immunomodulators that can be used include AS-101 (Wyeth-
15 Ayerst Labs.), bropiramine (Upjohn), gamma interferon (Genentech), GM-CSF (granulocyte macrophage colony stimulating factor; Genetics Institute), IL-2 (Cetus or Hoffman-LaRoche), human immune globulin (Cutter Biological), IMREG (from Imreg of New Orleans, La.), SK&F 106528, and TNF (tumor necrosis factor; Genentech).

Another common treatment for some types of cancer is surgical treatment, for example
20 surgical resection of the cancer or a portion of it. Another example of a treatment is radiotherapy, for example administration of radioactive material or energy (such as external beam therapy) to the tumor site to help eradicate the tumor or shrink it prior to surgical resection.

The following examples are provided to illustrate certain particular features and/or
25 embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

EXAMPLES

Example 1: Materials and Methods

30 This example describes the materials and experimental methods used for the studies described in Example 2.

Cell lines

The human mesothelioma cell line NCI-H226, ovarian cancer cell line OVCAR-3, and epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The embryonic kidney cell line, HEK-293, was purchased from Life Technologies (Grand Island, NY). H9 cells, which are transfected A431 cells stably expressing mesothelin have been described previously (Ho *et al.*, *Clin Cancer Res* 11:3814-3820, 2005). The cell lines were maintained as adherent monolayer cultures in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 1% L-glutamine, and 1% penicillin/streptomycin (Life Technologies) and incubated in 5% CO₂ with a balance of air at 37 °C. Media was changed twice a week to examine the binding properties of IL12-SS1 (Fv). Cells were confirmed to be negative for mycoplasma. The previously generated human mesothelioma cell line LMB-H226-GL was used for the mouse xenograft model (Feng *et al.*, *J Cancer* 2:123-131, 2011). Briefly, the NCI-H226 human mesothelioma cell line was fluorescently labeled by a lentiviral vector harboring a luciferase-GFP (Luc/GFP) fusion gene driven by the RNA polymerase II promoter. After single-cell cloning by flow cytometry, a clone (names LMB-H226-GL) that stably expresses high levels of Luc/GFP was obtained.

Plasmid construction and cloning of IL12-SS1 (Fv)

Baculovirus cloning, expression, and purification of IL12-SS1 (Fv) was performed by Protein Expression Laboratory, ATP, SAIC-Frederick. pDonr253 is a Gateway Donor vector modified from pDonr201 (Life Technologies). pDonr253 replaces the kanamycin resistance gene with a gene encoding spectinomycin resistance. The oligonucleotides used in this study are listed in Table 1.

Table 1. Primers used for cloning of IL12-SS1 (Fv)

Primer	Note	Sequence	SEQ ID NO:
9228	p40 Forward	ATGTGGGAGCTGGAGAAAGACGTTTATG	15
9229	p35 Forward	CCGATCCGGTGGCGGTGGCTCGGGCGGTGGTGGG TCGGGTGGCGGCGGATCTAGGGTCATTCCAGTCT CTGGACCTGCC	16
9230	p40 Reverse	CCGACCCACCACCGCCCGAGCCACCGCCACCGGA TCGGACCCTGCAGGGAACACATGCCC	17

Primer	Note	Sequence	SEQ ID NO:
9231	SSI Forward	GGCTATCTGAGTTCCGCCGGAAGCGCTGATGGAG GTATGGCCCAGGTGCAGCTGCAGCAG	18
9233	SSI Reverse	GCCCTTGTCGTCATCGTCCTTATAATCGCCCCGTT TTATTTCCAACCTTTGTCCCAGC	19
9234	p40 Forward	CCATCTCCTGGTTTGCCATCGTTTTGCTGGTGTCT CCACTCATGGCCATGTGGGAGCTGGAGAAAGACG	20
9235	p40 Reverse	GGGGACAACCTTTGTACAAGAAAGTTGATTAATGG TGATGGTGATGGTGATGGTGGCCCTTGTCGTCATC GTCC	21
9237	Adaptor	GGGGACAACCTTTGTACAAAAAAGTTGGCACCATG TGTCCTCAGAAGCTAACCATCTCCTGGTTTGCCAT CG	22
9330	p35 Reverse	CGCTTCCGGCGGAACTCAGATAGCCCATCACCT GTTGATGGTCACGACGCGGGTGCTGAAGGCG	23

Cloning for IL12-SS1 (Fv). IL12 p40: 9228 + 9230; IL12 p35: 9229 + 9330; scFv SSI: 9231 + 9233 and IL12-SS1 (Fv): 9234 + 9235 + 9237. Primer 9229 and 9230 contained linker (Ser₄Gly)₃ (SEQ ID NO: 24), 9231 and 9330 contained linker GSADGG (SEQ ID NO: 25), Adaptor 9237 included attB 1 sequence.

5

The subunits of IL12 with SSI were synthesized (Genescript, Piscataway, NJ) and cloned into pFuse vector (Invivogen, San Diego, CA). Plasmids pFuse-p40-SSI and pFuse-SSI-p35 containing p40 and p35 of IL12 with SSI, respectively, were generated. The IL12-SS1 (Fv) fragment was constructed using triple overlap PCR from pFuse-p40-SSI and pFuse-SSI-p35.

- 10 Three separate PCR reactions were carried out to amplify the IL12 p40, IL12 p35, and SSI Fv fragments containing 24-28 bp overlapping sequences. Initial PCRs were carried out using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) as noted in Table 1 and per manufacturer's instructions. The PCR products were purified using the QiaQuick™ PCR purification kit (Qiagen, Valencia, CA), and equal amounts of the three products were mixed together in a final PCR
- 15 reaction with the primers noted "IL12-SS1 (Fv)" in Table 1.

- The p40 and p35 subunits of murine IL12 were connected with the flexible linker (Ser₄Gly)₃ (SEQ ID NO: 24), and scFv (SSI) was fused to the p35 subunit of IL12 through another flexible linker (GSADGG; SEQ ID NO: 25). IL12-SS1 (Fv) also contains Flag and 8xHis tags at the C-terminus. After 5 cycles of amplification, 200 nM of 9237 adapter primer was added and
- 20 amplification was continued for 20 additional cycles. The adapter primer, which contains the attB 1 site and part of the signal sequence, was used to attach the attB 1 site during an adapter PCR process. Conditions were the same as for the original PCR but with an extension time of 1 minute. The final PCR products were flanked by Gateway recombination signal sequences, attB1 at the 5' end and attB2 at the 3' end.

The PCR products were purified using the QiaQuick™ PCR purification kit (Qiagen), and recombined into pDonr253 with the Gateway BP recombination reaction kit, following manufacturer's instruction (Life Technologies). *E. coli* DH10B cells were transformed with the BP reactions and colonies were isolated on LB plates containing 50 µg/ml spectinomycin. The sequence-verified entry clone was subcloned by Gateway LR recombination (Life Technologies) into pDest-8 for insect cell expression. Final expression clones were verified by size and restriction digest pattern. *E. coli* DH10Bac (Life Technologies) was then transformed with the expression clones and plated on selective media containing gentamycin, kanamycin, tetracycline, IPTG, and X-gal as per the manufacturer's protocol. White colonies were selected from these plates, and bacmid DNA was recovered by alkaline lysis plasmid preparation and verified by PCR amplification across the bacmid junctions.

The expression of IL12-SS1 (Fv) in insect cells using baculovirus

The IL12-SS1 (Fv) protein was expressed in baculovirus-infected insect cells. Sf-9 cells were maintained in suspension cultures of SFX-Insect medium (Thermo Scientific, Rockford, IL). One day prior to the large-scale direct transfection, the Sf-9 cells were fed to ensure that the cells were in logarithmic growth. On transfection day, 100 ml of Sf-9 cells per transfection were set at 1.5×10^6 cells/ml in a 490 cm² roller bottle (Corning, Tewksbury, MA) and incubated (27°C at 100 rpm) in an INNOVA 4430 shaker (New Brunswick, NJ) while the transfection mixtures were prepared (30 - 60 minutes). Fifty microliters of each bacmid DNA was added to 200 µl of saline in one microcentrifuge tube and 150 µl of transfection reagent (XpressNOW, Lonza, Germany) was added to 200 µl of saline in another tube. The diluted bacmid DNAs were added to the diluted transfection reagent and the combinations were mixed gently by pipetting. The mixture was incubated for 10 minutes at room temperature and then added to the 100 ml Sf-9 suspension cultures and the bottles were immediately returned to the 27°C shaking incubator. After four days the cultures were centrifuged at 2000 rpm. The supernatants containing the recombinant baculoviruses were stored at 4°C in the dark. For protein expression, High Five (H5) cells (Life Technologies) were fed the day before infection to insure the cells were in logarithmic growth phase the following day. One liter of H5 cells (1.5×10^6 cells/ml) in SFX medium was infected with 40 ml of the p40-p35-SS1 (Fv) virus. The culture was incubated at 21°C for 72 hours at an orbit speed of 100 rpm after which the supernatant was collected for purification.

Recombinant immunocytokine protein purification

One liter of clarified culture supernatant from baculovirus-infected H5 insect cells was concentrated to 200 ml using a 30 kDa normal molecular weight cutoff (MWCO) membrane (Milipore), buffer exchanged to PBS, pH 7.2 (using tangential flow filtration), amended with 50 mM imidazole, and applied to a 5 ml Histrap™ HP column, (GE Healthcare) that had been equilibrated in PBS, pH 7.2, 50 mM imidazole, at a flow rate of 2 ml/min. The column was washed to baseline with equilibration buffer and proteins eluted in a 20-column volume gradient from 50-500 mM imidazole in PBS, pH 7.2 positive fractions (as identified by SDS-PAGE/Coomassie staining) were pooled and dialyzed to PBS, pH 7.2. Protein concentration was determined by the Bradford assay and bovine IgG as standard (Bio-Rad, Hercules, CA).

Flow cytometry

To determine binding of IL12-SS1 (Fv) to mesothelin on the cell surface, cancer cells (H226, OVAC-3, H9, A431, and 293 F cells) were grown until confluent, cells were harvested, washed, and resuspended in ice-cold PBS containing 5 % bovine serum albumin (BSA). Cells were incubated with 5 µg/mL of MN (mesothelin mAb; Rockland) or isotype antibody and PE-conjugated Anti-Histidine (Abeam, Cambridge, MA). Binding was detected using goat anti-mouse IgG conjugated with phycoerythrin (Sigma-Aldrich, St. Louis, MO). The fluorescence associated with the cells was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

To evaluate inhibition of the binding of IL12-SS1 (Fv) to A431 cells by an anti-IL12 receptor antibody, 0.5×10^6 A431 cells were incubated with 25 µg/mL of anti-IL12R $\beta 2$ antibody (Thermo Scientific, Rockford, IL) and incubated on ice for 1 hour. Five µg/mL of SS1-IL12 was then added and incubated on ice for an additional hour. After washing, the cells were stained with goat anti-Flag-FITC conjugate (Abeam, Cambridge, MA) at a 1:200 dilution on ice for 1 hour. After washing, the fluorescence associated with the cells was measured by flow cytometry.

ELISA

Nunc MaxiSorp 96-well flat-bottomed plates were incubated overnight with 10 to 5000 ng/ml purified rabbit Fc MSLN (rFc-MSLN) or rabbit Fc control in PBS, followed by an 1 hour block with 5% BSA, 0.01% NaN₃ in PBS. Purified recombinant IL12-SS1 (Fv) was diluted to 1 µg/mL in ELISA buffer (0.01% Tween 20, 10% Pierce SuperBlock™, Thermo Scientific, Rockford, IL) and incubated on a plate for 1 hour at room temperature. Plates were then incubated with goat anti-rabbit IgG conjugated HRP for 1 hour at room temperature. The plates were washed four times with ELISA buffer between each coating. Visualization was achieved with 3,3',5,5'-

tetramethylbenzidine detection reagent (KPL, Gaithersburg, MD), and the absorbance was read at 450 nm with a SpectraMax™ Plus plate reader (Molecular Devices, Sunnyvale, CA). Any absorbance values equal to or less than 0.1 were considered negative.

5 The bioactivity of IL12-SS1(Fv)

PBMCs were isolated from buffy coat of health donors and activated with 10 µg/ml of PH-A for 4 days. Cell proliferation by IL12-SS1 (Fv) or IL12 was measured by a WST-8 colorimetric assay using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) according to the manufacturer's instructions. Two hundred microliters of PBMCs were seeded at 1 x 10⁴ cells per well in a 96-well plate, and recombinant IL12 or the IL12-SS1 (Fv) fusion proteins were added at the indicated concentrations. Cells were incubated at 37°C for 72 hours, followed by a proliferation assay using Cell Counting Kit-8. The IL12 activity assay was performed with PBMCs in the presence of murine recombinant IL12 or IL12-SS1 (Fv) fusion proteins for 4 days by assessing its ability to induce IFN-γ production using a Human IFN-γ Quantikine™ ELISA Kit (R&D Systems, Minneapolis, MN).

Mesothelioma mouse xenograft model

Eight-week old female athymic nude mice (ATHYMIC NCr-nu/nu) were housed in micro-isolator cages. To investigate the therapeutic effect of IL12-SS1 (Fv) against established mesothelioma, intraperitoneal mesothelioma tumors were established by intraperitoneal (i.p.) injection of 5 million LMB-H226-GL cells in 200 µl of growth media into the low abdomen or flank area of nude mice following a standard lab protocol (Feng *et al*, *J Cancer* 2:123-131, 201 1). Animals were imaged the following day and then once every week thereafter. The animals were divided into 4 groups: vehicle, SS1P (0.4 mg/kg), IL12-SS1 (Fv) (0.4 mg/kg), and IL12-SS1 (Fv) (1.6 mg/kg body weight)-treated mice.

Animal treatment

Tumor-bearing mice were treated with IL12-SS1 (Fv), SS1P, or vehicle at days 13, 15, 17, and 19. The day when the mice were injected with the tumor cells was set as day 1. SS1P, anti-MSLN scFv conjugated with *Pseudomonas* toxin, was used as a positive control. The treatment group and control group each contained 3 mice for initial experiments and 5 mice for repeated experiments. Each mouse in the treatment groups received 0.4 mg/kg body weight of SS1P, 0.4 mg/kg (low dose group), or 1.6 mg/kg (high dose group) of IL12-SS1 (Fv) every the other day (at

day 13, 15, 17, and 19). The control group received PBS as a vehicle control. Body weight and tumor growth were assessed twice a week.

Assessment of tumor growth

Two hundred microliters of 15 mg/mL D-luciferin (Caliper Life Sciences, Hanover, MD) in PBS was injected i.p. before imaging. The luciferase activity of the tumor was calculated using the Living Image 3.1.0 software (Caliper Life Sciences). Tumor growth was assessed in intraperitoneal using photon intensity, photons per second (ph/sec) as luciferase activity following a previously described protocol (Feng *et al*, *J Cancer* 2:123-131, 2011).

In vivo toxicology studies

At the end of the treatment, three mice in each group were euthanized. Blood was taken for whole blood complete blood counts (CBC) and serum chemistry analysis. A full necropsy was performed, in which organs and tissues were weighed and examined for gross findings.

Statistical analysis

Statistical analysis was performed with Prism (version 5) for Windows (GraphPad Software, La Jolla, CA). Raw data were analyzed by "analysis of variance" with Dunnett's and Newman-Keuls multiple comparison post tests. *p* values < 0.05 were considered statistically significant.

Example 2: Immunocytokine IL12-SS1 (Fv) inhibits mesothelioma tumor growth

This example describes the construction and characterization of a mesothelin-specific immunocytokine comprising IL12 fused to the SSI variable fragment. The IL12-SS1 (Fv) immunocytokine specifically binds mesothelin-expressing cells, retains IL12 biological activity and inhibits mesothelin-expressing tumor growth *in vivo*.

Construction of the IL12-SS1 (Fv) immunocytokine

To generate a new immunocytokine targeting mesothelin-expressing tumors, a monomeric IL12-SS1 (Fv) was constructed, which is a fusion of IL12 to the anti-mesothelin antibody fragment scFv (SSI). The p40 and p35 subunits of murine IL12 were connected by flexible linker (Ser₄Gly)₃ (SEQ ID NO: 24). The scFv (SSI) was fused to the p35 subunit of IL12 through a 6 amino acid flexible linker (GSADGG; SEQ ID NO: 25). IL12-SS1 (Fv) also contains Flag and 8XHis tags at the C-terminal end for detection. The final PCR products were flanked by Gateway recombination

signal sequences, attB1 at the 5' end and attB2 at the 3' end (FIG. 1A). FIG. 1B depicts the construct containing the p40 and p35 subunits of murine IL12 and the SSI scFv. IL12-SS 1 (Fv) was expressed in sf9 insect cells and fusion protein was purified to homogeneity by affinity chromatography. The production yields were 1.2 mg for IL12-SS 1 (Fv) per liter of cell culture supernatant. The resulting purified IL12-SS 1 (Fv) protein was characterized by SDS- PAGE analysis, confirming the presence of a single band of apparent molecular weight equal to 90 kDa under reducing and non-reducing conditions (FIG. 1C).

IL12-SS1 (Fv) specifically binds mesothelin and exerts bioactivity similar to IL12

To evaluate the binding properties and functionality of the IL12-SS 1 (Fv) immunocytokine, *in vitro* analysis was conducted using recombinant proteins and human cancer cells. The binding specificity of IL12-SS 1 (Fv) for MSLN was examined by ELISA (FIG. 2A). The recombinant IL12-SS 1 (Fv) bound to MSLN in a dose-dependent manner. A rFc fusion protein was used as a negative control and no binding to rFc was detected ($OD \leq 0.1$) and there was no dose-dependent correlation with rFc binding (FIG. 2A). To determine whether the binding of IL12-SS 1 (Fv) to the cells was specific, the *in vitro* binding capability of IL12-SS 1 (Fv) to MSLN-expressing cells was assessed by flow cytometry (FIG. 2B). Using IL12-SS1 (Fv) for detection, MSLN expression was determined to be strongly positive in NCI-H226, OVCA-3, and H9 cells, and weakly positive in A431 and HEK-293 cells. In the presence of an IL12 receptor blocking antibody, the binding of IL12-SS 1 (Fv) to A431 cells was inhibited, indicating that IL12-SS 1 (Fv) binds to the IL12 receptor on A431 cells (Fig. 2C). Gene expression of IL12 receptor was examined in those cells (NCI-H226, OVCA-3, H9, and HEK-293 cells) from the NCI-60 cell line panel and found that gene expression of IL12 receptor was low. The protein expression of the IL12 receptor was detected by western blot. Therefore, the IL12-SS1 (Fv) binds to IL12 receptor, not to MSLN in A431 cells. In particular, the binding was stronger in H9, an A431 line highly expressing MSLN on the cell surface, than in A431 which is mesothelin-negative. To determine whether IL12-SS1 (Fv) binds cell surface-associated MSLN, flow cytometric analysis was performed (FIG. 2D). SSIP was used as a positive control and it was determined that IL12-SS 1 (Fv) and SSIP bound to H9 in a dose-dependent manner. The binding affinity (equilibrium K_d) of IL12-SS 1 (Fv) and SSIP for mesothelin-expressing H9 cells were approximately 60 nM and 2.8 nM, respectively.

IL12 is a key immunoregulatory cytokine and plays an essential role in the interactions between the innate and adaptive arms of immunity by acting on natural killer (NK) cells and T cells and enhancing the generation and activity of cytotoxic lymphocytes (Geldhof *et al* , *Blood* 91:196-206, 1998; Manetti *et al* , *J Exp Med* 177: 1199- 1204, 1993; Gately *et al* , *Cell Immunol* 143: 127-

142, 1992). IL12 is responsible for the priming of Th1 cell responses and the secretion of large amounts of IFN- γ from T cells and NK cells (Colombo and Trinchieri, *Cytokine Growth Factor Rev* 13:155-168, 2002). IL12 induces an antitumor response in a murine model of malignant mesothelioma (Caminschi *et al.*, *Am J Respir Cell Mol Biol* 19:738-746, 1998). IL12 has exhibited
5 a potent antitumor and anti-metastatic activity in preclinical studies (Colombo and Trinchieri, *Cytokine Growth Factor Rev* 13:155-168, 2002; Brunda *et al.*, *J Exp Med* 178:1223-1230, 1993; Trinchieri, *Curr Opin Hematol* 4:59-66, 1997; Tsung *et al.*, *J Immunol* 158:3359-3365, 1997; Rodolfo and Colombo, *Methods* 19:114-120, 1999). Clinical trials in patients with cancer have revealed promising therapeutic activities, but have also shown that recombinant human IL12 is
10 extremely toxic to humans (Siddiqui *et al.*, *Mol Cancer Ther* 6:380-389, 2007). Since human IL12 has no biological activity in mice (Ling *et al.*, *J Immunol* 154:116-127, 1995), mouse IL12 was used in this study.

Several studies with tumor specific antibodies fused with IL12 have been reported, including anti-CD30 antibody for Hodgkin's lymphoma, anti-HER2 antibody for HER2-expressing
15 tumors (Heuser *et al.*, *Int J Cancer* 106:545-552, 2003; Helguera *et al.*, *Mol Cancer Ther* 5:1029-1040, 2006), and anti-extra-domain B (ED-B) of fibronectin for tumor vessels, IL12-huBC1 (Lo *et al.*, *Cancer Immunol Immunother* 56:447-457, 2007), and IL12-L19 (Gafner *et al.*, *Int J Cancer* 119: 205-2212, 2006). In order to confirm whether purified IL12-SS1 (Fv) maintains the biological activity of IL12, the stimulatory effect of murine IL12 and IL12-SS1 (Fv) were compared in a
20 lymphocyte proliferation assay; the ability of these proteins to induce INF- γ production in PBMC was also evaluated. Stimulation of PBMCs with doses of IL12 or IL12-SS1 (Fv) as low as 1 ng/ml caused an increase in proliferation (FIG. 3A). The specific binding affinity of IL12-SS1 (Fv) to MSLN was 60 nM. Somnavilla *et al.* have reported about immunocytokine consisting of the scFv (F8) specific to extra-domain A of fibronectin and human IL12, F8-IL12, lymphoproliferative
25 activity was 600 nM (Somnavilla *et al.*, *Protein Eng Des Sel* 23:653-661, 2010). A significant dose-dependent increase in the induction of IFN- γ by treatment with either IL12 or IL12-SS1 (Fv) was noted (FIG. 3B). IL12-SS1 (Fv) exhibited comparable biological activities, which was slightly lower than recombinant murine IL12 used as a standard, at high concentrations (100 ng/mL and 1000 ng/mL). At low concentrations (10 ng/mL or less), IL12-SS1 (Fv) exhibited 2- to 4-fold less
30 activity as compared to the control IL12. Taken together, these data indicate that the recombinant IL12-SS1 (Fv) fusion protein exerts its bioactivity by proliferation and induction of IFN- γ production in PBMC.

IL12-SS1 (Fv) inhibits mesothelioma tumor growth in nude mice

To investigate the therapeutic effect of IL12-SS1 (Fv) against established mesothelioma, a pilot study was performed in mice. Mice were inoculated with H226-GL mesothelioma cells (FIG. 4). In an initial animal experiment, mice were treated with two different doses of IL12-SS1 (Fv), 0.4 mg/kg and 1.6 mg/kg. After 13 days of tumor development, the tumor-bearing mice were treated at days 13, 15, 17, and 19 with 10 or 1.6 mg/kg of IL12-SS1 (Fv), 0.4 mg/kg body weight of SS1P, or a vehicle control. Tumor sizes were accessed via *in vivo* bioluminescence measurement using the IVIS Imaging System. FIG. 4A shows that the IL12-SS1 (Fv) and SS1P treatment groups exhibited significantly retarded tumor growth compared with the saline treatment group. In mice treated with vehicle, the photon ranges were gradually increased to 0.8, 5.7 and 8.3 X 10⁹ ph/sec at 18, 24, and 31 days, respectively after i.p. H226-GL injection. When photons were measured after injections with IL12-SS1 (Fv) or SS1P, the tumor size was significantly reduced. The photons were 0.4, 7.4, and 1.8 x 10⁸ at 31 days after treatment of SS1P (0.4 mg/kg), IL12-SS1 (Fv) (0.4 mg/kg and 1.6 mg/kg), respectively. At Day 18, the tumor sizes in either SS1P or IL12-SS1(Fv) treated groups were not significantly reduced as compared to the control group ($p > 0.05$). At Day 24 and Day 31, the tumor sizes in the mice treated with either SS1P or IL12-SS1(Fv) were significantly reduced ($p < 0.05$). The anti-tumor activity of IL12-SS1(Fv) was similar to that of SS1P ($p > 0.05$). High dose (1.6 mg/kg) or low dose (0.4 mg/kg) of IL12-SS1(Fv) had similar tumor growth inhibition ($p > 0.05$), indicating that a low dose of the IL12-SS1(Fv) immunotoxins may also be effective *in vivo*. No significant weight loss was observed in mice treated with vehicle, SS1P, or IL12-SS1 (Fv). The next animal experiment used five mice in each group and 0.4 mg/kg of IL12-SS1 (Fv) (FIG. 4B). As shown in the first experiment, both IL12-SS1 (Fv) and SS1P caused tumor remission at Day 21 while the control mice grew large tumors.

To examine the toxicity of IL12-SS1 (Fv), *in vivo* toxicology studies were performed at the end of the treatment (Table 2). Blood from mice in treatment and control groups (three mice per group) was analyzed by CBC, and sera isolated for serum chemistry analyses. All serum chemistry and CBC in the IL12-SS1 (Fv)-treated group were similar to those of the control group except white blood cells; the organ weights of IL12-SS1 (Fv) treated-mice were not significantly different from those of the control group mice, indicating that IL12-SS1 (Fv) has no serious toxicity in mice. It was noted that the white blood cells were elevated by about 2-fold in the IL12-SS1 (Fv) group (16.28 ± 2.47 K/ μ L) as compared to the control group (7.1 ± 2.16 K/ μ L) and the SS1P group (7.51 ± 0.97 K/ μ L).

Table 2. Selected *in vivo* toxicological results and organ weights

Complete blood counts and serum chemistry				
Selected Parameters	Control	SS1P	IL12-SS1 (Fv)	Normal Values
White blood cells (K/ μ L)	7.1 $\pm$ 2.16	7.51 $\pm$ 0.97	16.28 $\pm$ 2.47	1.80 - 10.70
Red blood cells (M/ μ L)	9.47 $\pm$ 0.32	9.51 $\pm$ 0.02	10.35 $\pm$ 0.84	6.36 - 9.42
Albumin (g/dL)	4.4 $\pm$ 0.42	4.27 $\pm$ 0.42	4.1 $\pm$ 0.3	1.6 - 2.8
Alkaline phosphatase (U/L)	60.5 $\pm$ 9.19	54 $\pm$ 15.13	73 $\pm$ 4.36	67 - 282
Alanine aminotransferase (U/L)	61 $\pm$ 16.5	46.33 $\pm$ 4.51	46.67 $\pm$ 12.50	29 - 181
Total bilirubin (mg/dL)	0.3	0.3	0.37 $\pm$ 0.06	0.0 - 0.6
Creatinine (mg/dL)	<0.2	<0.2	<0.2	0.2 - 0.4
Hemoglobin (g/dL)	14.53 $\pm$ 0.42	14.47 $\pm$ 0.65	14.83 $\pm$ 0.64	11.00 - 15.10
Total protein (g/dL)	6.67 $\pm$ 0.64	6.47 $\pm$ 0.15	6.13 $\pm$ 0.15	4.2 - 5.9
Blood urea nitrogen (mg/dL)	25.67 $\pm$ 3.06	25 $\pm$ 2.65	23 $\pm$ 1.73	12 - 52
Organ Weights (mg)				
Brain	0.45 $\pm$ 0.04	0.43 $\pm$ 0.02	0.43 $\pm$ 0.02	NA
Heart	0.12 $\pm$ 0.02	0.12 $\pm$ 0.01	0.14 $\pm$ 0.01	NA
Kidney	0.31 $\pm$ 0.04	0.29 $\pm$ 0.02	0.33 $\pm$ 0.02	NA
Liver	1.02 $\pm$ 0.29	1.01 $\pm$ 0.05	1.17 $\pm$ 0.09	NA
Lung	0.16 $\pm$ 0.03	0.16 $\pm$ 0.04	0.16 $\pm$ 0.01	NA
Spleen	0.14 $\pm$ 0.12	0.12 $\pm$ 0.02	0.12 $\pm$ 0.06	NA

In conclusion, described herein is an IL12-based immunocytokine that target cell surface-associated mesothelin proteins in mesothelioma and other cancers. The recombinant immunocytokine produced in baculovirus expression system is as biologically active as IL12 alone. Furthermore, animal testing showed that the immunocytokine exhibited inhibition of human mesothelioma grown in the peritoneal cavity of nude mice. These results suggest a new mesothelin-targeted antibody conjugate, which is different from other anti-mesothelin antibodies (such as chimeric antibody MORAb-009) or immunotoxins (SS1P) currently being evaluated in preclinical and clinical studies. IL12-SSI(Fv) is likely non-immunogenic, and therefore presents a significant advantage over currently immunotoxins. Local delivery of IL12 to tumor microenvironment by the anti-mesothelin antibody may enhance specific immune response against tumors.

Example 3: Mesothelin-specific immunocytokines for the treatment of cancer

This example describes the use of a mesothelin-specific immunocytokine, such as the immunocytokine disclosed herein (for example, IL12-SS1), for the treatment of cancers that exhibit overexpression of mesothelin (referred to herein as a "mesothelin-expressing" or "mesothelin-positive" cancer), including, but not limited to mesothelioma, prostate cancer, lung cancer, stomach cancer, squamous cell carcinoma, pancreatic cancer, cholangiocarcinoma, triple negative breast cancer or ovarian cancer. Patients diagnosed with a mesothelin-positive cancer are treated according to standard procedures in the art, for example with surgery to remove or partially remove tumors, chemotherapy, radiation therapy, or any combination thereof. The therapeutic approach will depend, for example, on the specific type of mesothelin-expressing cancer to be treated, the location of the tumor, the severity of the cancer and whether the cancer has metastasized. Exemplary chemotherapeutic agents for the treatment of mesothelin-expressing cancer include but are not limited to cisplatin, pemetrexed, carboplatin, gemcitabine, Navelbine™, Onconase™, 5-fluorouracil, doxorubicin, topotecan, etoposide, cyclophosphamide, epirubicin, taxotere, docetaxel, cabazitaxel, leucovorin and oxaliplatin.

In this example, patients diagnosed with a mesothelin-positive cancer are further treated by administration of an immunocytokine comprising a mesothelin-specific monoclonal antibody or antibody fragment comprising the CDR sequences (or complete VH and VL domains) of the SSI scFv, linked to the p35 and p40 subunits of IL12. In some patients, the immunocytokine is administered by intravenous bolus injection every other day or once a week for a total of three to six doses. In other patients, the immunocytokine is administered by continuous intravenous infusion over the course of three to seven days. Patients with stabilization or regression of disease may receive a second course of treatment four or five weeks later. The dose of immunocytokine administered to a patient varies depending on the weight and gender of the patient, and mode and time course of administration. Following treatment, patients are evaluated for cancer progression (including tumor growth and metastasis) and other clinical signs of illness.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. A conjugate comprising interleukin-12 (IL12) subunits p35 and p40 linked to a mesothelin- specific antibody or antibody fragment comprising a variable heavy (VH) domain and a variable light (VL) domain, wherein:

the VH domain comprises amino acid residues 31-35, 50-66 and 99-108 of SEQ ID NO: 2, or amino acid residues 26-33, 51-58 and 97-108 of SEQ ID NO: 2; and

the VL domain comprises amino acid residues 24-33, 49-55 and 88-96 of SEQ ID NO: 4, or amino acid residues 25-31, 49-51 and 88-96 of SEQ ID NO: 4.

2. The conjugate of claim 1, wherein the p35 subunit is linked to the VH domain of the antibody or antibody fragment.

3. The conjugate of claim 1 or claim 2, comprising in the N-terminal to C-terminal direction the p40 subunit, the p35 subunit, the VH domain and the VL domain.

4. The conjugate of claim 3, further comprising a linker between the p40 subunit and the p35 subunit.

5. The conjugate of claim 4, wherein the linker between the p40 subunit and the p35 subunit is about 4 to about 20 amino acid residues in length.

6. The conjugate of claim 5, wherein the linker between the p40 subunit and the p35 subunit is 15 amino acids in length.

7. The conjugate of any one of claims 4-6, wherein the linker between the p40 subunit and the p35 subunit comprises repeating serine and glycine residues.

8. The conjugate of claim 7, wherein the linker between the p40 subunit and the p35 subunit comprises the amino acid sequence (Ser₄Gly)₃ (SEQ ID NO: 24).

9. The conjugate of any one of claims 3-8, further comprising a linker between the p35 subunit and the VH domain.

10. The conjugate of claim 9, wherein the linker between the p35 subunit and the VH domain is about 4 to about 20 amino acids in length.

5 11. The conjugate of claim 10, wherein the linker between the p35 subunit and the VH domain is 6 amino acids in length.

12. The conjugate of any one of claims 9-11, wherein the linker between the p35 subunit and the VH domain comprises the amino acid sequence GSADGG (SEQ ID NO: 25).

10 13. The conjugate of any one of claims 1-12, wherein the IL12 is mammalian IL12.

14. The conjugate of claim 13, wherein mammalian IL12 is human or mouse IL12.

15 15. The conjugate of any one of claims 1-14, wherein the amino acid sequence of the VH domain comprises SEQ ID NO: 2.

16. The conjugate of any one of claims 1-15, wherein the amino acid sequence of the VL domain comprises SEQ ID NO: 4.

20 17. The conjugate of any one of claims 1-16, wherein the antibody fragment is a scFv.

18. The conjugate of claim 17, wherein the amino acid sequence of the scFv comprises SEQ ID NO: 6.

25 19. A composition comprising a therapeutically effective amount of the conjugate of any one of claims 1-18 in a pharmaceutically acceptable carrier.

20. An isolated nucleic acid molecule encoding the conjugate of any one of claims 1-18.

30 21. An isolated nucleic acid molecule encoding the conjugate of claim 17 or claim 18, wherein the nucleic acid molecule comprises the sequence of SEQ ID NO: 5.

22. A vector comprising the isolated nucleic acid molecule of claim 20 or claim 21.

23. An isolated cell comprising the nucleic acid molecule of claim 20 or claim 21, or the vector of claim 22.

24. A method of treating a subject having a mesothelin-expressing cancer, comprising
5 selecting a subject having a mesothelin-expressing cancer and administering to the subject a therapeutically effective amount of the conjugate of any one of claims 1-18 or the composition of claim 19.

25. A method of inhibiting tumor growth or metastasis in a subject having a mesothelin-
10 expressing cancer, comprising selecting a subject having a mesothelin-expressing cancer and administering to the subject a therapeutically effective amount of the conjugate of any one of claims 1-18 or the composition of claim 19.

26. The method of claim 24 or claim 25, wherein the mesothelin-expressing cancer is
15 mesothelioma, prostate cancer, lung cancer, stomach cancer, squamous cell carcinoma, pancreatic cancer, cholangiocarcinoma, triple negative breast cancer or ovarian cancer.

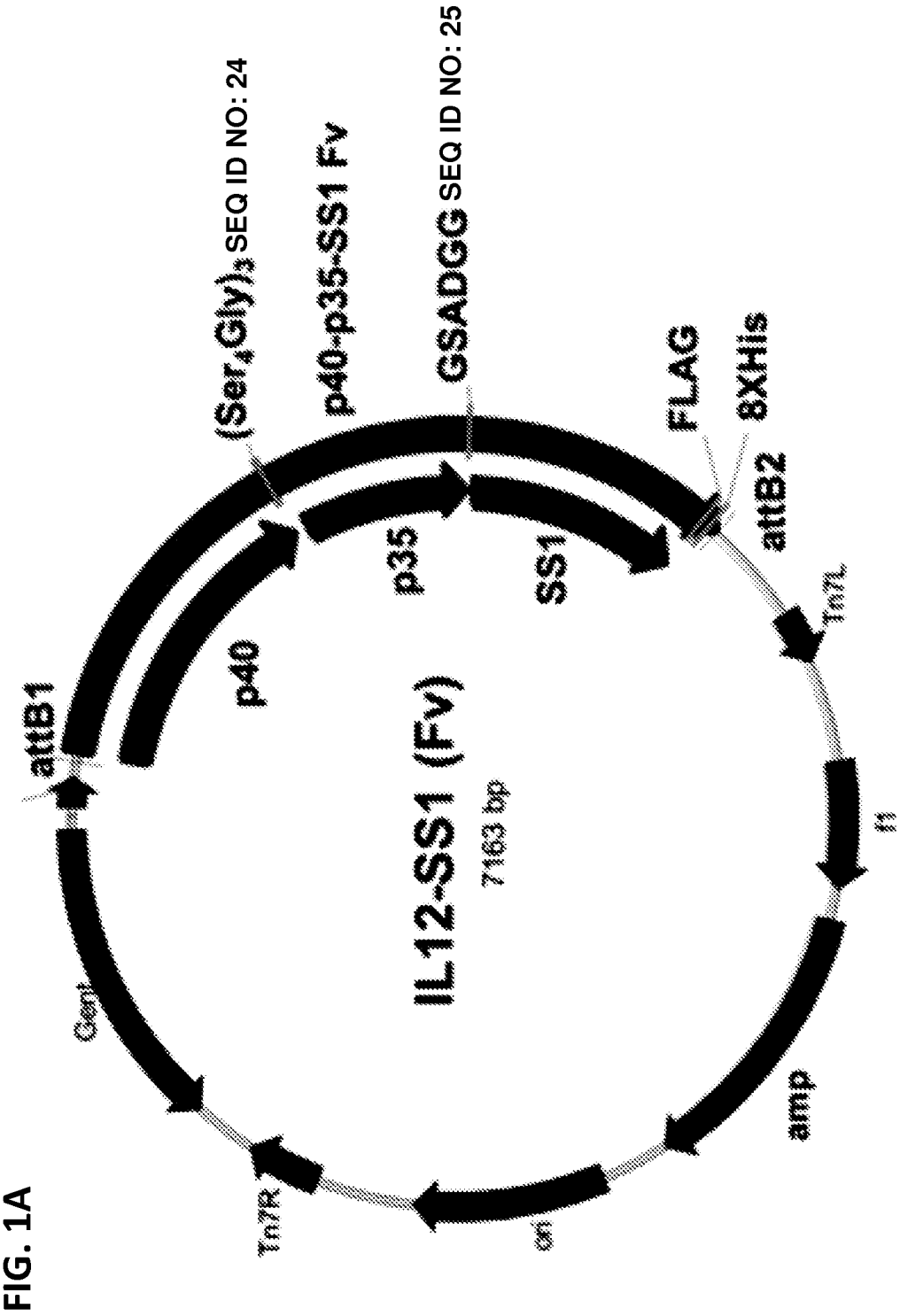


FIG. 1B

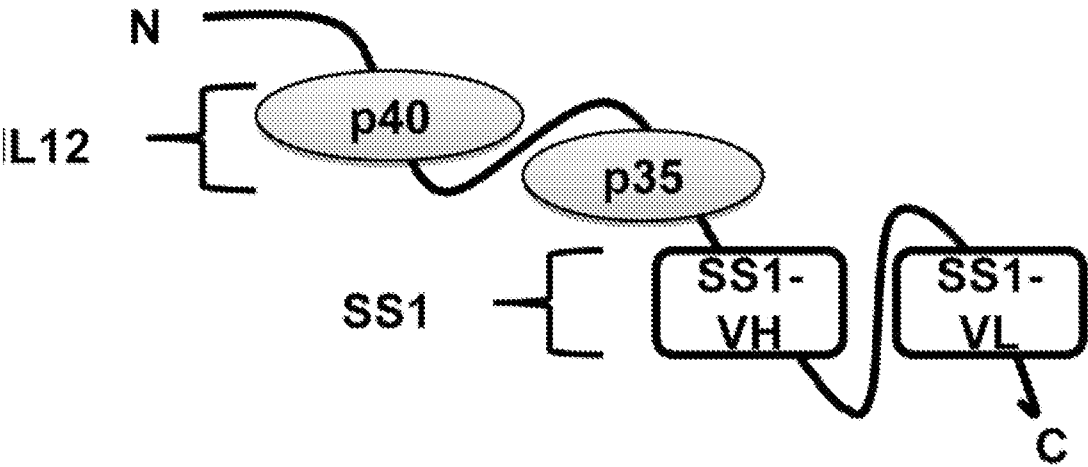


FIG. 1C

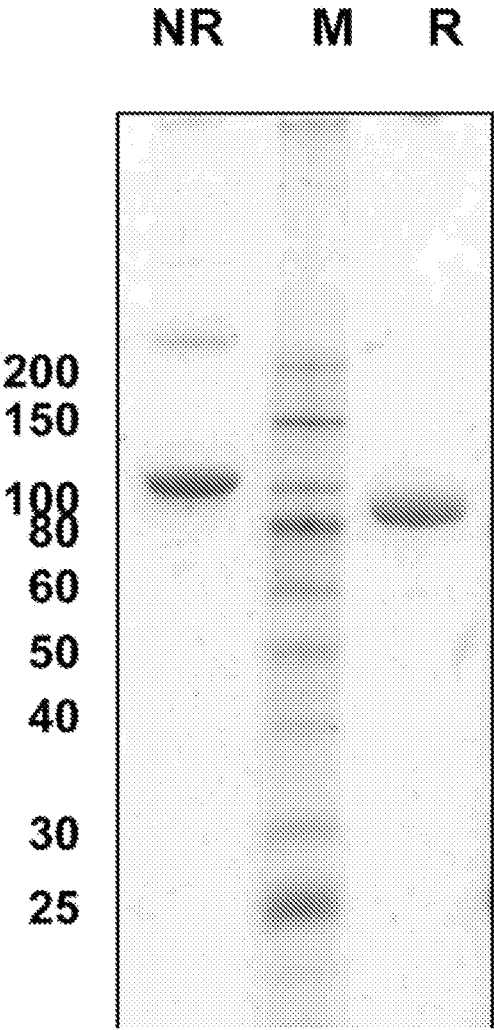


FIG. 2A

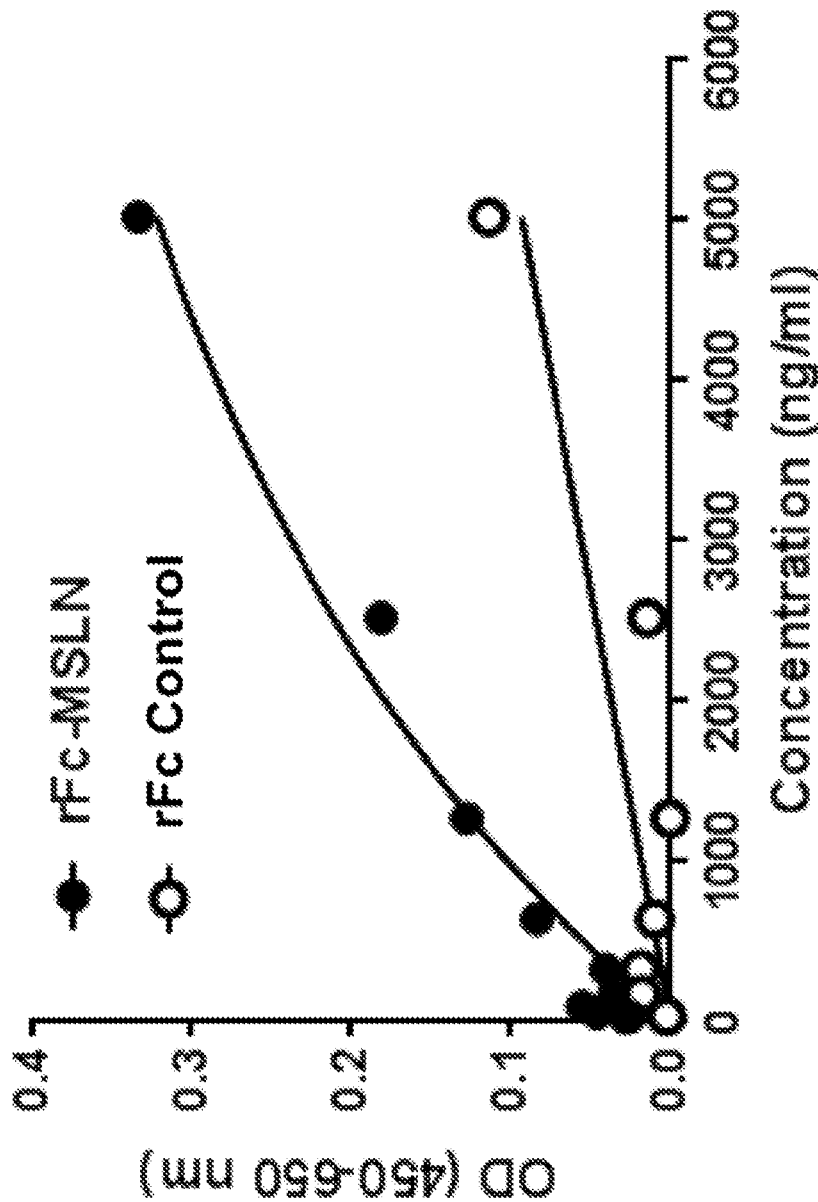


FIG. 2B

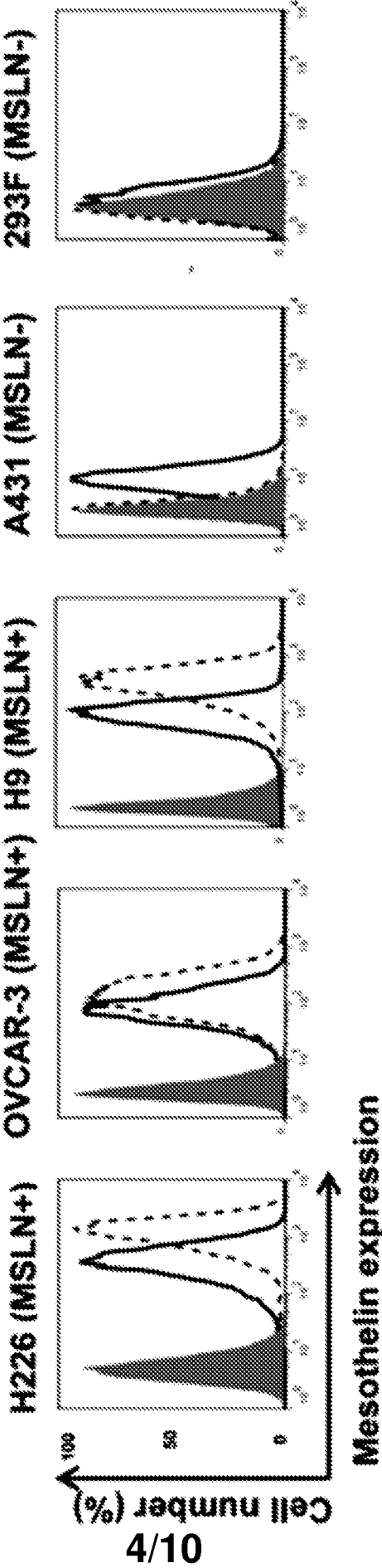


FIG. 2C

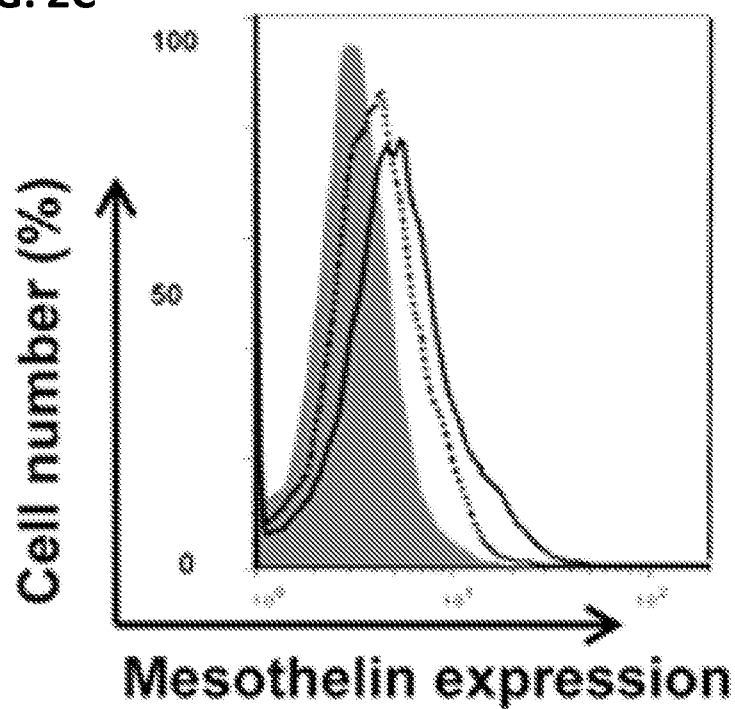


FIG. 2D

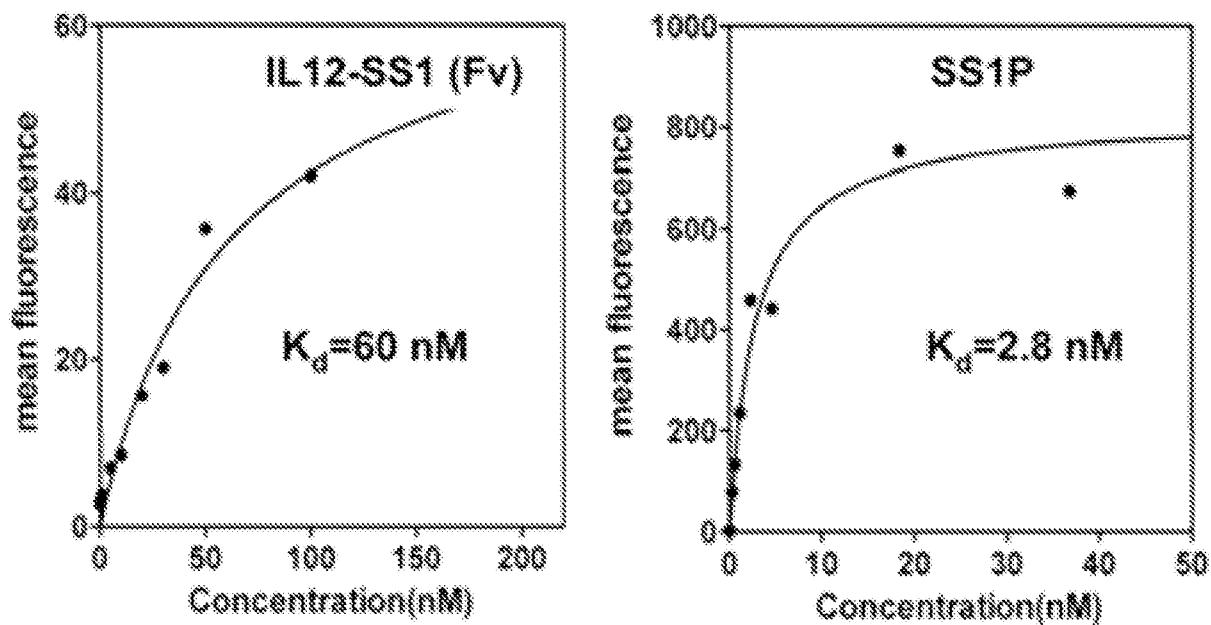


FIG. 3A

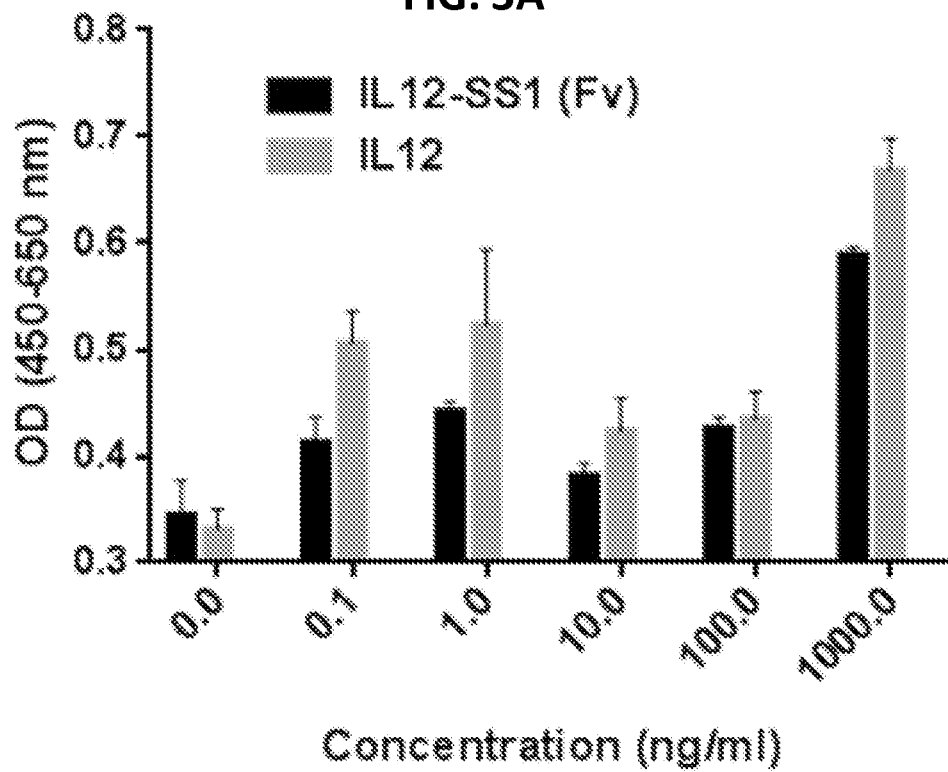


FIG. 3B

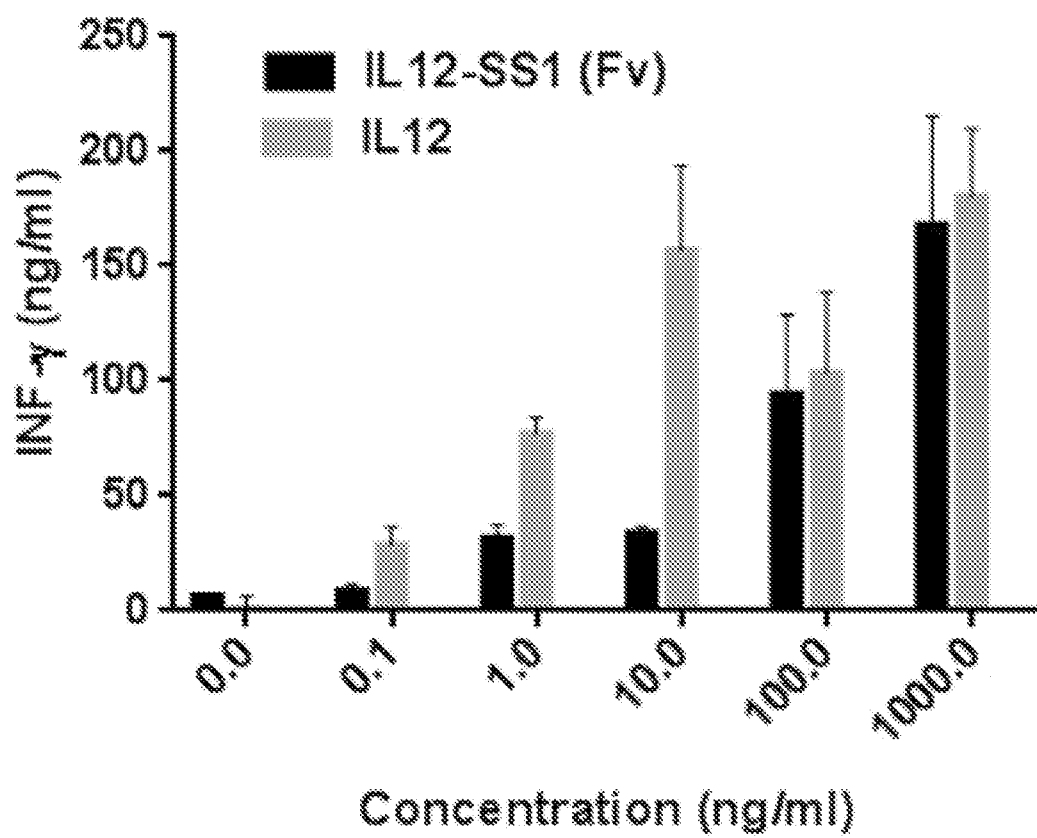


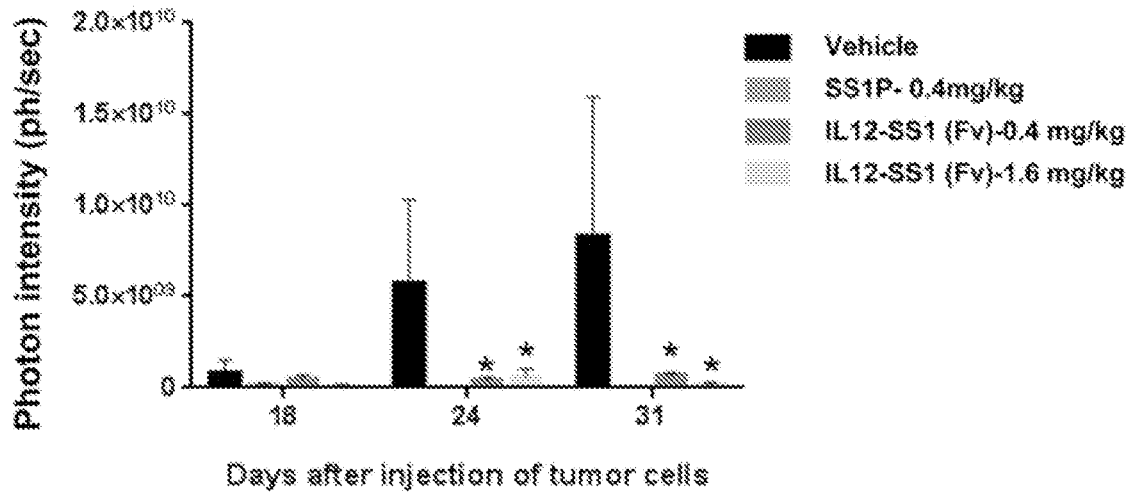
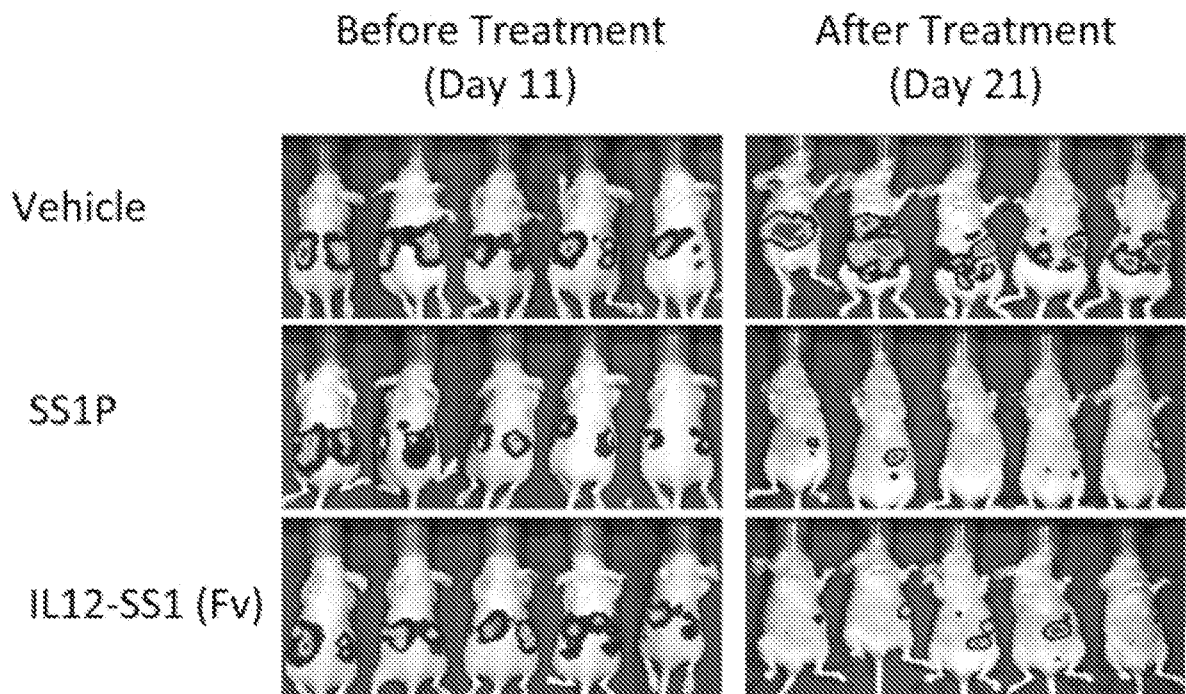
FIG. 4A**FIG. 4B**

FIG. 5A

SS1 VH

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Q V Q L Q Q S G P E L E K P G A S V K I S C K A S
CAGGTGCAGCTGCAGCAGTCTGGGCCTGAGCTGGAGAAGCCTGGCGCTTCAGTGAAGATATCCTGCAAGGCTTCT

CDR1

26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
G Y S F T G Y T M N W V K Q S H G K S L E W I G L
GGTTACTCATTCACTGGCTACACCATGAACTGGGTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGACTT

CDR2

51 52 ^{52a} 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
I T P Y N G A S S Y N Q K F R G K A T L T V D K S
ATTACTCCTTACAATGGTGCTTCTAGCTACAACCAGAAGTTCAGGGGCAAGGCCACATTAAGTGTAGACAAGTCA

75 76 77 78 79 80 81 82 ^{82a} ^{82b} ^{82c} 83 84 85 86 87 88 89 90 91 92 93 94 95 96
S S T A Y M D L L S L T S E D S A V Y F C A R G G
TCCAGCACAGCCTACATGGACCTCCTCAGTCTGACATCTGAAGACTCTGCAGTCTATTTCTGTGCAAGGGGGGGT

CDR3

97 98 99 100 ^{100A} ^{100B} 101 102 103 104 105 106 107 108 109 110 111 112
Y D G R G F D Y W G Q G T T V T V S S
TACGACGGGAGGGGTTTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA

FIG. 5B

SS1 VL

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
 D I E L T Q S P A I M S A S P G E K V T M T C S A
 GACATCGAGCTCACTCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCAGTGCC

CDR1 CDR2
 26 27 28 29 30 31 32 33 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51
S S S V S Y M H W Y Q Q K S G T S P K R W I Y D T
 AGCTCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGTCAGGCACCTCCCCCAAAGATGGATTTATGACACA

52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76
S K L A S G V P G R F S G S G S G N S Y S L T I S
 TCCAAACTGGCTTCTGGAGTCCCAGGTCGCTTCAGTGGCAGTGGGTCTGGAAACTCTTACTCTCTCACAATCAGC

CDR3
 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101
 S V E A E D D A T Y Y C Q Q W S K H P L T F G A G
 AGCGTGGAGGCTGAAGATGATGCAACTTATTACTGCCAGCAGTGGAGTAAGCACCTCTCACGTTCGGTGCTGGG

102 103 104 105
 T K L E
 ACAAAGTTGGA

FIG. 5C**SS1 ScFv**

atggcccaggtgcagctgcagcagctctgggcctgagctggagaagcctggcgcttcagtg
 M A Q V Q L Q Q S G P E L E K P G A S V
 aagatatcctgcaaggcttctgggttactcattcactggctacaccatgaactgggtgaag
K I S C K A S G Y S F T G Y T M N W V K
 cagagccatggaaagagccttgagtggattggacttattactccttacaatgggtgcttct
Q S H G K S L E W I G L I T P Y N G A S
 agctacaaccagaagttcaggggcaaggccacattaactgtagacaagtcacccagcaca
S Y N Q K F R G K A T L T V D K S S S T
 gcctacatggacctcctcagtcctgacatctgaagactctgcagtcctatcttctgtgcaagg
A Y M D L L S L T S E D S A V Y F C A R
 ggggggttacgacgggaggggttttgactactggggccaagggaccacgggtcaccgtctcc
G G Y D G R G F D Y W G Q G T T V T V S
 tcaggtgtaggcgggttcaggcggcggtggctctggcggtggcggtatcggacatcgagctc
S G V G G S G G G G S G G G G S D I E L
 actcagtcctccagcaatcatgtctgcacatctccaggggagaaggtcaccatgacctgcagt
T Q S P A I M S A S P G E K V T M T C S
 gccagctcaagtgttaagttacatgcactgggtaccagcagaagtcaggcacctccccaaa
A S S S V S Y M H W Y Q Q K S G T S P K
 agatggatttatgacacatccaaactggcttctggagtcccagggtcgcttcagtggcagt
R W I Y D T S K L A S G V P G R F S G S
 gggctctggaaactcttactctctcacaatcagcagcgtggaggctgaagatgatgcaact
G S G N S Y S L T I S S V E A E D D A T
 tattactgccagcagtgaggtaagcacctctcacgttcgggtgctgggacaaagttggaa
Y Y C Q Q W S K H P L T F G A G T K L E
 ataaaacgg
I K R

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/036296

A. CLASSIFICATION OF SUBJECT MATTER
 INV. A61K47/48 A61K39/00
 ADD.

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)

A61K

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)

EPO-Internal , BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PENG L S ET AL: "A single-chain IL-12 IgG3 anti body fusion protein retains anti body specificity and IL-12 bioactivity and demonstrates anti tumor activity", THE JOURNAL OF IMMUNOLOGY, THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol . 163, 1 January 1999 (1999-01-01) , pages 250-258, XP002967832 , ISSN: 0022-1767 abstract	1-26
Y	WO 2012/154530 A1 (US GOV HEALTH & HUMAN SERV [US] ; PASTAN I RA H [US] ; WELDON JOHN [US] ;) 15 November 2012 (2012-11-15) abstract ----- -/- .	1-26



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